Identification of plausible targets for antiproliferation by benzyl isothiocyanate in colorectal cancer cells 2016, March NAOMI ABE

# Identification of plausible targets for antiproliferation by benzyl isothiocyanate in colorectal cancer cells

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NAOMI ABE

The Graduate School of Environmental and Life Science

(Doctor Course)

OKAYAMA UNIVERSITY, JAPAN

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# LIST OF TABLES AND FIGURES

## ABBREVIATIONS USED

AITC, allyl isothiocyanate

APC, adenomatous polyposis coli

ATCC, American Type Culture collection

BITC, benzyl isothiocyanate

BSA, bovine serum albumin

DUB, deubiquitinating enzyme

FBS, fatal bovine serum

GSs, glucosinolates

IFN-γ, interferon-γ

IKK, IκB kinase

ITCs, isothiocyanates

JNK, c-Jun N-terminal kinase

LATs, L-type amino acid transporters

LDH, lactate dehydrogenase

MAPK, mitogen-activated protein kinase

mTORC1, mammalian target of rapamycin complex 1

NF-κB, nuclear factor-κB

*O*-GlcNAc, *O*-linked β-N-acetyl glucosamine

ORFs, open reading frames

PAP, Peroxidase anti-peroxidase soluble complex

PBS, phosphate buffered saline

PEITC, phenethyl isothiocyanate

PFA, paraformaldehyde

PI, propidium iodide

PP2A, protein phosphatase type 2A

SC-His, Synthetic complete without His

SFN, sulforaphane

TBE0, TCF-binding element0

TCF, T-cell factor

UCH, ubiquitin C-terminal hydrolases

USP, ubiquitin-specific protease

# CHAPTER 1

# General Introduction

## 1.1. Cancer

Cancer (malignant neoplasm or malignant tumor) is the cell group which becomes out of control by gene mutations and spreads throughout the body. Tumor frequently occurs with getting old. Tumor that the location is restricted in a part of body is called a benign tumor and “wart” is the example. On the other hand, tumor spreading throughout the body is called malignant tumor and it becomes “tumor”. The benign tumor is usually harmless for the host. However, it becomes a medically problem when it physically presses the peripheral brain or brain nerve and it excessively secretes the biologically active substance like hormone. Unlike the benign tumor, the malignant tumor has the invasive ability to invade into the peripheral tissue and the metastatic ability to enter the circulatory system and to settle on the other tissue after the invasion. Also the cancer cell has the characteristics frequently found in cells with high growth rate such as the big nucleus compared with cytoplasm, the conspicuous nucleolus and the frequent cell division, so it is identifiable under the microscope. The normal cell arises from one of three embryo cell layers: endoblast (it differentiates into a digestive organ, respiratory organs and urinary tract), ectoderm (it differentiates into skin, nervous system and sense organs) or mesoderm (it differentiates into skeletal system, muscular system and circulatory system), and the malignant tumor that arises from endoblast or an ectoderm is called “carcinoma”, the malignant tumor that arises from mesoderm is called “sarcoma” (分子生物学（下）第 3 版、Lodish et al. p.1167-1168)

According to the newest demographic statistics of Japan, the first cause of death is cancer (28.9%), the second is heart disease (15.5%), the third is pneumonia (9.4%), the fourth is cerebrovascular disease (9.0%) and the fifth is old age (5.9%), which indicates

one third of people dies of cancer. Based on the Annual change of the death rate of the main cause of death, cancer consistently keeps increasing and has been the first cause of death since 1981 (平成 26 年人口動態統計月報年計（概数）の概況・ http://www.mhlw.go.jp/toukei/saikin/hw/jinkou/geppo/nengai14/index.html).

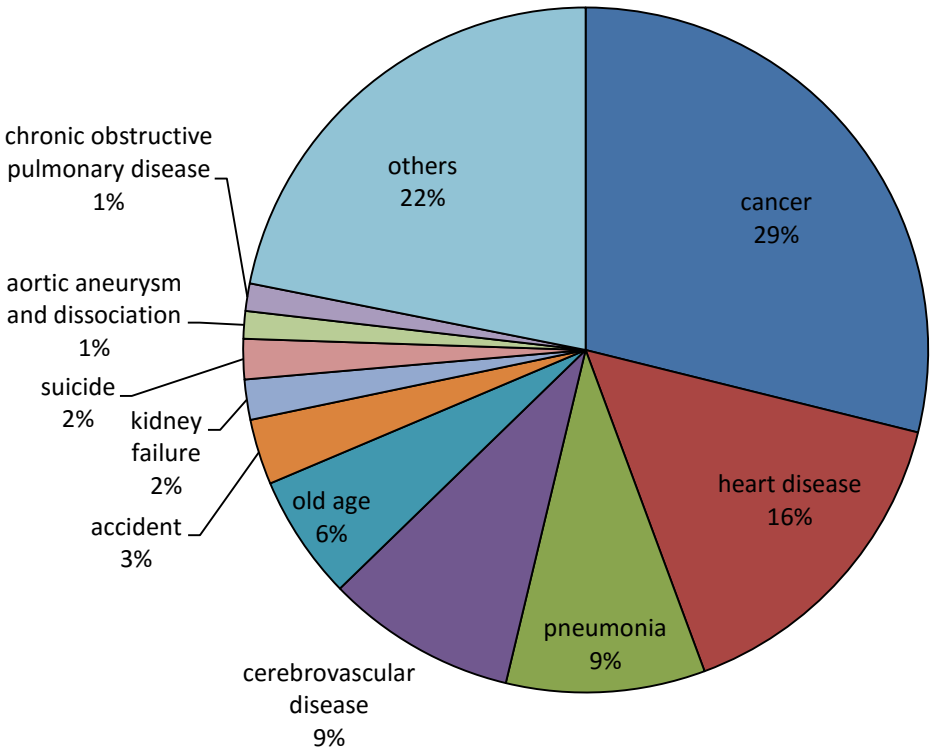



**Figure 1.1.** The rate of number of main causes of death in Japan in 2014

It is expected that by 2020, the world population reaches 75 billion and among them, 15 million people have a diagnosis of cancer newly and 12 million cancer patients die of cancer (Bray and Moller 2006). The first common cancer worldwide is lung cancer, the second is breast cancer and the third is colorectal cancer. The first high death rate cancer is lung cancer, the second is stomach cancer and the third is liver cancer. The ranking of common cancer is different between developed countries and developing countries. For example, the incidence of colorectal cancer in developed countries, including Japan, is three times as high as that in developing countries (Ferlay et al. 2010).

## 1.2. Colorectal cancer

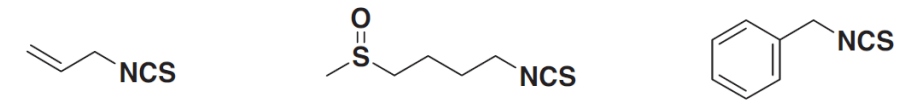
Colorectal cancer is the third cancer commonly diagnosed in men and second in women worldwide (Ferlay et al. 2010). Various factors are related to the development of colorectal cancer including high risk luminal environment, gene mutations and lifestyle such as the cigarette smoking and the consumption of processed meats, animal fats and alcohol (Raskov et al. 2014).

Adenomatous Polyposis Coli (APC) and/or β-catenin are mutated in more than 90% of human colorectal cancers. These mutations lead to stabilization and accumulation of β-catenin in cells. Once in nucleus, β-catenin engages DNA-bound LEF/T-cell factor (TCF) transcription factors on promoters and activates the expression of target genes including cyclin D1 that is required for cell cycle $G_1$/S transition (Tetsu and McCormick 1999). Because cyclin D1 positively regulates APC-dependent intestinal tumorigenesis in mice and cell proliferation in colorectal cancer cells (Arber et al. 1997, Hulit et al. 2004, Mermelshtein et al. 2005), it is believed that inhibition of cyclin D1 expression is important for prevention of colorectal cancer onset. Recently transcription factor nuclear factor-κB (NF-κB) has gathered great attention as a novel regulator of β-catenin/cyclin D1 pathway (Hwang et al. 2010).

Treatment of colorectal cancer may involve surgery, radiation therapy and chemotherapy. Despite the significant advances in conventional therapeutic approaches, about half of patients ultimately die of colorectal cancer (Ferlay et al. 2010). Therefore, the prevention is important for colorectal cancer. Epidemiological study demonstrated that the diet rich in fruits and vegetables correlates with a reduced risk of cancers including colorectal cancer (Slattery et al. 1997). Numerous classes of compounds, including isothiocyanates (ITCs), present in fruits and vegetables are believed to act as the cancer preventive agents.

## 1.3. Isothiocyanates

Isothiocyanates (ITCs) are compounds with ITC group (-N=C=S) and naturally stored mainly in cruciferous vegetables in a form of glucoside called glucosinolates (GSs). GSs, low molecular weight secondary metabolites in plants, are synthesized from several amino acids, including Met, Trp, and Phe in plants (Grubb and Abel 2006, Halkier and Gershenzon 2006). In nature, there are over 120 GSs that differ in side-chain structures (Fahey et al. 2001). For example, allyl ITC (AITC), sulforaphane (SFN) and benzyl ITC (BITC) are formed from allyl glucosinolate in black mustard (*Brassica nigra L.*), glucoraphanin in broccoli (*Brassica oleracea L. var. italica*) and benzyl glucosinolate (glucotropaeolin) in papaya (*Carica papaya*), respectively (Figure. 1.2). GSs have long been known to be precursors of insect deterring molecules generated in a tissue damage-dependent manner by the activity of β-thioglucoside glucohydrolase called myrosinase. In undamaged plants, GS and myrosinase are physically separated at cellular or subcellular levels (Husebye et al. 2002, Koroleva et al. 2000, Thangstad et al. 2004). When the plant tissue is damaged, GSs are hydrolyzed by myrosinase and then various species of molecules including ITCs are generated. ITCs possess high chemical reactivity and have been shown to have antimicrobial, antioxidative and anticarcinogenic activity (Fahey et al. 2002, Talalay and Fahey 2001, Zhang 2004).




**Allyl ITC (black mustard)** **Sulforaphane (broccoli)** **Benzyl ITC (papaya)**

**Figure 1.2.** Structures and natural sources of the representative ITCs

## 1.4. Cancer prevention by isothiocyanates

Recent meta-analysis study concluded that high intake of ITC-containing foods is inversely related with the risk of colorectal cancer in human (Wu et al. 2013). Thus, ITCs are explored as potential cancer preventive chemicals. ITCs have the ability to inhibit the formation of cancer cells (anti-carcinogenic activity) and suppress the survival and proliferation of existing cancer cells (anti-cancer activity) *in vitro* and *in vivo* in various organs including colon (Zhang et al. 2006b, Nakamura and Miyoshi 2010). The effects of ITCs are through various pathways: inhibition of carcinogen-activating enzymes, induction of carcinogen-detoxifying enzymes, inhibition of cell cycle progression and induction of apoptosis. The detailed molecular mechanisms of these pathway remain to be clarified. Previous studies revealed that BITC, a ITC isolated from papaya as a strong inducer of phase 2 enzyme (Nakamura et al. 2007), exerts its antiproliferative effects by inducing cell cycle arrest and apoptosis through mitogen-activated protein kinases (MAPKs) pathway in human leukemia Jurkat cells (Miyoshi et al. 2004). Also, BITC induces caspase-3-dependent apoptosis through mitochondrial death pathway in rat liver epithelial RL34 cells (Nakamura et al. 2002). Recent study revealed that BITC inhibits cell proliferation in human colorectal cancer cells. Also, it has been shown that that HT-29 (tumor suppresser gene p53 mutant) is more sensitive to antiproliferative effect of BITC compared with HCT-116 (p53 wild type) (Sakai et al. 2012). However, the molecular mechanism of antiproliferation by BITC in human colorectal cancer cells remains unclear. Although constitutive overexpression of β-catenin is observed in almost all colorectal cancer cells, whether BITC regulates the proliferation of colorectal cancer cell by targeting NF-κB, a negative regulator of β-catenin/cyclin D1 pathway, is unknown.

## 1.5. Purposes of the study

To clarify the molecular mechanism of antiproliferation by BITC in human colorectal cancer

cells. The present study was set to solve the following objectives:

- To clarify the mechanisms of antiproliferation by BITC in human colorectal cancer cells using yeast screening system
- To clarify the role of NF-κB in antiproliferation by BITC in human colorectal cancer cells

# CHAPTER 2

## Benzyl isothiocyanate inhibits cell proliferation through down-regulation of Mis12: identification of the molecular mechanism of antiproliferation using yeast screening system

### 2.1. Abstract

BITC is a naturally occurring isothiocyanate derived from cruciferous vegetables. BITC has been reported to inhibit the proliferation of various cancer cells, which is believed to be important for the inhibition of tumorigenesis. However, the detailed mechanism of action remains unclear. In this study, I employed a budding yeast *Saccharomyces cerevisiae* as a model organism for the screening to identify the factors related in antiproliferative effect of BITC. I identified 12 genes including *MTW1* as the overexpression suppressors for antiproliferative effect of BITC by the screening using genome-wide multi-copy plasmid collection for *S. cerevisiae.* Overexpression of kinetochore protein Mtw1 counteracts the antiproliferative effect of BITC in yeast. Consistently, the inhibitory effect of BITC on the proliferation of human colorectal cancer HCT-116 cells was suppressed by overexpression of Mis12, a human orthologue of Mtw1, and enhanced by knockdown of Mis12. I also found that BITC proteasome-dependently reduced Mis12 protein level. Cell cycle analysis showed that Mis12 overexpression completely impaired BITC-induced $G_2$/M arrest and partly attenuated BITC-induced apoptosis. Furthermore, Mis12 knockdown induced $G_2$/M arrest and enhanced BITC-induced apoptosis. These results suggest that BITC suppresses cell proliferation through the post-transcriptional regulation of kinetochore protein Mis12.

## 2.2. Introduction

ITCs mainly derived from cruciferous vegetables are regarded as promising chemopreventive agents. Naturally occurring ITCs such as BITC and SFN have been demonstrated to block the tumor formation initiated by chemicals in experimental animals, and dietary consumption of ITCs has been shown to strongly correlate with reduced risk of various cancers in human (Tse and Eslick 2014, Fowke et al. 2003, Zhao et al. 2001, Hecht 2000). The antiproliferative effect of ITCs through the induction of cell cycle arrest and apoptosis is believed to be important for the inhibition of tumorigenesis. For example, BITC induces cell cycle arrest at $G_2$/M phase through p38 MAPK-dependent pathway in human leukemia Jurkat cells (Miyoshi et al. 2004) and through the modification of checkpoint proteins such as cyclin B1, Cdc2 and Cdc25B and extracellular signaling-regulated kinase-dependent pathway and in human pancreatic cancer Capan-2 cells (Zhang et al. 2006a, Sahu et al. 2009). Also, BITC induces apoptosis through mitochondrial death pathway in rat liver epithelial RL34 cells (Nakamura et al. 2002) and through c-Jun-N-terminal kinase (JNK)-dependent pathway in Jurkat cells (Miyoshi et al. 2004). However, the detailed molecular mechanism involved in antiproliferative effect of ITCs remains to be clarified.

The identification of molecular targets of ITCs conferring their chemopreventive ability contributes to the understanding of molecular basis of ITCs. As electrophiles, ITCs can covalently bind to cellular nucleophilic amino acids. Mi *et al*. found a correlation between the covalent binding to cellular protein and antiproliferative effect of ITCs (Mi et al. 2007). Consistent with this report, the direct binding of ITCs to several proteins such as tubulin (Mi et al. 2008), macrophage migration inhibitory factor (Brown et al. 2009) and DNA topoisomerase II alpha (Lin et al. 2011) are likely to be associated with the chemopreventive ability of ITCs. Recent proteomics analysis by 2-dementional gel electrophoresis was used to screen the binding targets of ITCs and identified 30 proteins as the binding targets of ITCs in vitro (Mi et al. 2011). However, the binding targets of ITCs

identified by proteomics include many proteins abundantly existing in cell such as actin, tubulin and vimentin (Lenstra and Bloemendal 1983) possibly due to low binding specificity of ITCs, which makes it difficult to find the molecular targets specifically contributing to the chemopreventive effect of ITCs. Therefore, the screening system based on the phenotype induced by ITCs is required.

Budding yeast *S. cerevisiae* is a eukaryotic model organism which has been frequently used in scientific study due to the easy and cheap cultivation, short generation time and the ease of the application of molecular techniques for its genetic manipulation (Bjornsti 2002, Forsburg 2001), and widely employed for the identification of drug target and mechanism of action studies (Hartwell et al. 1997). The yeast screening system would be especially useful for the identification of target molecules contributing to antiproliferation by ITCs. That is because ITCs exert antiproliferative effect in yeast as well as human cancer cells (Mochida et al. 1988) and antiproliferative agents often target the components of cell division and DNA repair machineries which are highly conserved between human and yeast. One of the approaches to the screens for small-molecule target identification is a multi-copy suppression screen for genes that give resistance to a drug on overexpression. This screen is based on the principle that cells overexpressing small-molecule target should tolerate higher drug levels (Luesch et al. 2005). In addition, yeast genome has been entirely sequenced and includes about 6000 open reading frames (ORFs) (Goffeau et al. 1996, Winzeler et al. 1999). Based on the genome, Moriya *et al*. previously developed pRS423ks, a genome-wide multi-copy plasmid collection of *S. cerevisiae* (Makanae et al. 2013). Then, I tried to identify the molecular targets for antiproliferative effect of BITC by the introduction of the plasmid collection to wild-type yeast strain and the following examination of individual transformants for resistance to BITC.

Here, to investigate the mechanisms of antiproliferation by BITC in human colorectal cancer cells, I conducted a genome-wide overexpression screen in yeast and applied the results obtained from yeast to human cancer cells. By the screening, 12 genes

including MTW1 encoding an essential component of MIND kinetochore complex were identified as overexpression suppressors of antiproliferation by BITC in yeast. I found that down-regulation of Mis12, a human orthologue of Mtw1, plays an important role in antiproliferation by BITC in human colorectal cancer HCT-116 cells. My data indicate that the proteasome-dependent decrease of Mis12 by BITC induces $G_2$/M arrest and enhances BITC-induced apoptosis, which contributes to the suppression of colorectal cancer cell proliferation.

## 2.3. Materials and Methods

### *2.3.1. Chemicals and antibodies*

BITC was purchased from LKT Laboratories, Inc. (St Paul, MN). Antibodies against Mis12 and actin, and horseradish peroxidase-linked anti-rabbit and anti-mouse IgGs, Mis12-specific siRNA, control siRNA, siRNA transfection medium and siRNA transfection reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase anti-peroxidase soluble complex (PAP) antibody, propidium iodide (PI) and RNase A were purchased from Sigma-Aldrich (St. Louis, MO). Antibody against HA-Tag was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Alexa Fluor-linked anti-rabbit and anti-mouse IgGs, McCoy's 5A, penicillin/streptomycin, trypan blue stain, FastDigest EcoRI, FastDigest HindIII, Pierce® BCA Protein Assay Kit and Trizol® reagent were purchased from Thermo Fisher Scientific (Waltham, MA). Fatal bovine serum (FBS) was purchased from Nichirei Corporation (Tokyo, Japan). Immobilon-P membrane was purchased from Merck Millipore (Billerica, MA). Taq polymerase and PrimeScript™ RT Master Mix (Perfect Real Time) were purchased from Takara Bio Inc. (Shiga, Japan). All other chemicals were purchased from Nakalai Tesque Inc. (Kyoto, Japan).

### *2.3.2. Yeast strain, growth conditions and yeast transformation*

*S. cerevisiae* strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Brachmann et al.

1998) was used for yeast screening system. *S. cerevisiae* MTW1-TAP strain obtained from Funakoshi Co., Ltd. (Tokyo, Japan) was used for Western blot analysis. Yeast cultivation and transformation were performed as previously described (Amberg 2005). Synthetic complete without His (SC-His) media was used for the selection of yeast transformed with plasmid.

### *2.3.3. Generation of gene overexpression library of yeast*

To generate the gene overexpression library of *S. cerevisiae*, BY4741 strain was transformed with pRS423ks and plated on minimal media lacking His. Because BY4741 strain harbors inactivated *his3* genes, it cannot grow on His-lacking medium. However, the genomic DNA of the yeast strain from which the library was constructed has the functional *HIS3* genes. Thus, the presence of a plasmid in the library that contains *HIS3* genes in BY4741 strain will restore growth to a transformant on medium lacking His.

### *2.3.4 Measurement of maximum growth rate*

Yeast growth was measured by monitoring OD595 every 30 min at 30ºC using a microplate reader (Model 680XR, Bio-Rad Laboratories, Hercules, CA). Maximum growth rate was calculated as described previously (Moriya et al. 2006, Moriya et al. 2012).

### *2.3.5 Screening for overexpression suppressors of antiproliferation by BITC in yeast*

The entire scheme of yeast screening system is described as follows. A genome-wide multi-copy plasmid library of *S. cerevisiae* cloned into pRS423ks (Makanae et al. 2013) was divided to 8 groups for convenience and introduced into BY4741 cells. The 8 groups of transformants were cultivated in SC-His medium until mid-log phase, seeded to 96 well-plate and cultured in YPD liquid medium with 100 μM BITC for 3 days at 30ºC. The cell suspension in each well was plated onto SC-His agar plate and incubated for 3 days at

30ºC. Relatively big colonies were picked up and subjected to colony PCR using primers 5'-AAGATCAACACTGCCAAAGCG-3' and 5'-AGCCATATCAATACGGCGAACATC-3' to amplify DNA sequence of gene inserted in plasmid. PCR product was digested with EcoRI and HindIII for 1 h at 37ºC and separated by 1% agarose gel electrophoresis. Plasmid DNA from colony with the same band pattern frequently seen here (more than 2 times) was isolated by Yeast DNA miniprep as previously described (Amberg 2005). *Escherichia coli* XL1-Blue Electroporation Competent Cell (Agilent Technologies, Santa Clara, CA) was transformed with the isolated yeast DNA by electroporation and plated on ampicillin-containing LB plate. Plasmid DNA was isolated by *E. coli* DNA miniprep as previously described (Amberg 2005). DNA sequence of insert in the plasmid was determined by the service of Macrogen japan (Tokyo, Japan) using primers 5'-CGGCCGCTCTAGAACTAGTGGATCC-3' and 5'-ATTGGGTACCGGGCCCCCCCTCG AG-3'. To re-evaluate the suppressor activity of the identified candidate gene, suppressor plasmid was transfected to yeast again and performed spot assay.

### *2.3.6 Spot assay*

Yeast cells were cultured in YPD liquid medium with BITC (100, 500 and 1000 μM) or 0.1 % DMSO for 30 min. The cell suspension was diluted 1, 100 and 500 times with YPD liquid medium and the diluent of cell suspension (10 μl) was spotted onto YPD agar plate. After 2 days cultivation, the number of colonies was counted.

### *2.3.7 Human cell line and cell culture*

Human colorectal cancer cell line HCT-116 was obtained from the American Type Culture collection (ATCC) (Manassas, VA). HCT-116 cells were maintained in McCoy's 5A medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were grown at 37°C in an atmosphere of 95% and 5% $CO_2$.

### *2.3.8 Western blot analysis*

The experiment for yeast cells was performed as previously described (Makanae et al. 2015). For human cells, cells were washed with ice-cold phosphate buffered saline (PBS). Whole cell lysates were prepared in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM DTT, 10 mM NaF, 1 mM $Na_3VO_4$, 1 mM PMSF, 1% SDS and 1% Triton-X100) containing protease inhibitor cocktail and left on ice for 20 min. After sonication, lysates were centrifuged and the supernatant was used as whole cell lysates. Protein concentration in the supernatant was determined by the Bio-Rad protein assay. Equal quantities of protein were subjected to SDS-PAGE and transferred to Immobilon-P membrane. The membranes were blocked and then incubated with the primary antibody overnight at 4°C followed by an appropriate secondary antibody. Secondary antibody binding was visualized using a Chemi-Lumi One Super. Densitometric analysis of the bands was carried out using the Image J Software Program.

### *2.3.9 RNA interference*

Cells were cultured in 6-well plates ($2\times10^5$ cells per well) in normal growth medium without antibiotic and transfected with siRNA. Predesigned siRNA targeting Mis12 or nonspecific control siRNA were transfected to the cells according to the manufacturer's instructions using siRNA transfection medium and siRNA transfection reagent. After the incubation for appropriate time, cells were assayed using the appropriate protocol.

### *2.3.10 Trypan blue dye exclusion assay*

Trypan blue dye exclusion assay was carried out for quantitative analysis of cell viability. Cell suspensions were mixed with 0.4% Trypan blue stain. Viable cells (cells that excluded blue dye) were counted using a hemocytometer (Bürker-Türk) under a light microscope.

### *2.3.11 RT-PCR*

Cells were washed with ice-cold PBS. Total cellular RNA was isolated using Trizol reagent according to the manufacturer's recommendations. RNA was quantified by measuring absorbance at 260 nm. Total RNA (0.5 μg) was reverse transcribed using PrimeScript™ RT Master Mix (Perfect Real Time). PCR amplification was then performed with Taq polymerase and specific primers. Primers used in PCR amplification are as follows: *MIS12*, 5'-CAGGCCGTTGAACAGGTTAT-3' and 5'-TCAGCTGCAAAAACAGTTGC-3' (160 bp); and *β-ACTIN*, 5'-GTCACCCACACTGTGCCCATCTA-3' and 5'-GCAATGCCAGGGTACATGGTGGT-3' (455 bp). The PCR products were then subjected to agarose gel electrophoresis (3%), stained with ethidium bromide and photographed. Densitometric analysis of the bands was carried out using the Image J Software Program.

### *2.3.12 Plasmid and stable cell line*

Plasmid to express Mis12 was kindly provided by Drs. Mitsuhiro Yanagida and Takeshi Hayashi (Okinawa Institute of Science and Technology Graduate University, Okinawa, Japan). Mis12 cDNA fused with HA-tag sequence at N-terminal was produced by PCR and subcloned into pQCXIP (Clontech, Mountain View, CA). HCT-116 cells stably expressing HA-Mis12 were established as previously described (Koreishi et al. 2013).

### *2.3.13 Cell cycle analysis*

After treatment, the culture supernatant was collected, and the cells were detached by trypsin treatment. The detached cells were suspended in the collected culture supernatant. After the centrifugation at 1,500 rpm for 5 min at 4°C, cells were stained with PI solution (20 μg/ml PI, 0.1% Triton X-100, 0.2 μg/ml RNase A in PBS) for 30 min under dark condition at room temperature. Stained cells were analyzed by a Tali™ Image Based Cytometer (Thermo Fisher Scientific, Waltham, MA).

### *2.3.14 Immunofluorescence microscopy*

Cells on coverslips were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. The cells were blocked with 4% bovine serum albumin (BSA) in PBS for 15 min, and then incubated for 15 min with primary antibodies diluted in 4% BSA in PBS. The cells were washed three times with PBS, and incubated for 15 min with secondary antibodies conjugated to Alexa fluorophors. After washing the cells, the coverslips were mounted on microscope slides and imaged using a IX71 fluorescent microscope equipped with a 60× objectives (Olympus, Tokyo, Japan). Image data were processed and quantified using Image J software. The signal intensity of HA-Mis12 was determined by measuring the integrated fluorescence intensity within a 3 × 3 pixel square positioned over a single HA-Mis12 signal and subtracting the background intensity of a 3 × 3 pixel square positioned in a corresponding background. A minimum of 3 cells and 10 kinetochore foci per cell were measured for each condition each experiment (each replicated a minimum of three times).

### *2.3.15 Statistical analysis*

All values are expressed as mean ± SD. Statistical analysis was performed with the Student's *t*-test compared to control or between the indicated groups. A level of $*P<0.05$ was considered significant in all statistical tests.

## 2.4. Results

### *2.4.1. BITC dose-dependently suppresses yeast cell growth*

To determine the concentration for yeast screening, I examined the effect of BITC on yeast cell growth by calculating maximum growth rate in yeast BY4741 strain. As shown in Figure 2.1, maximum growth rate was decreased with increasing concentration of BITC, which suggests that BITC dose-dependently suppresses the proliferation of yeast. Since the treatment of BITC at too low or too high concentration makes it difficult to detect the

recovery of maximum growth rate by overexpressing genes, I decided to use 100 μM BITC for the screening.

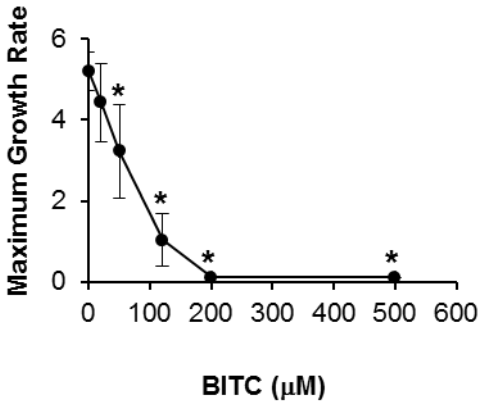



**Figure 2.1.** BITC inhibits cell growth in yeast. Yeast BY4741 cells were incubated in the YPD medium with different concentrations of BITC in 96 well-plate. The time-lapse change of absorbance at 595 nm was measured using microplate reader. Based on the data, maximum growth rate was calculated. The values represent means ± SD of three separate experiments (*$P$< 0.05 compared with 0 μM BITC; Student's $t$-test).

### *2.4.2. Overexpression of 12 genes contributes to BITC resistance in yeast*

The scheme of yeast screening by BITC is shown in Figure 2.2. First is BITC treatment step: transformants of BY4741 cells with 8 groups of pRS423ks, a genome-wide multi-copy plasmid collection of yeast (Makanae et al. 2013) (about 750 ORFs/group), were treated with 100 μM BITC at 30ºC for 3 days in YPD liquid medium. Second is colony formation step: cell suspension from each well was plated and cultivated on SC-His agar plate at 30ºC for 3 days. Third is major genes finding step: inserted DNA in plasmid was amplified by colony PCR, digested by restriction enzyme and separated by gel electrophoresis to find the same inserted DNA. Plasmid DNA of sample with same band patterns frequently seen (more than 2 times) was selected and isolated. Fourth is DNA sequencing step: 15 candidate genes were identified by DNA sequencing using isolated plasmids and primers. To validate the screening, pRS423ks plasmids with the sequences of identified 15 candidate genes were cloned by *E. coli* and introduced to yeast again, and then the transformants were subjected to spot assay. As shown in Figure 2.3, overexpression of 12 genes (*ATP10, BAP2, COX23, MTW1, RCM1, RRT5, RTS3, STP1, UBP7, YDR262W, YGR017W and YML100W-A*) but not overexpression of 3 genes (*SQT1, TAT1 and YOL019W*) significantly weakened antiproliferative effect of BITC compared with control (empty vector), which suggests that the overexpression of these 12 genes contributes to BITC resistance in yeast (Table 2.1).

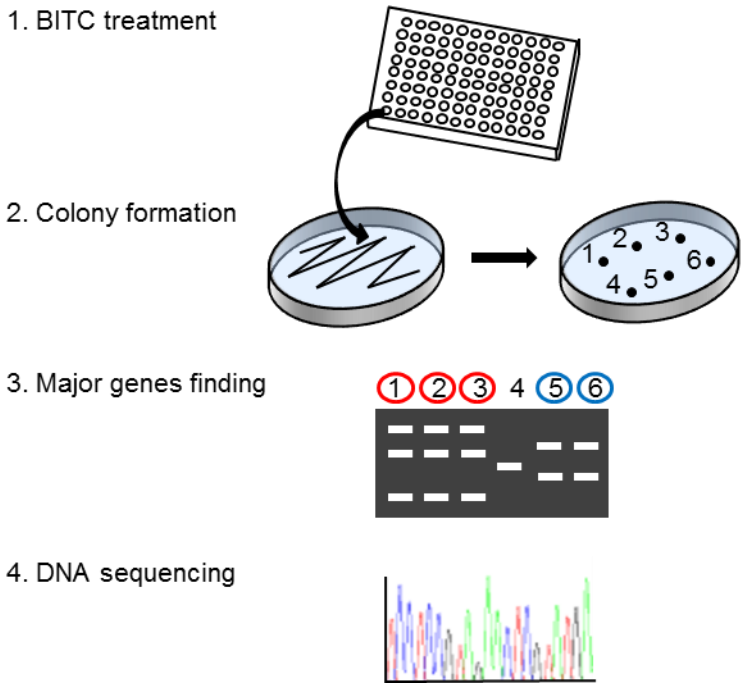



**Figure 2.2.** Scheme of screening for overexpression suppressors of antiproliferation by BITC in yeast. Transformants of BY4741 cells with pRS423ks were treated with 100 μM BITC for 3 days in YPD medium. Yeasts from each well were cultured on SC-His agar plate. Formed colonies were subjected to colony PCR to amplify inserted DNA into plasmid. The PCR products were digested with restriction enzymes and separated by gel electrophoresis. Only the plasmid DNA of samples with major band fragmentation pattern was subjected to DNA sequencing.

vector
ATP10
BAP2
COX23
MTW1
RCM1
RRT5
RTS3
SQT1
STP1
TAT1
UBP7
YDR262W
YGR017W
YML100W-A
YOL019W
BITC concentration (μM)
0
100
500
1000
1 100 500
Dilution ratio (times)


Empty vector
ATP10 OE
BAP2 OE
COX23 OE
MTW1 OE
RCM1 OE
RRT5 OE
RTS3 OE
SQT1 OE
STP1 OE
TAT1 OE
UBP7 OE
YDR262W OE
YGR017W OE
YML100W-A OE
YOL019W OE
BITC (μM)
0
100
500
1000
Cell viability
(% of 0 μM BITC)
0
20
40
60
80
100
120

**Figure 2.3.** Validation of screening by spot assay. BY4741 cells were transformed with pRS423ks with ORF of each 15 gene identified by screening and empty vector (control). Transformants were treated with indicated concentrations of BITC for 30 min in YPD medium. Cell suspensions were spotted onto YPD agar plate. The number of colony was counted to determine the cell viability. The values represent means ± SD of three separate experiments (*$P$< 0.05 compared between overexpression (OE) group of each gene and control at the same concentrations of BITC; Student's $t$-test).

**Table 2.1.** Overexpression suppresser genes of antiproliferation by BITC in yeast

| Gene | Brief description* |
|---|---|
| *ATP10* | Assembly factor for mitochondrial F1F0 ATP synthase |
| *BAP2* | Branched-chain amino acid permease |
| *COX23* | Copper chaperone for cytochrome c oxidase |
| *MTW1* | Essential component of the MIND kinetochore complex |
| *RCM1* | rRNA m5C methyltransferase |
| *RRT5* | Unknown |
| *RTS3* | Putative component of protein phosphatase 2A complex |
| *STP1* | Transcriptional regulator of amino acid transporter genes |
| *UBP7* | Ubiquitin-specific protease |
| *YDR262W* | Unknown |
| *YGR017W* | Unknown |
| *YML100W-A* | Unknown |

*_Saccharomyces_ genome database: http://www.yeastgenome.org

### *2.4.3. Change of Mis12 level affects the sensitivity to BITC in human colorectal cancer HCT-116 cells*

I focused on *MTW1* among 12 identified genes because the function and structure of Mtw1, an orthologue of human Mis12, are highly similar between yeast and human (Goshima et al. 2003). Mis12, an essential component of Mis12 kinetochore complex in human, is required for appropriate chromosome segregation during mitosis (Kline et al. 2006). In human colorectal cancer HCT-116 cells, I examined the effects of overexpression and knockdown of Mis12 on antiproliferation by BITC. The Mis12 protein level in HCT-116 cells stably overexpressing Mis12 (Mis12 OE cells) was about 1.7 times higher than that in vector (Figure 2.4A). Mis12 overexpression didn't affect cell proliferation (Figure 2.4B). As shown in Figure 4C, the antiproliferative effect of BITC in Mis12 OE cells was significantly attenuated compared with vector control, which is consistent with result of yeast in Figure 2.3. The transfection of HCT-116 cells with 30 nM Mis12-specific siRNA depleted the Mis12 protein level by 16% compared with control (Figure 2.4D). Notably, Mis12 knockdown significantly suppressed cell proliferation by itself (Figure 2.4E). As shown in Figure 2.4F, BITC alone dose-dependently suppressed cell proliferation in control siRNA-treated group, whereas Mis12 knockdown enhanced the antiproliferative effect of BITC. These results suggest that expression level of Mis12 in human as well as Mtw1 in yeast affects the antiproliferative effect of BITC.

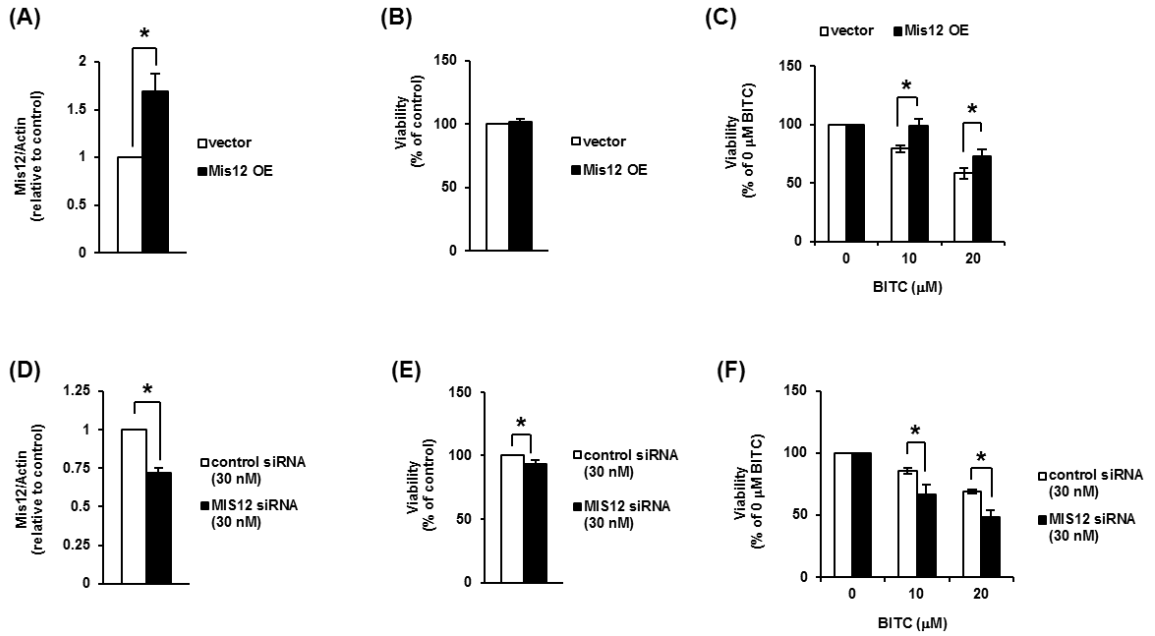



**Figure 2.4.** Change of Mis12 protein level affects the sensitivity of cells to antiproliferative effect of BITC. Mis12 protein level was determined by Western blot analysis. Actin was used as a loading control. Viability was determined by trypan blue dye exclusion assay. (A) Mis12 protein level in HCT-116 cells stably expressing HA-Mis12 (Mis12 OE cells) was determined. pQCXIP (vector) was used for a control. (B) Mis12 OE and control cells ($2\times10^6$) were cultured for 24 h and the viability was determined. (C) Mis12 OE and control cells ($2\times10^6$) were treated with BITC for 24 h and cell viability was determined. (D&E). HCT-116 cells were transfected with control siRNA or Mis12-specific siRNA. After that, Mis12 protein level (D) and viability (E) were determined. (F) HCT-116 cells were transfected with control siRNA or Mis12-specific siRNA. After the treatment with BITC for 24 h, cell viability was determined. The values represent means ± SD of three separate experiments (*$P$< 0.05 compared between the indicated groups; Student's $t$-test).

### *2.4.4. BITC induces proteasome-dependent decrease of Mis12 in HCT-116 cells*

I examined the effect of BITC on the protein expression of Mtw1 in yeast and Mis12 in HCT-116 cells. For the detection of Mtw1 protein expression, I used the yeast strain whose *MTW1* sequence on its genome is replaced with *MTW1-TAP* sequence by homogenous recombination. Western blot analysis showed that BITC significantly decreased Mtw1-TAP level in yeast (Figure 2.5A) and Mis12 level in HCT-116 cells (Figure 2.5B). The decrease of Mis12 by BITC started at 1 h and remained until 24 h (Figure 2.5C). Next, to check whether BITC-decreased Mis12 protein expression is involved in the transcriptional regulation or the proteasome-dependent degradation, I investigated the effect of BITC on mRNA expression of *MIS12* and the effect of proteasome inhibitor MG132 on BITC-decreased Mis12 protein level. As shown in Figure 2.5D, BITC didn't decrease the *MIS12* mRNA expression in HCT-116 cells. On the other hand, the co-incubation with MG132 impaired BITC-decreased Mis12 protein expression (Figure 2.5E). Previous study reported that Mis12 localizes on DNA throughout the cell cycle (Goshima et al. 2003). Immunofluorescence showed that BITC didn't affect the subcellular localization of Mis12 on DNA but significantly decreased the Mis12 fluorescent signal (Figure 2.5F). These data suggest that BITC proteasome-dependently decreased Mis12 level without affecting its gene expression or subcellular localization. In agreement with the report that siRNA-mediated knockdown of Mis12 induces misalignment of chromosomes in HeLa cells (Kline et al. 2006), I found that BITC induced chromosome misalignment in HCT-116 cells (Figure 2.5G). The collapse of microtubules was also observed by the treatment of BITC as previously reported (Mi et al. 2009).

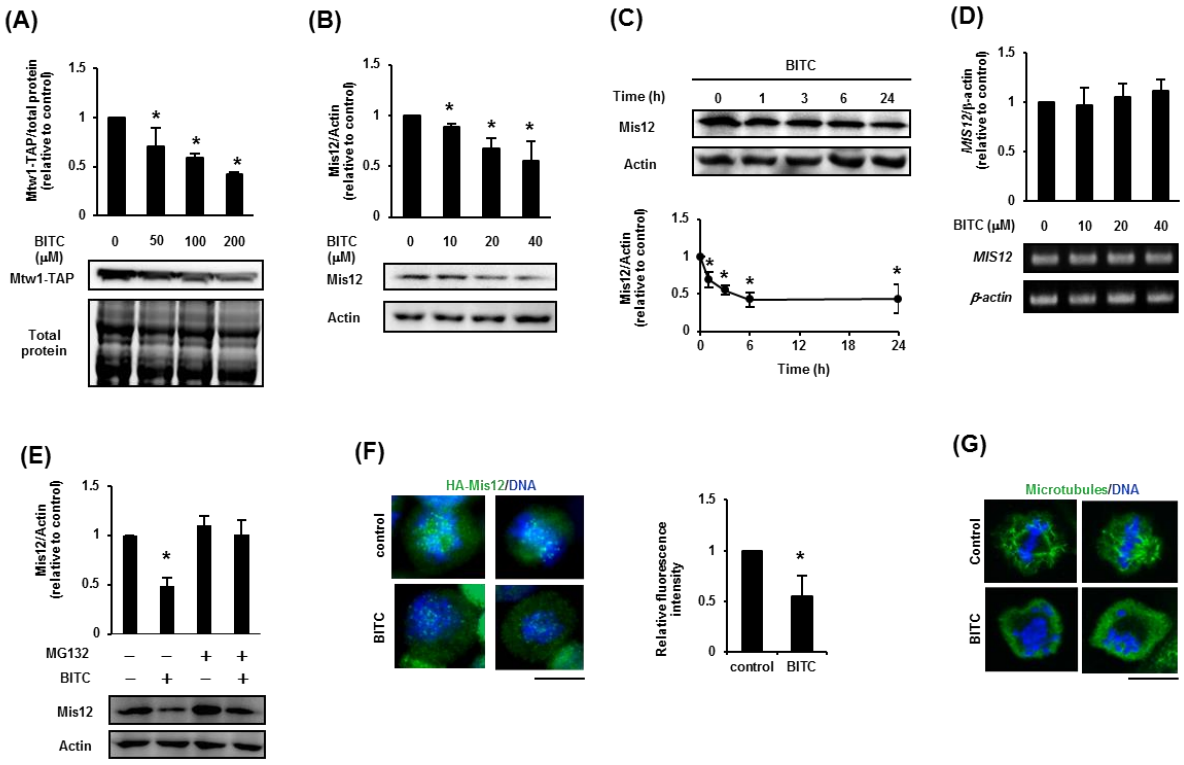



**Figure 2.5.** BITC decreases Mis12 protein expression in a proteasome-dependent manner. Mis12 protein level was determined by Western blot analysis. Total protein staining or actin was used as a loading control. (A) BY4741 cells expressing Mtw1-TAP were treated with BITC for 24 h and Mtw1-TAP protein level was determined. (B) HCT-116 cells were treated with BITC for 24 h and Mis12 protein level was determined. (C) HCT-116 cells were treated with 20 μM BITC for indicated hours and Mis12 protein level was determined. (D) HCT-116 cells were treated with BITC for 24 h and Mis12 mRNA level was determined by RT-PCR. (E) HCT-116 cells were co-treated with 10 μM MG132 and 20 μM BITC for 24 h, and Mis12 protein level was determined. (F) HCT-116 cells stably expressing HA-Mis12 were treated with 20 μM BITC for 1 h. The signals of HA-Mis12 (green) and DNA (blue) was detected by immunofluorescence. The signal intensity of HA-Mis12 at each kinetochore was measured relative to an adjacent background signal. Bar, 10 μm. Fluorescence intensity was quantified using Image J software. (G) HCT-116 cells were treated with 20 μM BITC for 1 h. The signals of microtubules (green) and DNA (blue) were detected by immunofluorescence. Bar, 10 μm. The values represent means ± SD of three

separate experiments (*$P$< 0.05 compared with control; Student's $t$-test).

### *2.4.5. Down-regulation of Mis12 increases $G_2$/M cell population and enhances BITC-induced apoptosis in HCT-116 cells*

BITC-induced chromosome misalignment may cause delay in mitosis. Moreover, BITC has been shown to arrest cell cycle progression at $G_2$/M phase and induce $G_2$/M phase-specific apoptotic cell death in human leukemia Jurkat cells (Miyoshi et al. 2004). Thus, I hypothesized that the decrease of Mis12 enhances BITC-induced apoptosis by increasing $G_2$/M cell population in HCT-116 cells. To test this hypothesis, I examined the effect of overexpression and knockdown of Mis12 on cell cycle regulation by BITC. In vector group, BITC increased $G_2$/M and Sub-$G_1$ cell population (Figure 2.6A and 2.6B). The apoptotic cells give a 'sub-$G_1$' peak in the DNA histogram (Ormerod et al. 1992). On the other hand, Mis12 overexpression cancelled BITC-increased $G_2$/M cell population and partly but significantly attenuated BITC-increased Sub-$G_1$ cell population. Compared with control siRNA-transfected group, Mis12 knockdown alone increased $G_2$/M cell population but not sub-$G_1$ cell population (Figure 2.6C and 2.6D). Moreover, the increase of sub-$G_1$ cell population by BITC was enhanced by knockdown of Mis12. These data suggest that BITC increases $G_2$/M cell population by reducing Mis12 level, which sensitizes the cells to BITC-induced apoptosis.

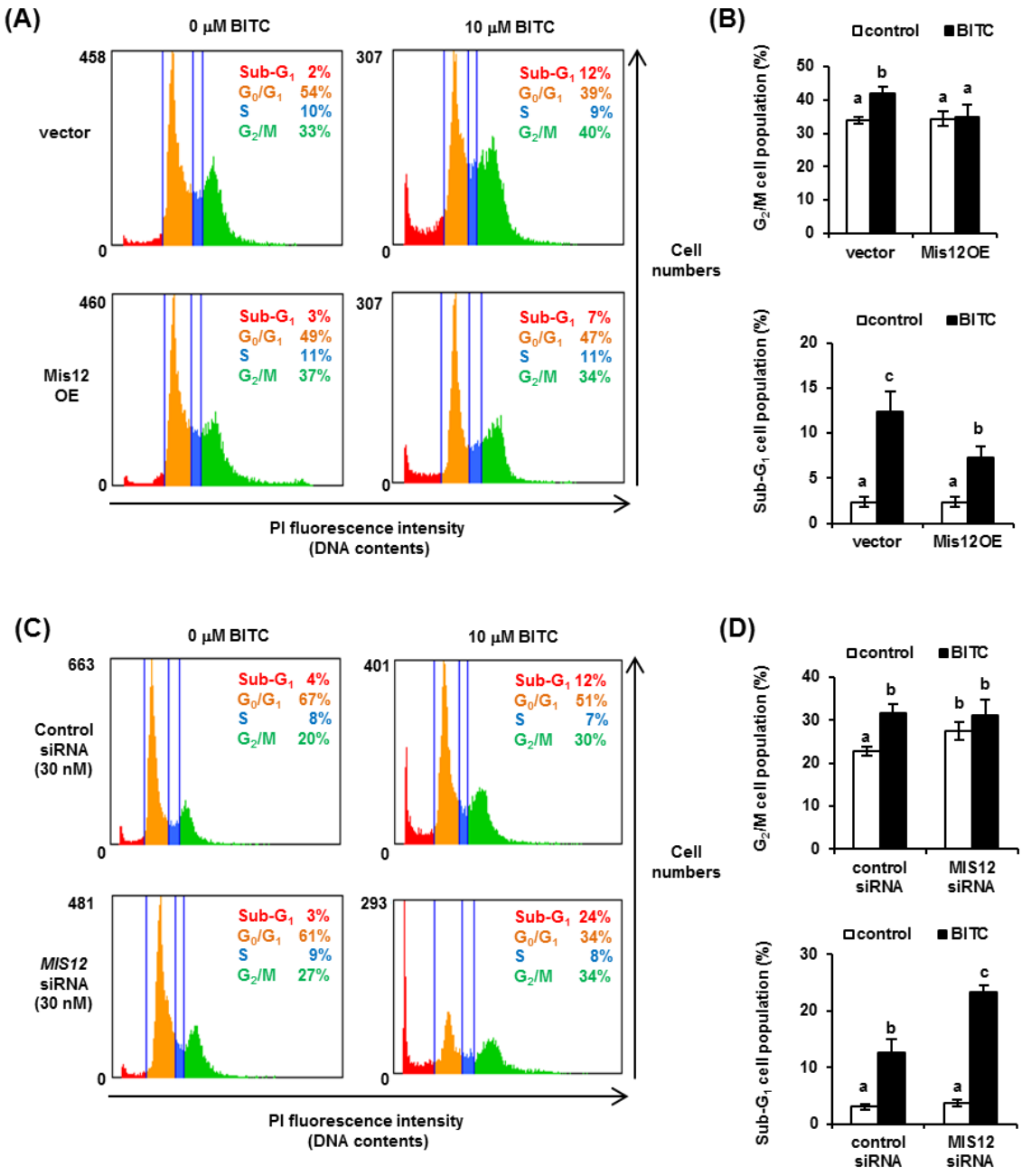



**Figure 2.6.** Change of Mis12 protein level affects the sensitivity of cells to BITC-induced apoptosis. (A&B) Cells in Mis12 OE and vector group were treated with BITC for 24 h and subjected to cell cycle distribution analysis using Tali™ image-based cytometer. Representative histogram is shown in (A). $G_2$/M and Sub-$G_1$ cell populations were statistically analyzed in (B). (C&D) HCT-116 cells were transfected with control siRNA or Mis12-specific siRNA. After the treatment with BITC for 24 h, cell cycle distribution was analyzed as (A). Representative histogram is shown in (C). $G_2$/M and Sub-$G_1$ cell populations were statistically analyzed in (D). Data were analyzed by a one-way analysis

of variance (ANOVA) followed by multiple comparisons among means (Tukey's HSD) using XLSTAT software (Addinsoft, Paris, France). Different letters above the bars indicate significant differences among treatments for each compound ($P < 0.05$).

## 2.5. Discussion

In this study, I identified a kinetochore protein Mtw1, an orthologue of human Mis12, as one of the overexpression suppressors for antiproliferative effect of BITC using yeast screening system and revealed that down-regulation of Mis12 contributes to antiproliferative effect of BITC in human colorectal cancer HCT-116 cells. My data showed that the antiproliferative effect of BITC was inhibited by Mis12 overexpression and enhanced by Mis12 knockdown in HCT-116 cells (Figure 2.4C and 2.4F). Consistent with the report that BITC suppresses cancer cell proliferation through $G_2$/M arrest and apoptosis (Miyoshi et al. 2004, Sahu et al. 2009, Nakamura et al. 2002), the induction of $G_2$/M arrest and apoptosis by BITC was also inhibited by Mis12 overexpression and enhanced by Mis12 knockdown (Figure 2.6). Furthermore, BITC decreased the protein expression of Mis12 in a proteasome-dependent manner (Figure 2.5E), which suggests that the post-transcriptional regulation of Mis12 plays a pivotal role in antiproliferative effect of BITC.

My results also indicate that down-regulation of Mis12 by BITC sensitizes cells to BITC-induced apoptosis by increasing $G_2$/M cell population. Mis12 knockdown induced $G_2$/M arrest but not apoptosis in HCT-116 cells and synergistically enhanced antiproliferative effect of BITC accompanied with acceleration of BITC-induced apoptosis (Figure 2.4F and 2.6). These observations suggest that the accumulation of $G_2$/M cell population is positively related with increased sensitivity to BITC-induced apoptosis. The idea is supported by the report that BITC induces $G_2$/M phase-specific apoptosis in human leukemia Jurkat cells (Miyoshi et al. 2004).

Human Mis12 kinetochore complex, a heterotetramer composed of Mis12, Dsn1, Nnf1 and Nsl1, connects the centromere region on chromosome to mitotic spindle microtubule and is essential for kinetochore assembly and proper chromosome segregation (Figure 2.7) (Kline et al. 2006). Mis12 depletion induces the high frequency of misaligned metaphase chromosomes and lagging chromosomes in anaphase, followed by

the frequent appearance of micronuclei in interphase. These mitotic and interphase abnormalities should cause chromosome missegregation and aneuploidy, an aberrant number of chromosomes (Goshima et al. 2003, Sen 2000). Consistent with this finding, I also observed chromosome misalignment induced by BITC (Figure 2.5G), which shows the possibility that BITC induces aneuploidy through Mis12 down-regulation. Targeting the components involved in mitotic regulation is major strategy of cancer therapy, whereas the induction of delay in mitosis causes not only cell death but also aneuploidy (Sudakin and Yen 2007). Recent study revealed that paclitaxel, a first-line anti-cancer agent inhibiting the depolymerization of spindle microtubules, induces aneuploidy to suppress proliferation at clinically relevant concentration range in breast cancer (Zasadil et al. 2014). These findings suggest that the induction of aneuploidy is effective as a strategy for cancer therapy, while aneuploidy has ever been recognized to be a hallmark of cancer. Some researchers argue that aneuploidy is simply a by-product of tumorigenesis (Zimonjic et al. 2001), others argue that aneuploidy is a driving force of tumorigenesis (Duesberg et al. 1998). This confusion may be due to various factors including the nature and heterogeneity of cancers and the individual genetic alteration in human (Wood et al. 2007). Thus, whether the down-regulation of Mis12 by BITC causes carcinogenesis or not should be examined cautiously in various types of cell line. Although the agents targeting the components of kinetochore complex including Ndc80 and Nuf2 have been shown to have anti-cancer effects (Wu et al. 2008, Kaneko et al. 2009, Hu et al. 2015), to my best knowledge, this is the first report to show the mechanism to suppress cancer cell proliferation through the degradation of Mis12. This finding is being expected to contribute to the development of new anti-cancer drug targeting Mis12 as well as other kinetochore complex.

Here, I originally developed the yeast screening system as a reliable and convenient tool to identify overexpression suppressors for the antiproliferative effect of BITC. As shown in Table 2.1, the identified 12 genes in yeast are involved in ATP synthase (*ATP10*), amino acid transport system (*BAP2* and *STP1*), cytochrome c oxidase Cu

chaperon (*COX23*), kinetochore complex (*MTW1*), deubiquitinating enzymes (DUBs) (*UBP7*), ribosomal RNA (rRNA) methyltransferase (*RCM1*) and protein phosphatase type 2A (PP2A) complex (*RTS3*). Among them, ATP synthase, cytochrome c oxidase Cu chaperon and DUB are reported to be directly bound by phenethyl ITC (PEITC), an aromatic ITC like BITC, *in vitro* by proteomics study using human cancer cell lysate (Mi et al. 2011). The existence of such common hits between my screening and proteomics analysis further verified my yeast screening system and also implies that the down-regulation or dysfunction of these proteins through the direct binding of BITC to them suppresses cancer cell proliferation. The involvement of mitochondrial death pathway in BITC-induced apoptosis has been already reported (Nakamura et al. 2002). The present study showed Mis12 depletion was not essential for the apoptosis induction by BITC. Therefore, the collapse of the mitochondrial electron transport system through direct binding of BITC to ATP synthase or cytochrome c oxidase Cu chaperon might induce apoptosis. Dysregulation of the ubiquitin-proteasome system has been related with the pathogenesis of many human diseases including cancer (Hoeller et al. 2006). DUBs, a large group of proteases that cleave ubiquitin from proteins and other molecules including ubiquitin-specific proteases (USPs) and ubiquitin C-terminal hydrolases (UCHs), are promising targets for cancer treatment through the interference with ubiquitin regulation machinery (Pal et al. 2014). For example, knockdown of USP7 exerts antiproliferative effect through induction of cell cycle arrest in HCT-116 cells (Colland 2006). ITCs-bound proteins are degraded in proteasome dependent manner (Mi et al. 2009). Since my data showed the proteasome-dependent degradation of Mis12 by BITC, DUB dysfunction through BITC binding might positively regulate Mis12 degradation by ubiquitin-proteasome system. In fact, a very recent study revealed that BITC and PEITC inhibits the activity of USP9x and UCH37 possibly through the direct binding of ITCs to Cys residue at catalytic domain of DUBs (Lawson et al. 2015), which supports my hypothesis.

Overexpression suppressors for antiproliferative effect of BITC identified by my screening system contain the components of amino acid transport system (*BAP2* and

*STP1*) which hasn't been shown to have the relationship with ITCs signaling. Bap2 is the branched-amino acid transporter that mediates the uptake of leucine, isoleucine and valine to cells (Grauslund et al. 1995). Stp1 regulates the transcription of amino acid transporters including Bap2 (Nielsen et al. 2001, Ljungdahl 2009). Increased expression of L-type amino acid transporters (LATs), the major transporters that regulate uptake of leucine to cells, is the hallmark of many kinds of human cancers (Wang and Holst 2015). There is now strong evidence that leucine enhances mammalian target of rapamycin complex 1 (mTORC1) signaling which modulates protein synthesis to regulate cell growth, proliferation and cellular differentiation (Laplante and Sabatini 2012), which makes LATs attractive targets for cancer therapeutics. In budding yeast, BITC decreased Bap2 protein expression and the increased leucine level in cells made yeast tolerant to antiproliferative effect of BITC (data not shown). Thus, the inhibition of mTORC1 pathway through the down-regulation of LATs might contribute to antiproliferative effect of BITC in human cells. In relation to that, the attenuation of the antiproliferative effect of BITC by overexpressing rRNA methyltransferase might be attributed to the facilitation of protein synthesis because the methylation of rRNA regulates ribosome maturation (Grummt 1977).

PP2A is a family of serine/threonine protein phosphatases that regulates many cellular processes including cell cycle regulation, apoptosis, protein synthesis, cell morphology and development (Janssens and Goris 2001, Virshup 2000). Down-regulation of PP2A-B55 complex accelerates entry into mitosis and inhibits exit from mitosis. Inactivation of PP2A induces apoptosis in many cancer cells (Kiely and Kiely 2015). Based on these reports, overexpression of PP2A component might weaken the antiproliferative effect of BITC by inhibiting BITC-induced M arrest and apoptosis. However, PP2A positively as well as negatively regulates cell growth possibly due to the activity of different PP2A complexes with distinct substrate specificity and diverse subcellular localization (Schonthal 2001). Thus, the role in each PP2A complexes in antiproliferative effect of BITC should be independently analyzed.

In conclusion, my data suggest that Mis12 degradation by BITC induces $G_2$/M

arrest and enhances BITC-induced apoptosis, resulting in the suppression of proliferation in HCT-116 cells (Figure 2.8). The yeast screening system which I originally developed takes lower cost, shorter time and simpler operations compared with human cells. My screening system seems to be reliable because I found here the pivotal role of Mis12 in antiproliferative effect of BITC and the commonality between my screening results and possible binding targets of BITC reported previously (Mi et al. 2011). So it should be good strategy to clarify the molecular mechanism of chemical-induced antiproliferation by applying the results obtained by yeast screening system to human cells. Here, my results don't provide the evidence that BITC targets Mis12 directly or indirectly. Direct modification of Mis12 with BITC can be detected by MS using BITC-treated recombinant Mis12 or by immunoprecipitation assay using the antibody recognizing Lys adduct of BITC (Nakamura et al. 2010). The inhibition of DUB activity through the direct binding of BITC is the strongest candidate of the indirect pathway. Therefore, whether the inhibitory effect of BITC on DUB activity contributes to proteasome-dependent Mis12 degradation should be examined.

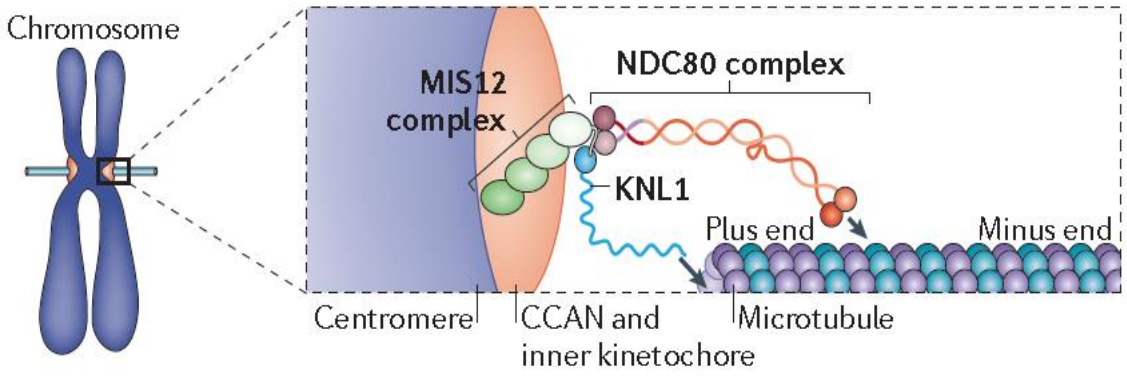



**Figure 2.7.** KMN (kinetochore null protein 1 (KNL1)–MIS12 complex–nuclear division cycle 80 (NDC80) complex) network at the kinetochore. The four-subunit MIS12 complex bridges KNL1 and the NDC80 complex to the constitutive centromere-associated network (CCAN) and centromeric DNA. Arrows indicate microtubule-binding activities of the NDC80 complex and KNL1. Note that picture is from Fig. 2a of Foley and Kapoor (2013).

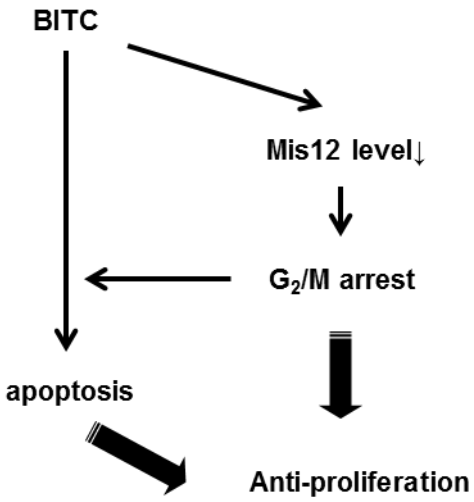



**Figure 2.8.** Proposed model of antiproliferation by BITC. BITC has been shown to induce $G_2$/M arrest and apoptosis in various cancer cells. BITC decreases Mis12 protein level. Reduced-Mis12 level causes $G_2$/M arrest but not apoptosis and enhances the apoptosis induction by BITC. Thus, down-regulation of Mis12 contributes to antiproliferative effect of BITC by inducing $G_2$/M arrest and sensitizing cells to BITC-induced apoptosis.

# CHAPTER 3

## Nuclear factor-kappaB sensitizes to benzyl isothiocyanate-induced antiproliferation in p53-deficient colorectal cancer cells

### 3.1. Abstract

BITC, a dietary isothiocyanate derived from cruciferous vegetables, inhibits the proliferation of colorectal cancer cells, most of which overexpress β-catenin as a result of mutations in the genes for adenomatous polyposis coli or mutations in β-catenin itself. Because NF-κB is a plausible target of BITC signaling in inflammatory cell models, I hypothesized that it is also involved in BITC-inhibited proliferation of colorectal cancer cells. siRNA-mediated knockdown of the NF-κB p65 subunit significantly decreased the BITC sensitivity of human colorectal cancer HT-29 cells with mutated-p53 tumor suppressor protein. Treating HT-29 cells with BITC induced the phosphorylation of IκB kinase, IκB-α and p65, the degradation of IκB-α, the translocation of p65 to the nucleus and the up-regulation of NF-κB transcriptional activity. BITC also decreased β-catenin binding to a positive *cis*-element of the cyclin D1 promoter and thus inhibited β-catenin-dependent cyclin D1 transcription, possibly through a direct interaction between p65 and β-catenin. siRNA-mediated knockdown of p65 confirmed that p65 negatively affects cyclin D1 expression. On the other hand, when human colorectal cancer HCT-116 cells with wild-type p53 were treated with BITC, translocation of p65 to the nucleus was inhibited rather than enhanced. p53 knockout increased the BITC sensitivity of HCT-116 cells in a p65-dependent manner, suggesting that p53 negatively regulates p65-dependent effects. Together, these results identify BITC as a novel type of antiproliferative agent that regulates the NF-κB pathway in p53-deficient colorectal cancer cells.

## 3.2. Introduction

Colorectal cancer is the third most common cancer in men and second in women worldwide (Ferlay et al. 2010). Colorectal cancer develops in a complex, multi-step process involving progressive disruption of the homeostatic mechanisms that control epithelial proliferation, inflammation and differentiation. One of these disruptions is activation of the Wnt/β-catenin signaling pathway, which plays an essential role in the tumorigenesis of colorectal cancer in human (Gilesvan Es and Clevers 2003). In the absence of a Wnt signal, cytosolic β-catenin is normally bound to a β-catenin degradation complex, which causes it to be phosphorylated and then degraded by the ubiquitin-proteasome system (Figure 3.1). The β-catenin degradation complex is composed of APC, axin, casein kinase Iα and glycogen synthase kinase 3. In response to the Wnt signal, β-catenin accumulates in the cytoplasm and is translocated to the nucleus, where it binds to TCF/lymphoid enhancer factor transcription factors and regulates the expression of target genes involved in the proliferation and invasiveness of cancer cells and angiogenesis (Behrens et al. 1996, Molenaar et al. 1996). Loss-of-function mutations in APC or mutations in β-catenin at the phosphorylation site, which are found in almost all human colorectal cancers, lead to stabilization of the β-catenin protein and aberrant activation of Wnt/β-catenin signaling. Accumulation of the overexpressed β-catenin in nucleus activates the expression of its target genes such as cyclin D1, which is required for the $G_1$/S transition in the cell cycle (Tetsu and McCormick 1999). This transition contributes to cell proliferation and tumorigenesis in colorectal cancers (Arber et al. 1997, Hulit et al. 2004, Mermelshtein et al. 2005). Thus, targeting the β-catenin/cyclin D1 pathway is a promising strategy for preventing the onset of colorectal cancer.

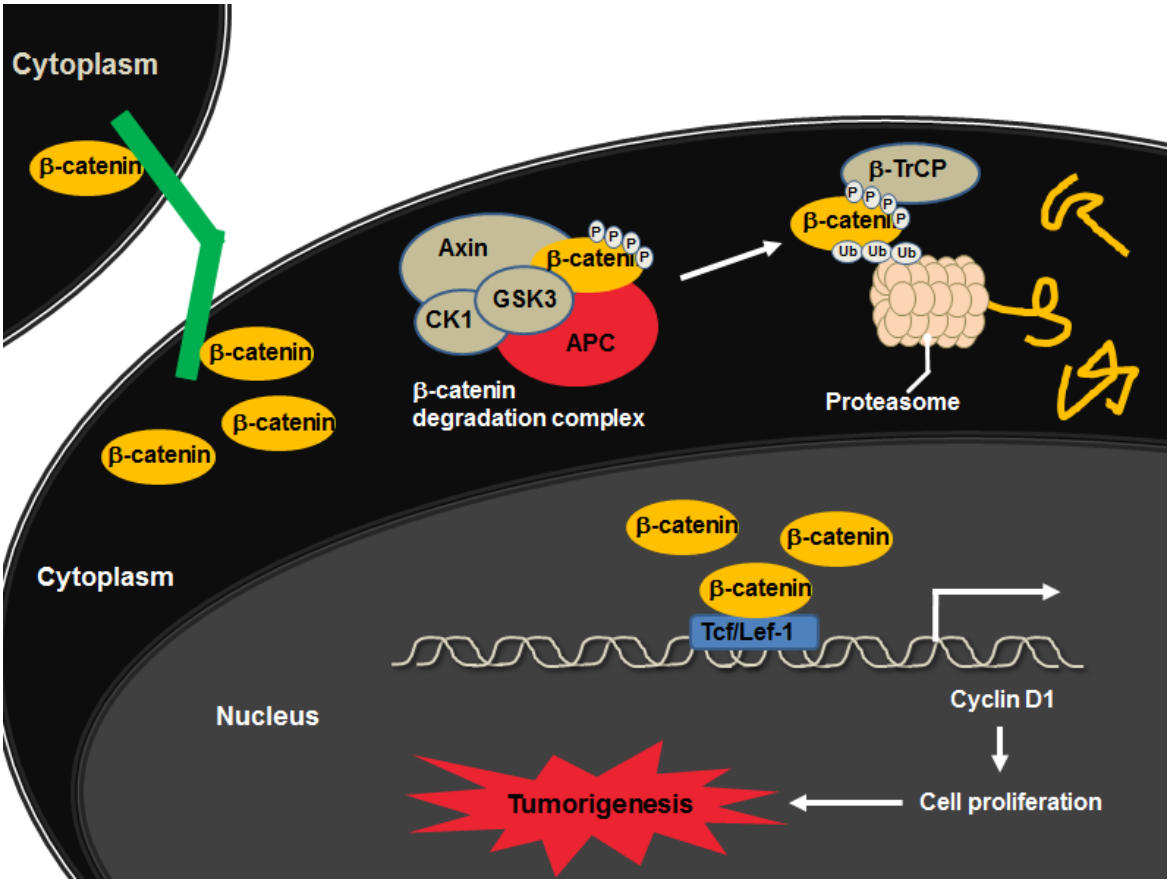



**Figure 3.1.** Molecular mechanism of tumorigenesis in colorectal cancer

In addition to β-catenin, a transcriptional factor nuclear factor-κB (NF-κB) controls the proliferation of epithelial cells by regulating cyclin D1 expression (Hinz et al. 1999, Guttridge et al. 1999). NF-κB regulates a wide variety of cellular genes involved in immunity, inflammation, cell proliferation and apoptosis. The p65 subunit of NF-κB is a potent transcriptional activator and can also directly interact with DNA (Baeuerle and Baltimore 1989). NF-κB in the cytosol of resting cells is bound to and inhibited by IκB-α protein (Figure 3.2). Various stimuli including tumor necrosis factor-α and lipopolysaccharide enhance the phosphorylation of IκB kinase (IKK), and then phosphorylated IKK (phospho-IKK) phosphorylates IκB-α and p65. Phosphorylation of IκB-α at Ser32/36 causes it to disassociate from NF-κB, leading to its degradation by the ubiquitin-proteasome system (Brown et al. 1995). This allows NF-κB to translocate to the nucleus where it regulates the expression of its target genes. Phosphorylation of p65 at

Ser536 is also an indicator of NF-κB activation (Sakurai et al. 2003). Crosstalk between β-catenin and NF-κB plays a significant role in regulating the expression of their target genes (Masui et al. 2002, Deng et al. 2002, Du and Geller 2010). For example, p65 is recently reported to inhibit β-catenin binding on the positive *cis*-element TBE0 site of the cyclin D1 promoter and thus its transcription (Hwang et al. 2010). Therefore, NF-κB has attracted much attention as a novel regulator of the β-catenin/cyclin D1 pathway in colorectal cancer cells.

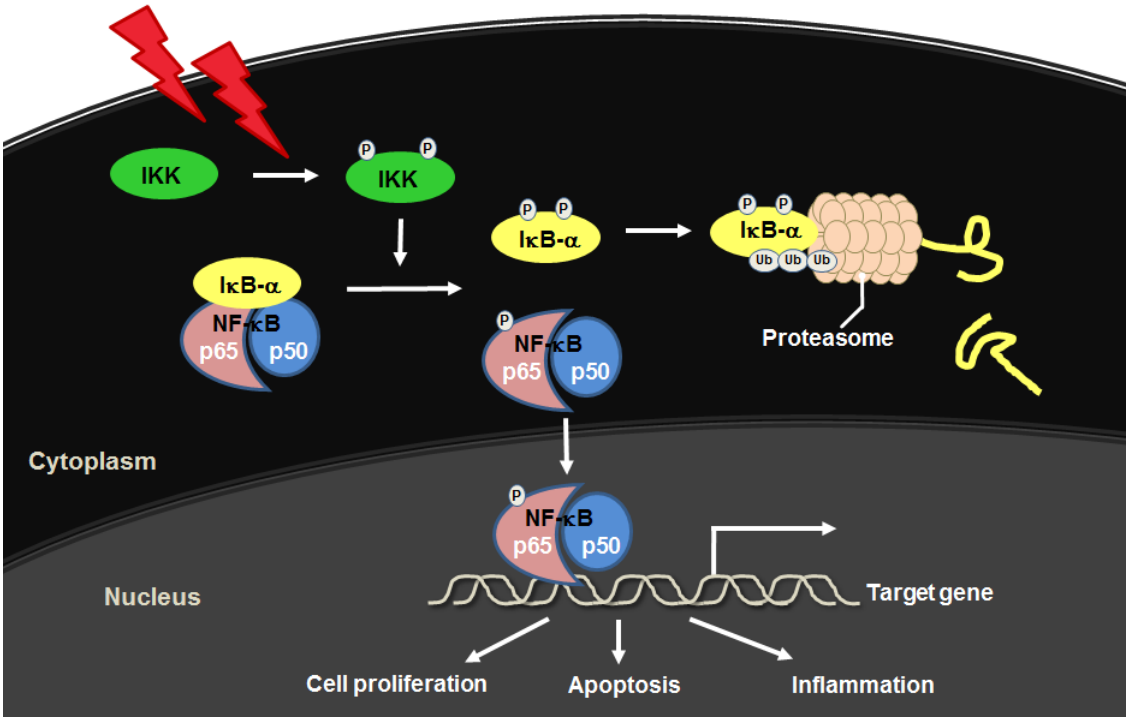



**Figure 3.2.** Molecular mechanism of NF-κB signaling pathway

ITCs, mainly derived from cruciferous vegetables such as broccoli, wasabi (Japanese horseradish) and watercress, are highly effective in chemoprevention and have anti-tumor activities *in vitro* and *in vivo* (Nakamura and Miyoshi 2010). Dietary consumption of ITC-containing foods has been inversely related to the risk of colorectal cancer in human (Wu et al. 2013). I previously demonstrated that BITC, an ingredient in papaya (Nakamura et al. 2007), inhibits cell proliferation by inducing cell cycle arrest and apoptosis through MAPK pathways in human T lymphocytic leukemia Jurkat cells (Miyoshi

et al. 2004). In human colorectal cancer cells, BITC also inhibits cell proliferation by stimulating apoptosis (Bonnesen et al. 2001). By regulating NF-κB, BITC reduces inflammation in RAW264.7 murine macrophages and reduces migration of MDA-MB-231 human breast cancer cells (Murakami et al. 2003, Kim et al. 2012). Although BITC might target NF-κB, it is unclear whether such targeting regulates colorectal cancer cell proliferation.

In the present study, I investigated the role of NF-κB in BITC-inhibited colorectal cancer cell proliferation. Here I demonstrate that NF-κB sensitizes to BITC-induced anti-proliferation in human colorectal cancer HT-29 cells. My results indicate that BITC inhibits β-catenin-dependent cyclin D1 transcription and cell proliferation by causing p65 to accumulate in the nucleus. Furthermore, experiments with two other colorectal cancer cell lines (HCT-116 $p53^{+/+}$ and HCT-116 $p53^{-/-}$) revealed that p53 tumor suppressor protein negatively regulates the effects of p65 in BITC signaling. This study provides the evidence showing that NF-κB represents a novel therapeutic target of the ITC-based prevention of colorectal cancer with p53 mutation and β-catenin overexpression.

## 3.3. Materials and Methods

### *3.3.1 Chemicals and antibodies*

BITC and SFN were purchased from LKT Laboratories, Inc. (St Paul, MN). Antibodies against phosphorylated NF-κB p65 (phospho-NF-κB p65, Ser536), phospho-IKKα/β (Ser176/180), phospho-IκB-α (Ser32/36) and IKK were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Protein A/G PLUS-Agarose Immunoprecipitation reagent, siRNAs for NF-κB p65 and p53, control siRNA, siRNA transfection medium, siRNA transfection reagent, antibodies against NF-κB p65, IκB-α, lamin B1, actin, β-catenin and p53, and horseradish peroxidase-linked anti-rabbit and anti-mouse IgGs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protease inhibitor cocktail was purchased from Sigma-Aldrich (St. Louis, MO). McCoy's 5A, RPMI1640, Leibovitz's L15

and HamF12 medium, penicillin/streptomycin, Trypan blue stain, Lipofectamine® 3000 and Trizol reagent were purchased from Life technologies (Carlsbad, CA). pNF-κB-Luc was purchased from Agilent Technologies, Inc. (Santa Clara, CA). pRL-TK vector and Dual-Luciferase® Reporter Assay System were purchased from Promega (Madison, WI). FBS was purchased from Nichirei Corporation (Tokyo, Japan). Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories (Hercules, CA). Chemi-Lumi One Super was purchased from Nakalai Tesque Inc. (Kyoto, Japan). Immobilon-P membrane was purchased from Merck Millipore (Billerica, MA). M-MLV reverse transcriptase and Taq polymerase were purchased from Takara Bio Inc. (Shiga, Japan). Salmon sperm DNA was purchased from BioDynamics Laboratory (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

### *3.3.2 Human colorectal cancer cell lines*

HT-29 cells and HCT-116 $p53^{+/+}$ cells were obtained from the ATCC (Manassas, VA). HCT-116 $p53^{-/-}$ cells were kindly provided by Dr. Bert Vogelstein (Johns Hopkins Medical Institute, Baltimore, MD). DLD-1 cells and SW480 cells were obtained from Tohoku University Cell Resource Center for Biomedical Research (Miyagi, Japan). LoVo cells were obtained from RIKEN BioResource Center Cell Bank (Ibaraki, Japan). HT-29 cells, HCT-116 $p53^{+/+}$ cells and HCT-116 $p53^{-/-}$ cells were maintained in McCoy's 5A medium. DLD-1 cells, SW480 cells and LoVo cells were maintained in RPMI1640, Leibovitz's L15 and HamF12 medium, respectively. All medium were supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were grown at 37°C in an atmosphere of 95% and 5% $CO_2$. Confluent cells were exposed to the test compounds (resolved in 0.2% DMSO) in the medium containing 0.5% FBS.

### *3.3.3 RNA interference*

Cells were cultured in 6-well plates ($2\times10^5$ cells per well) in normal growth medium without

antibiotic and transfected with siRNA. Predesigned siRNAs targeting p65 and p53 or nonspecific control siRNAs were transfected to the cells according to the manufacturer's instructions using siRNA transfection medium and siRNA transfection reagent. After 72 h incubation, cells were assayed using the appropriate protocol.

### *3.3.4 Trypan blue dye exclusion assay*

Trypan blue dye exclusion assay was performed as described in Chapter 2.

### *3.3.5 Lactate dehydrogenase (LDH) release assay*

LDH release assay was carried out for the quantitative determination of cytotoxicity. Cells were seeded in 96-well plates at a density of $2\times10^4$ cells/well in culture medium. After incubation, cells were treated with BITC for 24 h. LDH activity was measured by using an LDH-Cytotoxicity Test *Wako*, in accordance with the manufacturer's instructions. The absorbance was measured at 560 nm. Total LDH release (100%) was obtained by the treatment of 0.1% Tween20.

### *3.3.6 Western blot analysis*

Western blot analysis was performed as described in Chapter 2. For preparation of nuclear lysates, cells were washed with ice-cold PBS (-) and suspended with buffer-1 (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10 mM NaF, 1 mM $Na_3VO_4$ and 1 mM PMSF) containing protease inhibitor cocktail and left on ice for 15 min. After addition with 0.4% NP-40, the mixture was centrifuged at 500×g for 4 min. Pellets were washed with buffer-1 for three times and suspended with buffer-2 (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10 mM NaF, 1 mM $Na_3VO_4$ and 1 mM PMSF) containing protease inhibitor cocktail. The mixture was kept on ice for 15 min. After centrifugation, the supernatant was used as nuclear lysates.

### *3.3.7 NF-κB luciferase assay*

Cells were cultured in 24-well plates ($5\times10^4$ cells per well) in normal growth medium and co-transfected with 1 μg of pNF-κB-Luc and 1 μg of pRL-TK vector (internal control) for 48 h using Lipofectamine® 3000 and treated with BITC for 6 h. After treatment, cells were lysed and analyzed using a Dual-Luciferase® Reporter Assay System.

### *3.3.8 RT-PCR*

RT-PCR was performed as described in Chapter 2. Primers used in PCR amplification are as follows: cyclin D1, 5'-TCAAGTGTGACCGAGACTGC-3' and 5'-AGAGATGGAAGGGG GAAAGA-3' (355 bp); c-myc, 5'-GTCCTCGGATTCTCTGCTC-3' and 5'-GACTCTGACA CTGTCCAACT-3' (342 bp); COX-2, 5'-TTCAAATGAGATTGTGGA AAAATTGCT-3' and 5'-AGATCATCTCTGCCTGAGTATCTT-3' (305 bp); and interferon-γ (IFN-γ), 5'-GGTCATTC AGATGTAGCGGA-3' and 5'-GCGTTGGACATTCAAGTCAG-3' (270 bp); β-actin, 5'-GTC ACCCACACTGTGCCCATCTA-3' and 5'-GCAATGCCAGGGTACATG GTGGT-3' (455 bp).

### *3.3.9 MTT assay*

MTT assay was carried out for quantitative analysis of cell viability according to the manufacturer's instructions. Cells were pre-incubated for 24 h in 96-well plate and treated with BITC for 24 h at 37°C. MTT solution was added to each well, and the absorbance was measured at 570 nm after 2 h-incubation at 37°C. The obtained values were compared with each of the controls incubated with vehicle only.

### *3.3.10 Immunoprecipitation assay*

Whole cell lysates (800 μg), prepared as described above, were used for immunoprecipitation for 1 h at 4°C with β-catenin antibody, p65 antibody or goat IgG. Following immunoprecipitation, 20 μl Protein A/G PLUS-Agarose Immunoprecipitation

reagents were added and the mixture was incubated for 2 h at 4°C. Beads were washed with lysis buffer three times. Immunoprecipitated proteins were subjected to Western blot analysis.

### *3.3.11 Chromatin immunoprecipitation (ChIP) assay*

Cells were cross-linked with 1% formaldehyde at 37°C. Cells were washed with PBS (-) containing 1 mM PMSF and 1 mM protease inhibitor cocktail, lysed with SDS lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS, 1 mM PMSF and 1 mM protease inhibitor cocktail, pH 8.1) and sheared by sonication. The lysates were centrifuged and the supernatants were collected. Soluble chromatin was precleared with salmon sperm DNA and Protein A/G PLUS-Agarose Immunoprecipitation reagent for 30 min and immunoprecipitated with β-catenin antibody, p65 antibody or goat IgG overnight at 4°C. Control sample was omitted the inclusion of antibody. Immune complexes were collected with Protein A/G PLUS-Agarose beads for 2 h and washed once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl and 150 mM NaCl, pH 8.1), once with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl and 500 mM NaCl, pH 8.1), once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA and 10 mM Tris-HCl, pH 8.1) and twice with TE buffer and extracted with elution buffer (1% SDS, 0.1 M $NaHCO_3$ and 1 mM DTT). The eluent was added with 200 mM NaCl and heated at 65°C for 6 h to reverse the formaldehyde cross-linking. After digestion with RNase and proteinase K, DNA fragments were purified by phenol extraction and ethanol precipitation. Purified DNA fragments were used as a template for PCR amplification The PCR products were then subjected to agarose gel electrophoresis (5%), stained with ethidium bromide and photographed. Densitometric analysis of the bands was carried out using the Image J Software Program. Primers used for ChIP assay are as follows and referred to the previous report (Hwang et al. 2010): TCF-binding element0 (TBE0) site on cyclin D1 promoter (−551 to −433), 5’-GGTCCTCCCCGTCCTTGC-3’ and

5’-TGGCGTTCTTGGAAATGCG-3’, and NF-κB binding site on cyclin D1 promoter (−872 to −782), 5’-GCTTTCCATTCAGAGGTGTGTT-3’ and 5’-GTCAAGGTAGGAAGGCAGCC -3’.

## 3.4. Results

### *3.4.1. NF-κB sensitizes to antiproliferation by BITC in HT-29 cells*

To examine whether NF-κB is involved in BITC-suppressed proliferation of colorectal cancer cell, I examined the effect of knockdown of NF-κB p65 subunit on antiproliferation by BITC in HT-29 cells. The HT-29 cell line is commonly used as a colorectal cancer model because it has loss-of-function mutations in APC (Yang et al. 2006). An immunoblot analysis showed that the transfection of HT-29 cells with p65-specific siRNA depleted the p65 level by 68% compared with control (Figure 3.3a). BITC dose-dependently suppressed the viability of HT-29 cells transfected with control siRNA, whereas siRNA-mediated knockdown of p65 significantly counteracted the antiproliferation induced by 2.5 and 5 μM BITC but not by 10 μM BITC (Figure 3.3b). LDH release which is used as an index of cytotoxicity was drastically increased by the treatment of more than 10 μM BITC (Figure 3.3c). These results indicate that NF-κB plays a significant role in cell growth suppression rather than cell death by the lower concentrations of BITC in human colorectal cancer cells (Figure 3.3d).

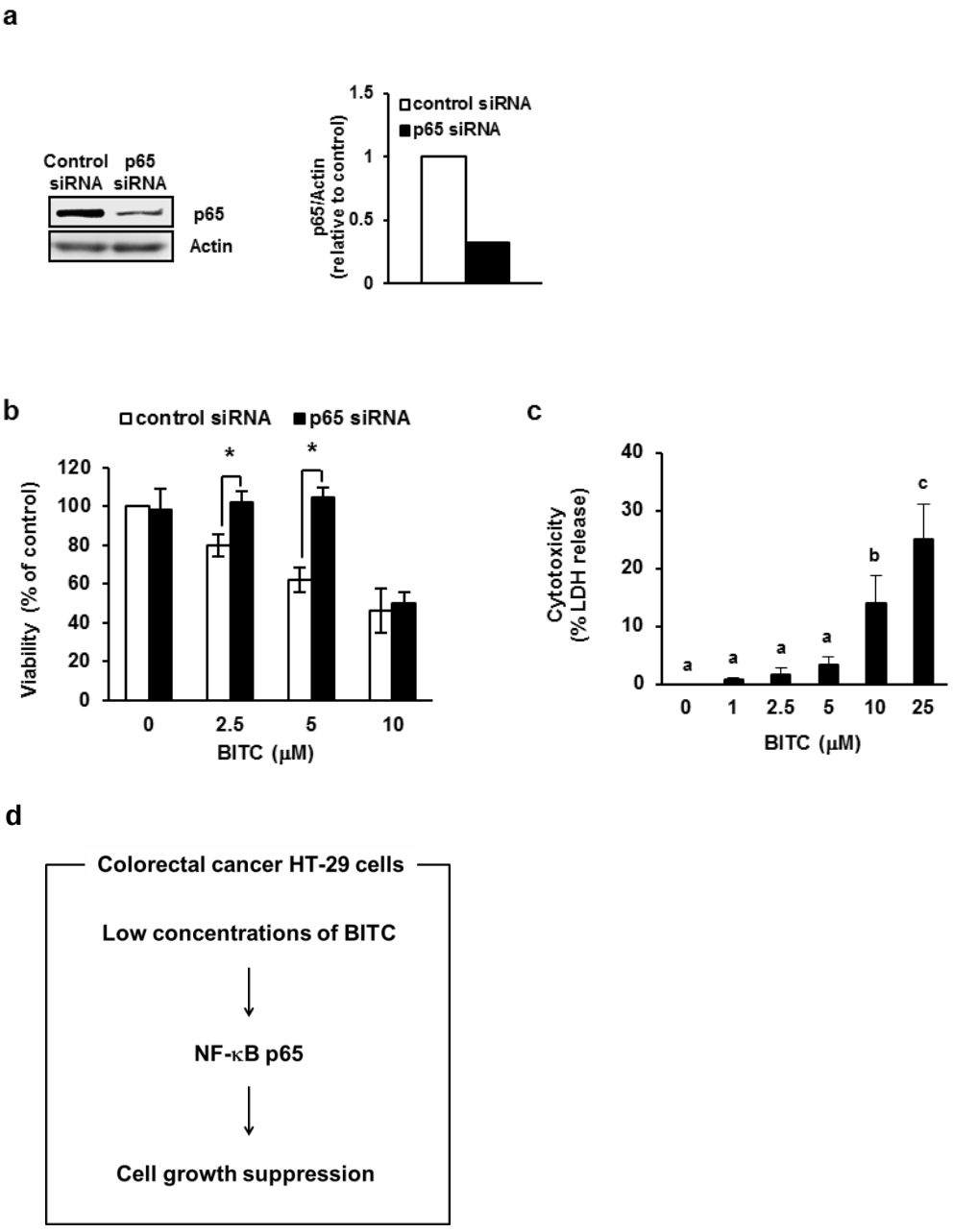



**Figure 3.3.** Effects of p65 knockdown on antiproliferation by BITC in HT-29 cells. **(a)** Knockdown of p65 by RNAi in HT-29 cells. HT-29 cells were transfected with control siRNA or p65 siRNA. Whole cell lysates were prepared and Western blot analysis was performed for p65 and actin. **(b)** Effects of p65 knockdown on antiproliferation by BITC in HT-29 cells. HT-29 cells were transfected with control siRNA or p65 siRNA and exposed to the indicated concentrations of BITC for 24 h, and cell viability was determined by trypan blue dye exclusion assay. The values represent means ± SD of three separate experiments (*$P$< 0.05 compared between the indicated groups; Student's $t$-test). **(c)**

Cytotoxicity of BITC to HT-29 cells. HT-29 cells were exposed to the indicated concentrations of BITC for 24 h, and cytotoxicity was determined by LDH release assay. Data were analyzed by a one-way analysis of variance (ANOVA) followed by multiple comparisons among means (Tukey's HSD) using XLSTAT software (Addinsoft, Paris, France). Different letters above the bars indicate significant differences among treatments for each compound ($P < 0.05$). **(d)** Proposed model based on Figure 3.3. Note that data (a-c) are from Fig. 1 of ABE et al. (2014).

### *3.4.2. BITC activates NF-κB signaling pathway in HT-29 cells*

To confirm whether the antiproliferative effect of BITC which I observed in HT-29 cells was attributed to modulation of the NF-κB signaling pathway, I investigated the effects of BITC on the protein expression of NF-κB signaling molecules using Western blot analysis. As shown in Figure 3.4a, 5 μM BITC increased the phosphorylation of IKK, IκB-α and p65 (at Ser176/180, Ser32/36 and Ser536, respectively), whereas a higher concentration of BITC (25 μM) decreased the phosphorylation of these residues. Consistently, 1-5 μM BITC significantly increased, but 25 μM BITC decreased, the nuclear translocation of p65 (Figure 3.4b). The increases of phospho-IKK and phospho-IκB-α and the decrease of total IκB-α were time dependent (Figure 3.4c). The increase in nuclear p65 was apparent 3 h post treatment of BITC and peaked at 6 h. These results suggest that BITC activates NF-κB signaling pathway at the lower concentrations and suppresses it at the higher concentrations.

To check whether the other ITCs as well as BITC activate NF-κB signaling pathway, I examined the effect of SFN, a naturally occurring aliphatic ITC in broccoli, on the nuclear translocation of p65. In contrast to BITC, SFN at concentrations from 1 to 25 μM, didn't increase the nuclear p65 level (Figure 3.4d), which suggests that not all ITCs are activators of NF-κB signaling in HT-29 cells.

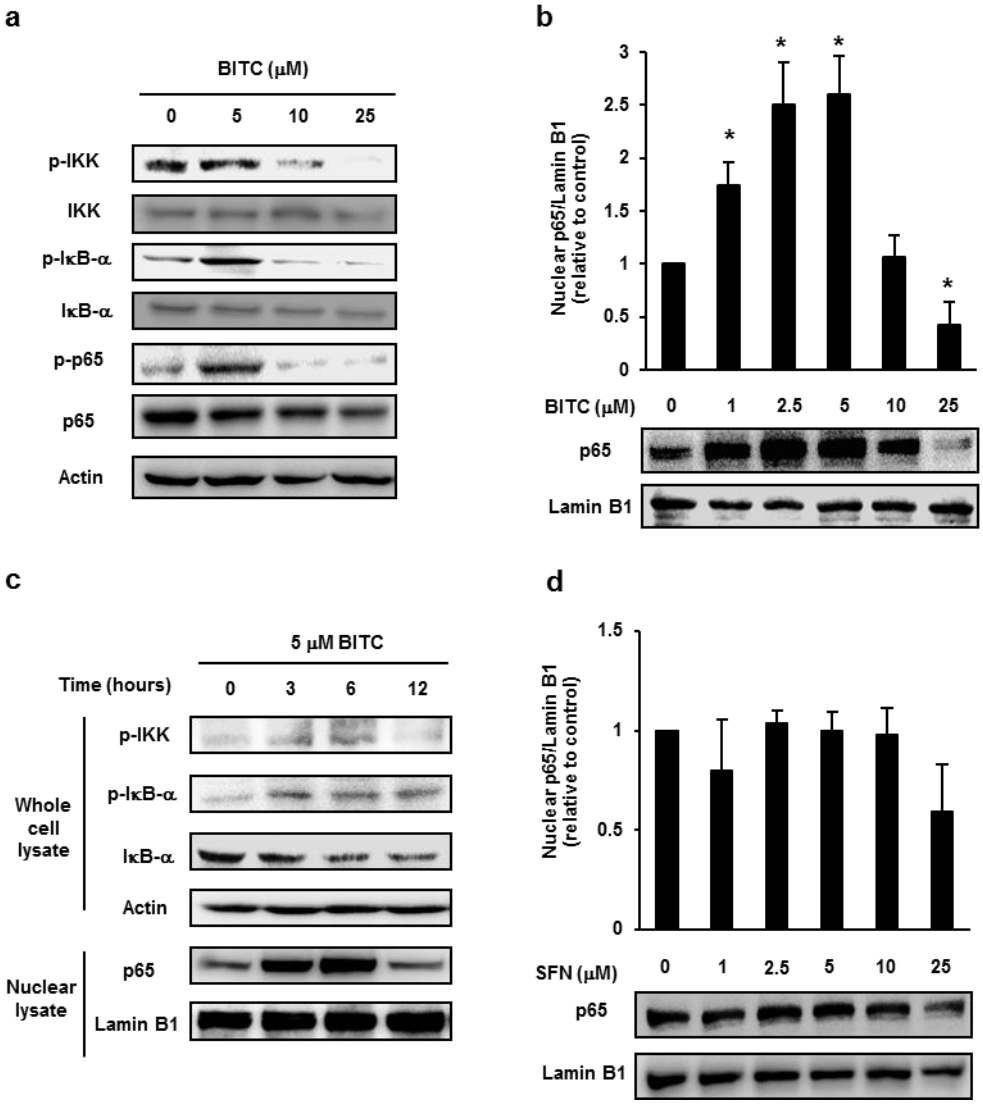



**Figure 3.4.** Effects of BITC and SFN on the expressions of NF-κB signaling proteins in HT-29 cells. HT-29 cells were treated with BITC or SFN and subjected to Western blot analysis. **(a)** Whole cell lysates, phospho-IKK, IKK, phospho-IκB-α, IκB-α, phospho-p65, p65 and actin. Three-hour BITC treatment. **(b)** Nuclear lysates, p65 and lamin B1. Six-hour BITC treatment. **(c)** Whole cell lysates (upper panel) and nuclear lysates (lower panel), p-IKK, p-IκB-α, IκB-α, actin, p65 and lamin B1. **(d)** Nuclear lysates, p65 and lamin B1. Six-hour SFN treatment. The values represent means ± SD of three separate experiments (*$P$< 0.05 compared with control; Student's $t$-test). Note that data are from Fig. 2 of ABE et al. (2014).

### *3.4.3. BITC modulates cyclin D1 transcription by modifying the promoter binding of β-catenin*

Consistent with the effects of BITC on NF-κB signaling pathway (Figure 3.4a and 3.4b), 1-5 μM BITC significantly increased the transcriptional activity of NF-κB, whereas 25 μM BITC decreased it (Figure 3.5a). At both the mRNA and protein levels, cyclin D1 was decreased by the treatment of BITC at 5 μM and 25 μM (Figures 3.5b and 3.5c). Moreover, siRNA-mediated knockdown of p65 canceled the suppression of cyclin D1 gene expression by 5 μM BITC (Figure 3.5d). These results suggest that BITC suppresses cyclin D1 transcription in NF-κB-dependent manner. I also examined the effects of BITC on mRNA levels of the NF-κB-targeted genes other than cyclin D1. The tendency of the dose-dependent effect of BITC on c-myc was quite similar to that on cyclin D1, even though the expression of IFN-γ was enhanced by 2.5 μM BITC (Figure 3.6).

Recently Hwang et al. demonstrated that NF-κB p65 represses β-catenin-activated transcription of cyclin D1 (Hwang et al. 2010). In this model, overexpression of β-catenin increased in β-catenin binding on the TBE0 site and then increased in cyclin D1 mRNA expression. On the other hand, when NF-κB p65 is overexpressed, p65 binding on the NF-κB binding site is increased, β-catenin binding on the TBE0 site is reduced and cyclin D1 mRNA expression is decreased. This model proposes that mRNA expression of cyclin D1 is negatively regulated by NF-κB p65 through the interference of β-catenin binding on the TBE0 site possibly through the protein-protein interaction between p65 and β-catenin. To determine whether BITC regulates cyclin D1 gene expression through the modification of promoter binding pattern of β-catenin as explained above, I performed ChIP assay to evaluate the protein-DNA interaction. The binding of β-catenin and p65 on the NF-κB binding site was enhanced by treatment of 5 μM BITC and the β-catenin binding on the TBE0 site was suppressed by treatment of 5 and 25 μM BITC (Figure 3.5f). In addition, 25 μM BITC decreased the nuclear β-catenin level in HT-29 cells (Figure 3.5g). I next performed immunoprecipitation assay to confirm the protein-protein interaction between

β-catenin and NF-κB. Treatment of 5 μM BITC enhanced the interaction between β-catenin and p65 (Figure 3.5h). These results suggest that the interference of β-catenin binding on the TBE0 site by p65 is involved in the suppression of cyclin D1 gene expression by BITC.

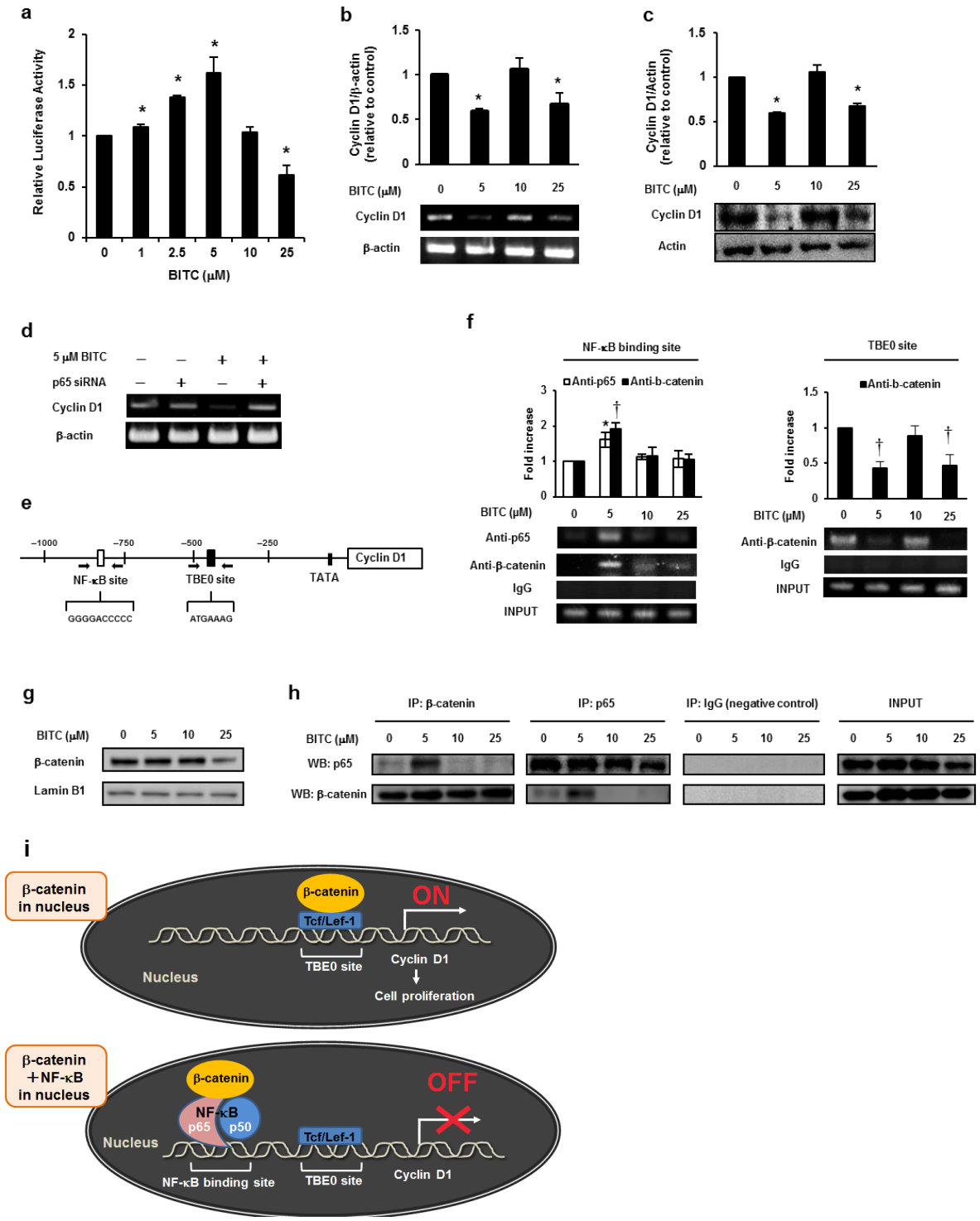



**Figure 3.5.** Involvement of NF-κB in the regulation of β-catenin-dependent cyclin D1 expression by BITC. **(a)** Effects of BITC on transcriptional activity of NF-κB in HT-29 cells. Cells were co-transfected with 1 μg of pNF-κB-Luc and 1 μg of pRL-TK vector for 48 h and treated with BITC for 6 h. After treatment, cells were analyzed using a Dual-Luciferase®

Reporter Assay System. The values represent means ± SD of three separate experiments (*$P$< 0.05 compared with control; Student's $t$-test). **(b)** Effects of BITC on the gene expression of cyclin D1. HT-29 cells were treated with BITC for 6 h. The mRNA levels of cyclin D1 and β-actin were determined by RT-PCR. The values represent means ± SD of three separate experiments (*$P$< 0.05 compared with control; Student's $t$-test). **(c)** Effects of BITC on the protein expression of cyclin D1. HT-29 cells were treated with BITC for 24 h. Whole cell lysates were prepared and Western blot analysis was performed for cyclin D1 and actin. The values represent means ± SD of three separate experiments (*$P$< 0.05 compared with control; Student's $t$-test). **(d)** Effects of p65 knockdown on BITC-decreased cyclin D1 gene expression. HT-29 cells were transfected with control siRNA or p65 siRNA and exposed to 5 μM BITC for 6 h. The mRNA expression of cyclin D1 and β-actin were determined using RT-PCR. **(e)** Schematic representation of the human cyclin D1 promoter. NF-κB binding site, TBE0 binding site and their sequences are shown. Small arrows indicate the positions and directions of the PCR primers used for the ChIP assay. **(f)** Binding of β-catenin or p65 to the NF-κB binding site and the TBE0 site on cyclin D1 promoter. After treatment with BITC for 6 h, HT-29 cells were cross-linked with 1% of formaldehyde for ChIP assay. Chromatin fragments were immunoprecipitated with antibodies against β-catenin, p65 or goat IgG (negative control), and the cyclin D1 promoter regions were amplified by PCR. The values represent means ± SD of three separate experiments (*$P$< 0.05 compared with control of anti-p65 group and †$P$< 0.05 compared with control of anti-β-catenin group; Student's $t$-test). **(g)** Effects of BITC on nuclear β-catenin level in HT-29 cells. HT-29 cells were treated with different concentrations of BITC for 6 h. Nuclear lysates were prepared and Western blot analysis was performed for β-catenin and lamin B1. **(h)** Detection of the interaction between β-catenin and p65 using immunoprecipitation assay. HT-29 cells were treated with BITC for 6 h and immunoprecipitated with antibodies against β-catenin, p65 or goat IgG (negative control). Western blot analysis was performed for p65 and β-catenin. **(i)** Model

that NF-κB p65 represses β-catenin-dependent cyclin D1 transcription proposed by Hwang et al. (2010). Note that data (a-h) are from Fig. 3 of ABE et al. (2014).

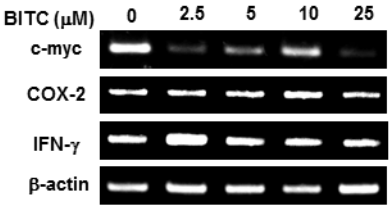



**Figure 3.6.** Effects of BITC on the mRNA level of c-myc, COX-2 and IFN-γ. HT-29 cells were treated with BITC for 6 h. The mRNA levels of c-myc, COX-2, IFN-γ and β-actin were determined by RT-PCR. Note that data are from Supplementary Fig. 1 of ABE et al. (2014).

### *3.4.4. p53 negatively regulates BITC-activated NF-κB signaling pathway*

To examine whether my finding in HT-29 cells that BITC activates NF-κB signaling pathway is also applicable to other colorectal cancer cell lines, I investigated the effect of BITC on the protein level of p65 in nucleus in HCT-116 $p53^{+/+}$ cells that are often used as colorectal cancer model because they overexpress β-catenin (Morin et al. 1997). In contrast to HT-29 cells, HCT-116 $p53^{+/+}$ cells show a decreased nuclear translocation of p65 in the presence of 2.5-25 μM BITC (Figure 3.7a), accompanied by a decreased phosphorylation of IκB-α at Ser32/36 (Figure 3.8). Since the p53 status is one of the differences between these cell lines, I hypothesized that p53 inhibits the BITC-activated NF-κB signaling pathway in HCT-116 $p53^{+/+}$ cells. To test this hypothesis, I examined whether p53 knockdown changes the effect of BITC on the nuclear level of p65 in HCT-116 $p53^{+/+}$ cells. Immunoblot analysis indicated that transfection of p53-specific siRNA depleted the p53 level by 80% and didn't affect the p65 level (Figure 3.7b). As shown in Figure 3.7c, 2.5-10 μM BITC significantly enhanced the nuclear translocation of p65 in p53 siRNA-treated HCT-116 $p53^{+/+}$ cells but not in control siRNA-treated cells. I further confirmed the dependency of BITC-activated NF-κB signaling on p53 status by using a p53 knockout cell line, HCT-116 $p53^{-/-}$. As shown in Figure 3.9a, 1-5 μM BITC significantly enhanced the nuclear translocation of p65 in HCT-116 $p53^{-/-}$ cells. I also found that the basal phosphorylation levels of IκB-α at Ser32/36 and p65 at Ser536 in HCT-116 $p53^{-/-}$ cells were high compared with those in HCT-116 $p53^{+/+}$ cells (Figure 3.9b). The negative regulating role of p53 in the NF-κB translocation was also confirmed in other colorectal cancer cells including LoVo cells (p53-wild type) and, DLD-1 and SW480 cells (p53-mutated) (Figure 3.9c).

To confirm the idea that p53 negatively regulates the antiproliferation by BITC in colorectal cancer cells, I compared the effects of BITC on cyclin D1 expression and cell viability in HCT-116 $p53^{+/+}$ and HCT-116 $p53^{-/-}$ cells. BITC (1-5 μM) significantly decreased cyclin D1 expression in HCT-116 $p53^{-/-}$ cells (Figure 3.9d) and significantly decreased their

viability (Figure 3.9e) relative to HCT-116 $p53^{+/+}$ cells. At BITC concentrations more than 10 μM, the decreases of cyclin D1 expression and cell viability were not significantly different between HCT-116 $p53^{+/+}$ and HCT-116 $p53^{-/-}$ cells. Moreover, 77% knockdown of p65 in HCT-116 $p53^{-/-}$ cells (see Figure 3.9f) significantly counteracted the antiproliferation induced by 1-5 μM BITC but not the antiproliferation induced by 10 μM BITC (Figure 3.9g). These results strongly suggest that p53 negatively regulates NF-κB-dependent antiproliferation by BITC in colorectal cancer cells.

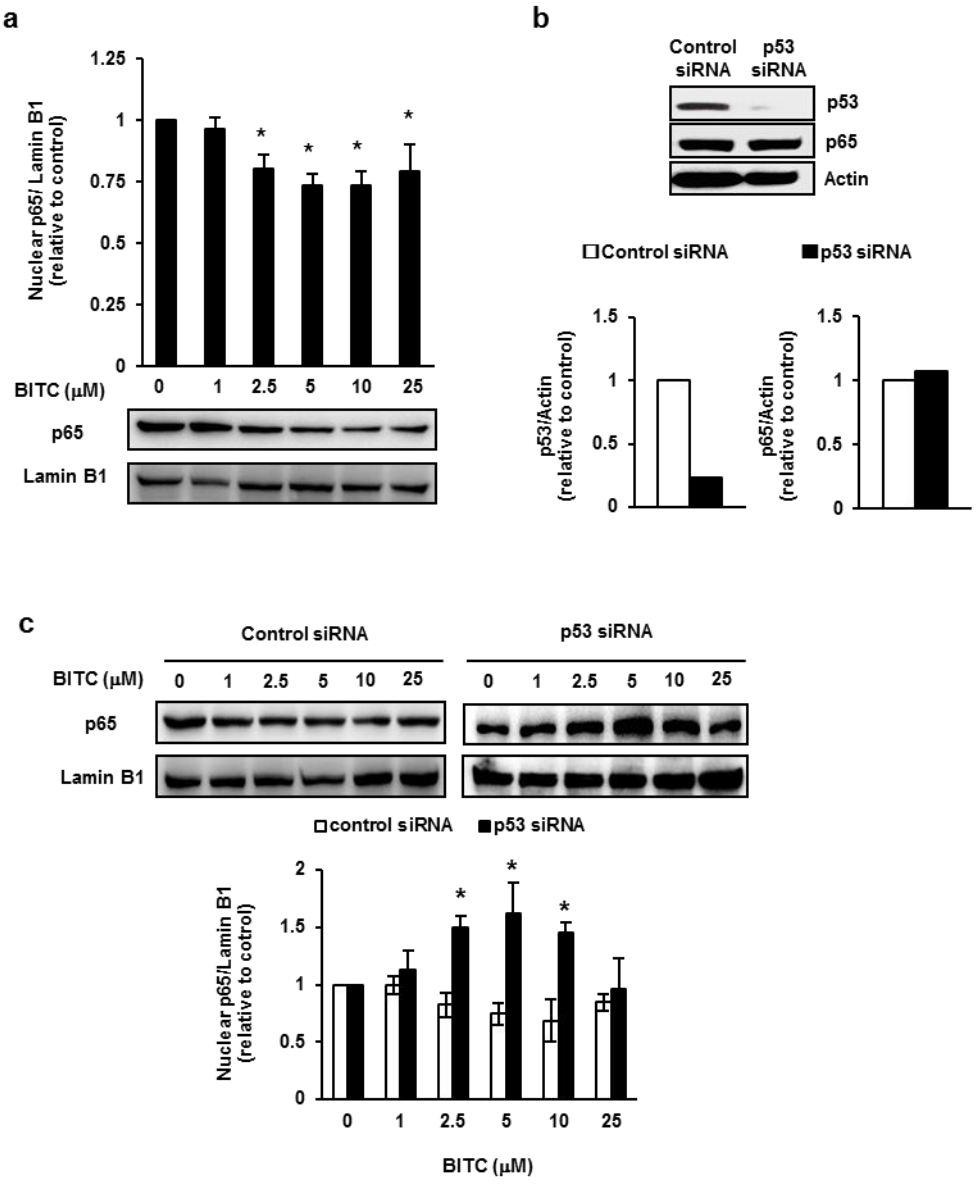



**Figure 3.7.** Effects of p53 knockdown on nuclear translocation of p65 by BITC in HCT-116 $p53^{+/+}$ cells. **(a)** Effects of BITC on nuclear p65 level in HCT-116 $p53^{+/+}$ cells. HCT-116 $p53^{+/+}$ cells were treated with the indicated concentrations of BITC for 6 h. Western blot analysis of nuclear lysates was performed for p65 and lamin B1. The values represent means ± SD of three separate experiments (*$P$< 0.05 compared with control; Student's $t$-test). **(b)** Knockdown of p53 by RNAi. HCT-116 $p53^{+/+}$ cells were transfected with control siRNA or p53 siRNA. Whole cell lysates were prepared and Western blot analysis was performed for p53, p65 and actin. **(c)** Effects of BITC on nuclear p65 level in p53 siRNA-transfected HCT-116 $p53^{+/+}$ cells. HCT-116 $p53^{+/+}$ cells were transfected with

control siRNA or p53 siRNA and treated with the indicated concentrations of BITC for 6 h. Western blot analysis of nuclear lysates was performed for p65 and lamin B1. The values represent means ± SD of three separate experiments (*$P$< 0.05 compared with control of p53 siRNA group; Student's $t$-test). Note that data are from Fig. 4 of ABE et al. (2014).

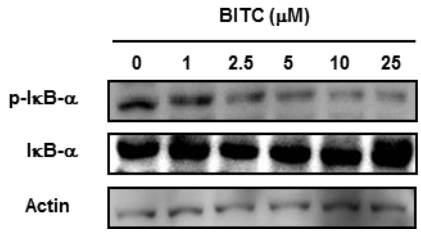



**Figure 3.8.** Effects of BITC on the phosphorylation of IκB-α in HCT-116 $p53^{+/+}$ cells. HCT-116 $p53^{+/+}$ cells were treated with the indicated concentrations of BITC for 3 h. Western blot analysis of whole cell lysates was performed for phospho-IκB-α (Ser32/36), IκB-α and actin. Note that data are from Supplementary Fig. 2 of ABE et al. (2014).

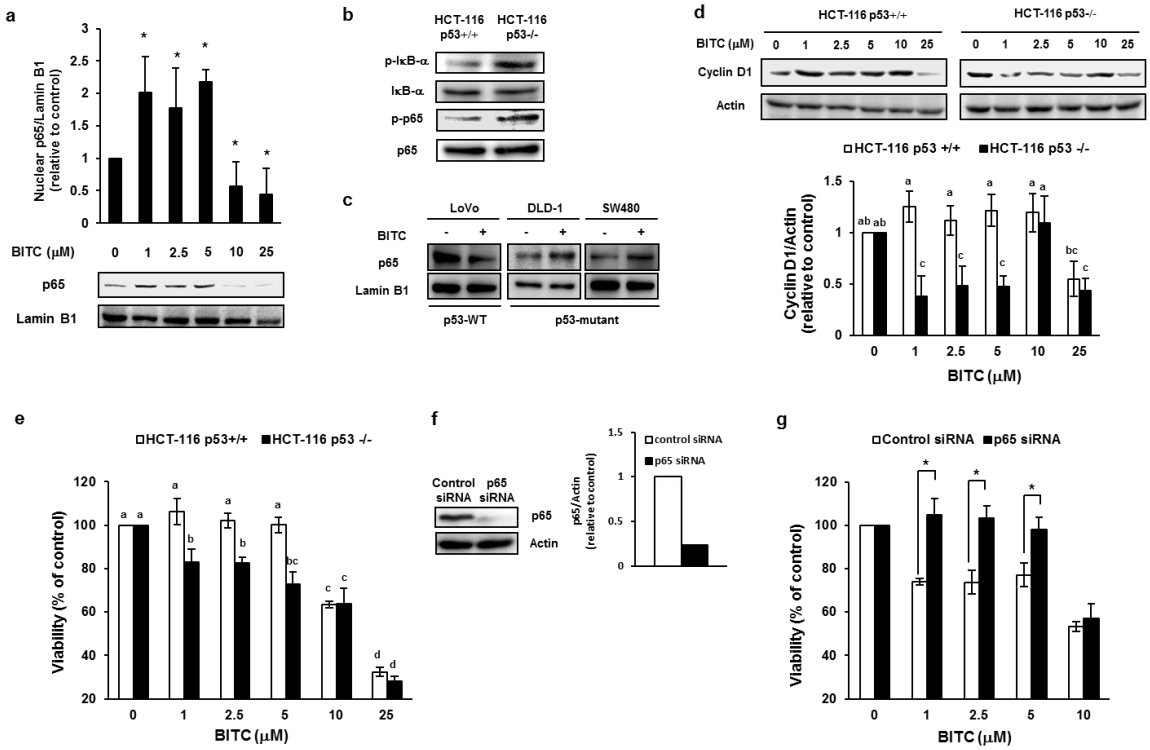



**Figure 3.9.** Effects of p53 deficiency on antiproliferation by BITC in colorectal cancer cells. **(a)** Effects of BITC on nuclear p65 level in HCT-116 $p53^{-/-}$ cells. HCT-116 $p53^{-/-}$ cells were treated with the indicated concentrations of BITC for 6 h. Western blot analysis of nuclear lysates was performed for p65 and lamin B1. The values represent means ± SD of three separate experiments (*$P$< 0.05 compared with control; Student's $t$-test). **(b)** The basal phosphorylation levels of IκB-α at Ser32/36 and p65 at Ser536 in HCT-116 $p53^{+/+}$ and HCT-116 $p53^{-/-}$ cells. Whole cell lysates of HCT-116 $p53^{+/+}$ and HCT-116 $p53^{-/-}$ cells were prepared and western blot analysis was performed for phospho-IκB-α (Ser32/36), IκB-α, phospho-p65 (Ser536) and p65. **(c)** Effects of BITC on nuclear p65 level in other colorectal cancer cells. Indicated colorectal cancer cell lines with different p53 statuses were treated with 2.5 μM BITC for 6 h. Western blot analysis of nuclear lysates was performed for p65 and lamin B1. **(d)** Effects of BITC on the protein expression of cyclin D1 in HCT-116 $p53^{+/+}$ and HCT-116 $p53^{-/-}$ cells. Both cells were treated with BITC for 24 h. Whole cell lysates were prepared and Western blot analysis was performed for cyclin D1 and actin. The

values represent means ± SD of three separate experiments. Data were analyzed by a one-way ANOVA followed by Tukey's HSD using XLSTAT software. Different letters above the bars indicate significant differences among treatments for each compound ($P < 0.05$). **(e)** Effects of BITC on cell viability in HCT-116 $p53^{+/+}$ and HCT-116 $p53^{-/-}$ cells. Both cells were exposed to different concentrations of BITC for 24 h, and cell viability was determined by MTT assay. Data were analyzed by a one-way ANOVA followed by Tukey's HSD using XLSTAT software. Different letters above the bars indicate significant differences among treatments for each compound ($P < 0.05$). **(f)** Knockdown of p65 by RNAi in HCT-116 $p53^{-/-}$ cells. HCT-116 $p53^{-/-}$ cells were transfected with control siRNA or p65 siRNA. Whole cell lysates were prepared and Western blot analysis was performed for p65 and actin. **(g)** Effects of p65 knockdown on antiproliferation by BITC in HCT-116 $p53^{-/-}$ cells. HCT-116 $p53^{-/-}$ cells were transfected with control siRNA or p65 siRNA and exposed to the indicated concentrations of BITC for 24 h, and cell viability was determined by trypan blue dye exclusion assay. The values represent means ± SD of three separate experiments (*$P$< 0.05 compared between the indicated groups; Student's *t*-test). Note that data (a-g) are from Fig. 5 of ABE et al. (2014).

## 3.5. Discussion

The present results demonstrate that NF-κB sensitizes to BITC-induced antiproliferation in p53-deficient human colorectal cancer cells. Knockdown of p65 decreased the BITC sensitivity of HT-29 cells (Figure 3.3b) and HCT-116 $p53^{-/-}$ cells (Figure 3.9g). BITC significantly induced the nuclear translocation of the NF-κB p65 subunit in HT-29 cells (Figure 3.4b), HCT-116 $p53^{-/-}$ cells (Figure 3.9a), DLD-1 cells and SW480 cells (Figure 3.9c). NF-κB-targeting anti-cancer agents include non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin. Aspirin has been reported to activate the NF-κB signaling pathway and induce apoptosis in xenografted HT-29 tumors and in adenomas from $APC^{Min+/-}$ mice (Stark et al. 2007). BITC is a food-derived compound that is known to regulate the proliferation of colorectal cancer cells. To my knowledge, this is the first report that its antiproliferation activity is sensitized by NF-κB. In human lung cancer A549 cells, both BITC and SFN exert their anti-cancer effects through binding to tubulin (Mi et al. 2008). However, in HT-29 cells, the nuclear translocation of p65 was induced by BITC (Figure 3.4b) but not by SFN (Figure 3.4d). BITC has a higher hydrophobicity than SFN due to its aromatic ring (Zhang 2001), which may cause it to target molecules other than tubulin. Although further study is needed to clarify the underlying mechanism, it is noteworthy that regulation of NF-κB depends on the structure of the ITC.

Although numerous studies have established NF-κB as a tumor-promoting transcription factor (Karin 2006), recent studies have shown that NF-κB can also act as a tumor suppressor (Karl et al. 2009, Chien et al. 2011, Jing et al. 2011, Jennewein et al. 2012). Because NF-κB activity enhances sensitivity to cytotoxic chemotherapy in certain cancer cell lines, the two opposing roles of NF-κB may be explained by the other oncogenic status (Klein and Ghosh 2011). For example, since β-catenin is overexpressed in colorectal cancers and functions as an oncogene, NF-κB could act as a tumor suppressor in colorectal cancer cells. Low concentrations of BITC (1-5 μM) increased the

transcriptional activity of NF-κB (Figure 3.5a), whereas they decreased or did not affect the expression of genes whose promoters contain binding sites for β-catenin/TCF and NF-κB: cyclin D1 (Tetsu and McCormick 1999, Hinz et al. 1999, Guttridge et al. 1999) (Figure 3.5b), c-myc (La Rosa et al. 1994, He et al. 1998) and COX-2 (Nunez et al. 2011, Inoue et al. 1994) (Figure 3.6). To the best of my knowledge, the IFN-γ promoter contains the binding sites of NF-κB (Sica et al. 1997) but not that of β-catenin/TCF. The mRNA level of IFN-γ was increased by a low concentration of BITC (2.5 μM; Figure 3.6). This is consistent with previous findings that crosstalk between β-catenin/TCF and NF-κB plays a pivotal role in regulating the expression of their targeted genes (Masui et al. 2002, Deng et al. 2002, Du and Geller 2010). Furthermore, p65-dependent effects of BITC are not observed in p53-positive cancer cells (Figure 3.9), and thus are presumably not observed in normal tissue. Since the possibility that BITC-induced NF-κB activation leads to invasion and metastasis of p53-deficient cancer cells couldn't be excluded in this study, further studies are needed to check the side effects of NF-κB activation.

Inhibiting the β-catenin/cyclin D1 pathway can prevent the onset of colorectal cancer. Little is known about the antiproliferating mechanism of the β-catenin targeting anti-cancer agents other than that it involves reducing the β-catenin level (Verma et al. 2003) and attenuating the transcriptional activity of β-catenin/TCF complex (Dihlmann et al. 2001). p65 was recently shown to repress β-catenin-dependent cyclin D1 transcription, possibly through a protein-protein interaction (Hwang et al. 2010). This model proposes that mRNA expression of cyclin D1 is negatively regulated by p65 through the interference of β-catenin binding on the TBE0 site. Consistent with this repression model of p65, 5 μM BITC enhanced the interaction between β-catenin and p65 (Figure 3.5h), inhibited the β-catenin binding to the TBE0 site on the cyclin D1 promoter (Figure 3.5f) and decreased cyclin D1 expression (Figures 3.5b and 3.5c). On the other hand, at a concentration of 25 μM, BITC decreased cyclin D1 expression independently of p65 (Figures 3.5b and 3.5c) possibly by decreasing the binding of β-catenin to the TBE0 site by down-regulating the

expression of nuclear β-catenin (Figures 3.5f and 3.5g). An epidemiological study suggested that patients with a combination of cyclin D1 A870G polymorphism, low dietary ITC consumption and high activity glutathione *S*-transferase profile have an increased risk of colorectal cancer (Probst-Hensch et al. 2006). In colorectal cancer model Apc(Min/+) mice, PEITC, a dietary ITC with an aromatic ring like BITC, decreased cyclin D1 expression and polyposis formation but not β-catenin total expression (Khor et al. 2008). These findings strongly support the idea that dietary aromatic ITCs elicit chemopreventive effects, possibly by regulating cyclin D1 expression in colorectal cancers.

My data indicate that p53 weakens the inhibition of cyclin D1 expression and cell proliferation by BITC by blocking the activation of NF-κB signaling pathway in colorectal cancer cells. HCT-116 $p53^{+/+}$ cells have a wild-type p53, whereas the p53 of HT-29 cells has a loss-of-function mutation at codon 273 (Rodrigues et al. 1990). The present results show that BITC increases the level of IκB-α and nuclear p65 in (p53-mutated) HT-29 cells (Figures 3.4a and 3.4b), whereas both are decreased in HCT-116 $p53^{+/+}$ cells (Figure 3.7a and Figure 3.8). I also found that in HCT-116 cells with p53 knockout, BITC increased nuclear translocation of p65 (Figure 3.9a) and decreased cyclin D1 expression and cell viability (Figures 3.9d and 3.9e). Tumor suppressor protein p53 plays a key role in cellular responses to DNA damage. p53 inactivates NF-κB signaling by reducing the catalytic activity of IKK through the inhibition of *O*-linked β-*N*-acetyl glucosamine (*O*-GlcNAc) modification (Kawauchi et al. 2009). Thus, p53 might block BITC-activated NF-κB signaling pathway by inhibiting *O*-GlcNAc modification of IKK. This idea is also supported by the observation that the basal phosphorylation levels of IκB-α at Ser32/36 and p65 at Ser536 in HCT-116 $p53^{-/-}$ cells were much higher than those in HCT-116 $p53^{+/+}$ cells (Figure 3.9b). Previous study reported that p53 negatively regulates the cytotoxicity by BITC in normal colorectal CCD-18Co cells (Miyoshi et al. 2007). Consistent with this report, I showed in Figure 3.9e that HCT-116 $p53^{+/+}$ cells are more resistant to antiproliferation by BITC than HCT-116 $p53^{-/-}$ cells. BITC might decrease phospho-IκB-α level and nuclear

p65 level through the decrease of phospho-IKK catalytic activity by increasing p53 level in p53 positive cells. Taken together, these findings suggest that p53 is a negative regulator of antiproliferation of colorectal cancer cells by BITC. Although BITC did neither significantly affect cyclin D1 expression in HCT-116 $p53^{+/+}$ cells nor significantly increase their viability, further studies are needed to check whether BITC increases cancer risk in the other p53-positive cell lines and tissues.

My results also indicate that the antiproliferation effects of BITC depend on its concentration. NF-κB-dependent antiproliferation effects of BITC were only observed at the limited concentrations (1-5 μM). More than 10 μM of BITC drastically increased cell death in HT-29 cells (Figure 3.3c) without NF-κB dependency on antiproliferation effect (Figure 3.3b). In a preliminary study, I also found that 10 and 25 μM of BITC induced apoptotic cell death but not necrosis (unpublished data). These results suggest that low concentrations of BITC mainly induce cell growth inhibition through an NF-κB-dependent pathway only in p53-deficient cells, whereas higher concentrations of BITC induce apoptotic cell death with the decrease of nuclear NF-κB in both p53-positive and -deficient cells. Such dose-specific cellular responses to BITC were also observed in human T lymphocytic leukemia Jurkat cells: BITC induced the activation of JNK at 5 μM but not at more than 25 μM (Chen et al. 1998) and induced apoptosis at low concentration but induced necrosis at high concentration (Nakamura et al. 2002). The electrophilic moiety of BITC is reported to covalently bind to cysteine and lysine residues *in vitro* (Nakamura et al. 2010) and to cysteine residues *in vivo* (Mi et al. 2008). The number of species of cellular proteins that are modified by ITCs increases with increasing their concentration (Mi et al. 2011). Therefore, high doses of BITC may non-specifically target various proteins to inhibit cell proliferation independently of NF-κB signaling.

In conclusion, I have demonstrated that NF-κB sensitizes to BITC-induced antiproliferation in p53-deficient colorectal cancer cells. BITC enhances the interaction between p65 and β-catenin to block the β-catenin binding to the positive *cis*-element of the

cyclin D1 promoter and then inhibits cyclin D1 expression and cell proliferation. Furthermore, p53 blocks BITC-induced nuclear translocation of p65 and downregulates BITC-inhibited cyclin D1 expression and cell proliferation. Taken together, my results suggest that BITC inhibits β-catenin-dependent cyclin D1 transcription and cell proliferation through the nuclear translocation of p65 in human colorectal cancer cells (Figure 3.10). Thus, I identify NF-κB as a novel therapeutic target in p53-deficient colorectal cancer cells, which contributes to our understanding of the complex intracellular signaling cascades that regulate cell proliferation. After consumption of cruciferous vegetables, plasma concentrations of ITC metabolites peak at a few µmol/l (Ye et al. 2002, Platz et al. 2013). However, a recent preclinical evaluation revealed that the concentrations of ITCs in the gastric lumina temporally reached 600-2000 µM, after the consumption of broccoli extract (Cornblatt et al. 2007). Therefore, the concentrations of BITC used in this study are locally achievable at the colorectum, but it is unclear whether ITC metabolites such as ITC-glutathione conjugates have antiproliferative effects. Further studies are needed to determine the *in vivo* effects of ingested ITCs on colorectal cancer cells as well as the primary target to activate the NF-κB pathway by BITC.

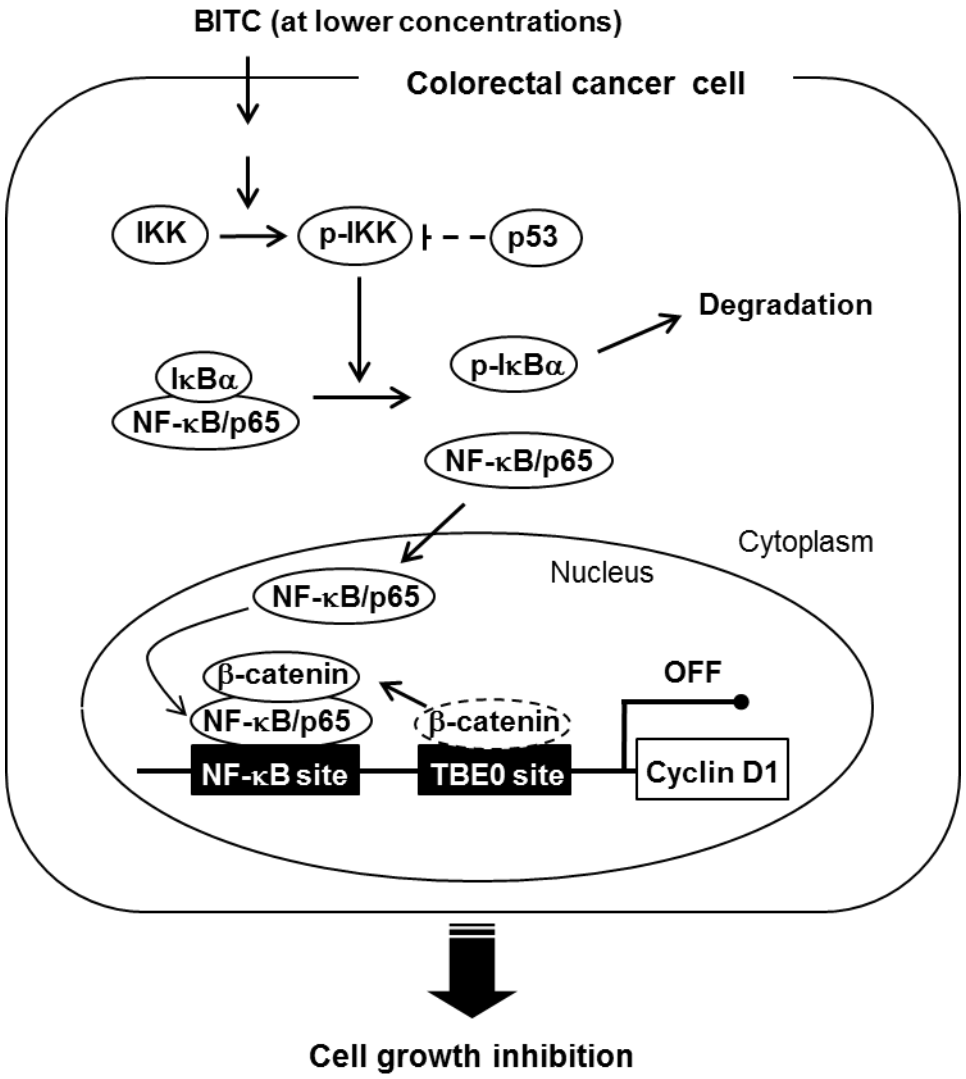



**Figure 3.10.** Proposed mechanism for the antiproliferation by BITC. BITC activates NF-κB signaling pathway. p53 inhibits catalytic activity of IKKβ. Lower concentrations of BITC enhance the interaction between p65 and β-catenin, which blocks the binding of β-catenin to the TBE0 site (a positive *cis*-element of the cyclin D1 promoter). BITC then inhibits cyclin D1 expression and cell growth in colorectal cancer cells. Note that picture is from Fig. 6 of ABE et al. (2014).

# SUMMARY

Colorectal cancer is the third most common cancer in men and second in women worldwide. ITCs are electrophilic compounds mainly derived from cruciferous vegetables and dietary consumption of ITC-containing foods has been inversely related to the risk of colorectal cancer in human. Therefore, ITCs are explored as promising cancer preventive phytochemicals. BITC is a main ITC compound derived from garden cress and papaya. BITC suppresses cell proliferation in human colorectal cancer cells. However, the underlying molecular mechanism is not fully understood. The present study is set to further elucidate the molecular mechanism of antiproliferation by BITC in human colorectal cancer cells. Identification of molecular targets of ITC greatly contributes to understanding the molecular mechanism of ITC effects. Proteomics was not an effective way for identification of the molecular targets of ITCs because the protein thiol conjugates of ITC which is mainly formed in our bodies are unstable. Then, I employed budding yeast as a model organism to do the screening based on phenotype induced by ITCs. In Chapter 2, I tried to clarify the mechanism of antiproliferation by BITC in human colorectal cancer cells using yeast screening system. I found that overexpression of 12 genes, including *MTW1* which encodes an essential component of the MIND kinetochore complex, contributes BITC resistance in yeast. Moreover, my results suggest that more than 10 μM BITC (higher concentrations of BITC) proteasome-dependently decreased Mis12, a human orthologue of Mtw1, to suppress the proliferation in human colorectal cancer cells by the induction of cell cycle arrest at $G_2$/M phase accompanied with the sensitization of cells to BITC-induced apoptosis.

Next I especially focused on colorectal cancer cell-specific property to further clarify the mechanisms of antiproliferation by BITC in human colorectal cancer cells in Chapter 3. In almost all the colorectal cancer cell, β-catenin is accumulated in nucleus by gene mutations and it activates the transcription of cyclin D1, a positive regulator of cell cycle

progression at $G_1$/S phase. Thus, the up-regulation of this β-catenin/cyclin D1 pathway which contributes to cell proliferation finally leads to tumorigenesis in colorectal cancer. Recently NF-κB has been reported to inhibit this pathway, so I focused on NF-κB. Previous study reported that BITC reduces inflammation by regulating NF-κB in murine macrophage, which indicates that NF-κB signaling is a possible target of BITC. Then I investigated the role of NF-κB in antiproliferation by BITC in human colorectal cancer cells in Chapter 3. My results reveal that less than 5 μM BITC (lower concentrations of BITC) suppressed colorectal cancer cell proliferation in a NF-κB-dependent manner without inducing cell death. Also, my data suggest that the lower concentrations of BITC suppress the proliferation through the repression of β-catenin/cyclin D1 pathway by activating NF-κB signaling in p53-deficient human colorectal cancer cells.

In conclusion, here I identified Mis12 and NF-κB as the novel molecular targets of the antiproliferation by BITC in human colorectal cancer cells. Furthermore, I clarified the dose-dependent molecular mechanisms of antiproliferation by BITC in human colorectal cancer cells as follows:

- At the higher concentrations of BITC: the induction of $G_2$/M arrest and the sensitization to BITC-induced apoptosis by decreasing Mis12 level.
- At the lower concentrations of BITC: the repression of β-catenin-dependent cyclin D1 transcription by activating NF-κB pathway in p53-deficient cells.

These findings are expected to contribute to the development of new drugs for colorectal cancer treatment and further understanding of the safety of food-derived compounds. Taking no phytochemicals like BITC doesn't cause the deficiency diseases, whereas the intake of some food-derived xenobiotics can enhance the resistance to the phenomena disturbing health maintenance. The cellular response to BITC changes complexly up to the concentration range and excess amount of BITC induces necrosis, one of the cause of carcinogenesis. Thus, the guideline for the intake of phytochemicals need to be enriched.

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# LIST OF PUBLICATIONS


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