

Figure 3. Schematic representation of in vitro selection of Q15-binding protein by mRNA display. (A) A cDNA library derived from SW480 cells was transcribed, ligated with PEG-Puro spacer (1) and translated in vitro (2) to form a protein-mRNA conjugates library. The library was incubated with biotinylated Q15-immobilized beads (3) and unbound molecules were washed away. The bound molecules were eluted and their mRNA portion was amplified by RT-PCR (4). The resulted DNA was used for the next round of selection and analyzed by cloning and sequencing (5). (B) The chemical structure of biotinylated Q15.
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entirely *in vitro* process, both toxic and nontoxic interacting proteins can be characterized. This is a distinct advantage of this method, because toxic proteins are not amenable to characteriza-

tion by assays that require *in vivo* steps, such as Y2H [26] and TAP-MS [27].

Application of this approach here identified a HEAT-repeat domain of hCAP-G2, which is one of the subunits of condensin II

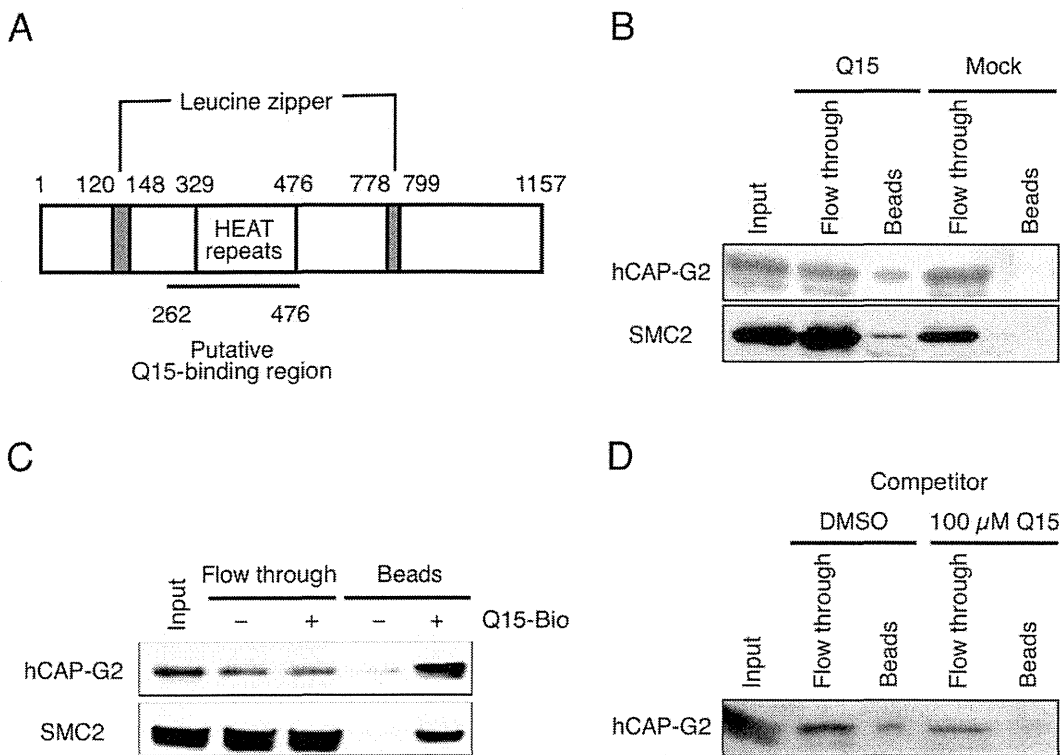


Figure 4. hCAP-G2 protein interacts directly with Q15. (A) Domain structure of hCAP-G2. The region (262–476) selected by mRNA display is underlined. (B and C) Lysates from KMS34 (B) and SW480 (C) cells were incubated with Q15-immobilized beads for 2 h. Each fraction was subjected to 10% SDS-PAGE. Protein bands were detected by immunoblotting with antibodies against hCAP-G2 and SMC2. (D) Cell lysates from KMS34 cells were incubated with Q15-immobilized beads for 2 h in the absence or presence of 100 μ M free Q15. Each fraction was subjected to 10% SDS-PAGE and protein bands were detected by immunoblotting with antibodies against hCAP-G2.
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complex, as a Q15 binder that might be related to inhibition of cell proliferation. The condensin II complex is composed of SMC2 and SMC4 ATPases and three auxiliary subunits (hCAP-G2, hCAP-D3 and hCAP-H2) [9,10,14,28]. In binding assay using Q15-immobilized beads, SMC2 was co-precipitated with hCAP-G2, suggesting that Q15 interacts not with monomeric hCAP-G2, but with the holo-complex of condensin II. In condensin I, the HEAT-repeat domains of hCAP-G and hCAP-D2 are suggested to play important roles in their interaction with hCAP-H [29]. Therefore, this might also be the case for the interaction between hCAP-G2 and hCAP-H2. In fact, our data suggested that Q15 binds to HEAT repeats of hCAP-G2, which are thought to facilitate protein-protein interaction [11]. It is therefore possible that Q15 inhibits interactions between hCAP-G2 and hCAP-H2 or between hCAP-G2 and any other chromosomal proteins.

Previous studies showed that siRNA-mediated depletion of hCAP-G2 from HeLa cells results in severe defects in metaphase chromosome morphology [9], chromosome alignment and anaphase chromosome segregation [30]. Other studies also suggested that condensins regulate mitotic chromosome segregation in many different organisms [10,12,13]. Here, we have observed distortion of metaphase chromosome morphology, as well as incomplete alignment of metaphase chromosomes, in Q15-treated cells. These features are similar, if not identical, to the phenotypes observed in hCAP-G2-depleted cells, implying that binding of Q15 to hCAP-G2 inhibits condensin II function. Electron microscopic observations further indicated that abnormal cell division induced by Q15 resulted in formation of multinucleated cells.

In conclusion, we have identified a novel anilinoquinazoline derivative Q15 as a growth inhibitor of several intractable cancer cell lines. Using our mRNA display technology, we identified hCAP-G2, a subunit of condensin II complex, which is regarded as a key player in mitotic chromosome condensation, as a Q15 binding partner. We further showed that Q15 induces apoptosis and abnormal chromosome segregation during cell division. Taken together, our results indicate that Q15 may induce mitotic failure in tumor cells by interfering with condensin II function, and this leads to apoptosis. Therefore, inhibition of condensin function could be a novel strategy for the development of antitumor drugs for a range of tumors that are unresponsive to existing drugs.

Materials and Methods

Cell Lines

Multiple myeloma cell lines (KMM1, KMS11, KMS26, KMS27, KMS34 and RPMI8226) were generous gifts from Prof. T. Otsuki (Kawasaki Medical College, Kurashiki, Japan) [31] and were maintained in RPMI1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin. HeLa cells (RIKEN Cell Bank, 2002) and SW480 cells (ATCC, 2005) were maintained in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin. The sources of other human cancer cell lines are described elsewhere [32]. All cell lines were expanded immediately upon receipt, and multiple vials of low-passage cells were maintained in liquid N₂. No vial of cells was cultured for more than 3 months. The cells were tested routinely for Mycoplasma

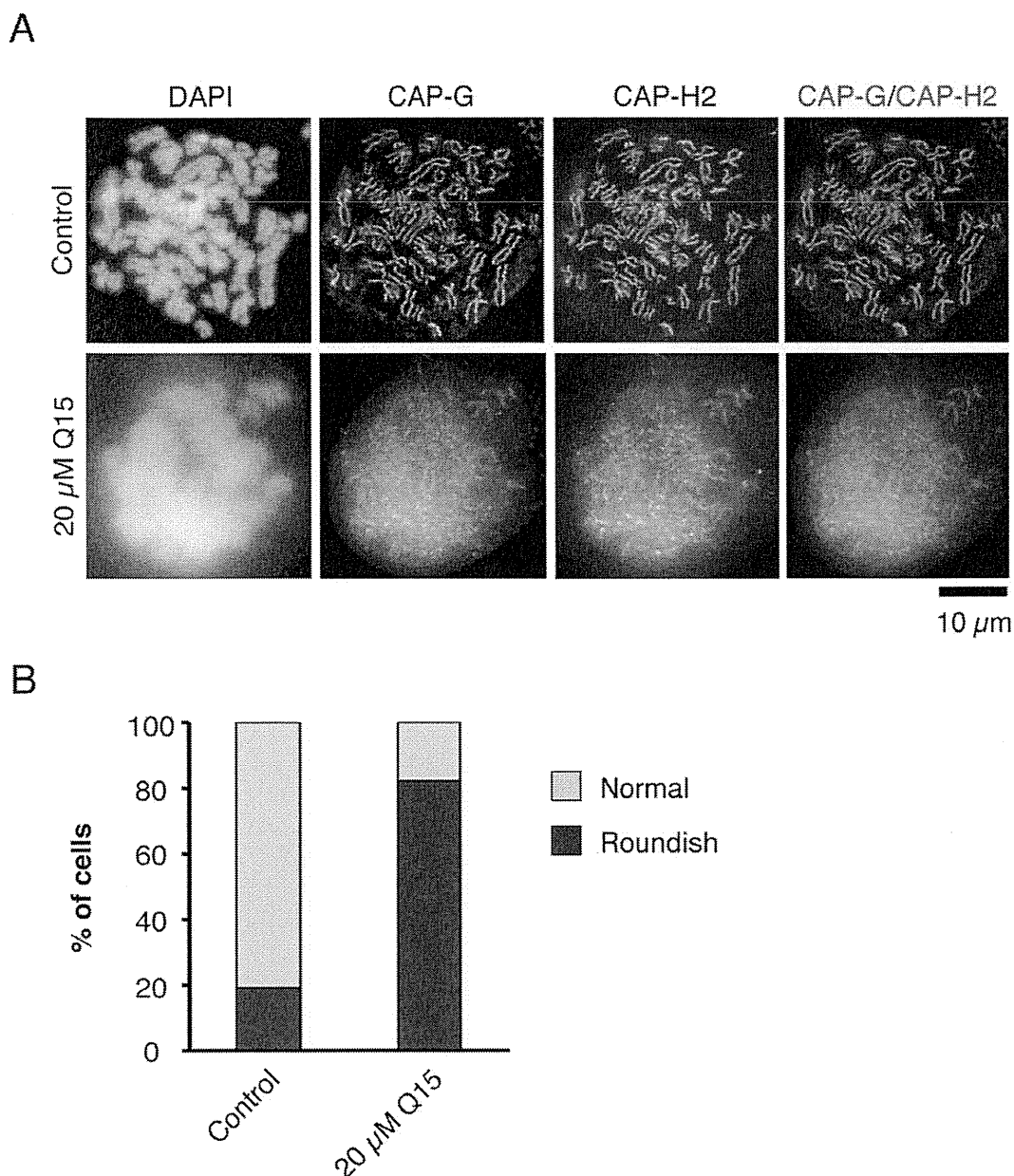


Figure 5. Q15 induces structural aberration of chromosomes in mitosis. (A) HeLa cells were treated with DMSO or 20 μ M Q15 for 24 h. Then, immunofluorescence staining with antibodies against hCAP-G (green) and hCAP-H2 (red) was performed. DNA was stained with DAPI. Bar, 10 μ m. (B) At least 100 cells were counted, and the percentages of normal and roundish cells were quantified. doi:10.1371/journal.pone.0044889.g005

and purity. The identification of cell lines was performed based on an STR Multiplex method that uses 9 different loci: D5S818, D13S317, D7S820, D16S539, vWA, TH01, Amelogenin, TPOX and CSF1PO (Powerplex 1.2 system, Promega Corporation) in 2011.

Compounds

The syntheses of Q15 (6-amino-4(N)-(3-chloro-4-fluorophenyl)-7-[4-[4-phenoxy(phenylcarbamoyl)]piperazin-1-yl]quinazolin-4-amine) and biotinylated Q15 (Q15-Bio) are described in the Methods S1. All other anilinoquinazoline derivatives were synthesized from the corresponding quinazoline derivatives and aniline derivatives. The chemical structures of all synthetic

compounds were confirmed by 1 H-NMR spectroscopy and mass spectrometry.

Cell Proliferation Assay

The cytotoxicity of each compound was assessed by MTS cell survival assays according to the instructions provided by the manufacturer (Promega, Madison, WI). Briefly, SW480 cells (1×10^4 cells), KMS11, KMS21, KMS26, KMS28 or KMS34 cells (2.5×10^4 cells, respectively) were plated in 96-well plates at a density of 20,000 cells per well. Synthetic anilinoquinazoline derivatives were dissolved in DMSO (Sigma) to make 20 mM stock solutions. The stock solutions were then diluted to 0.078–50 μ M in medium and distributed in 96-well plates. After 48 or

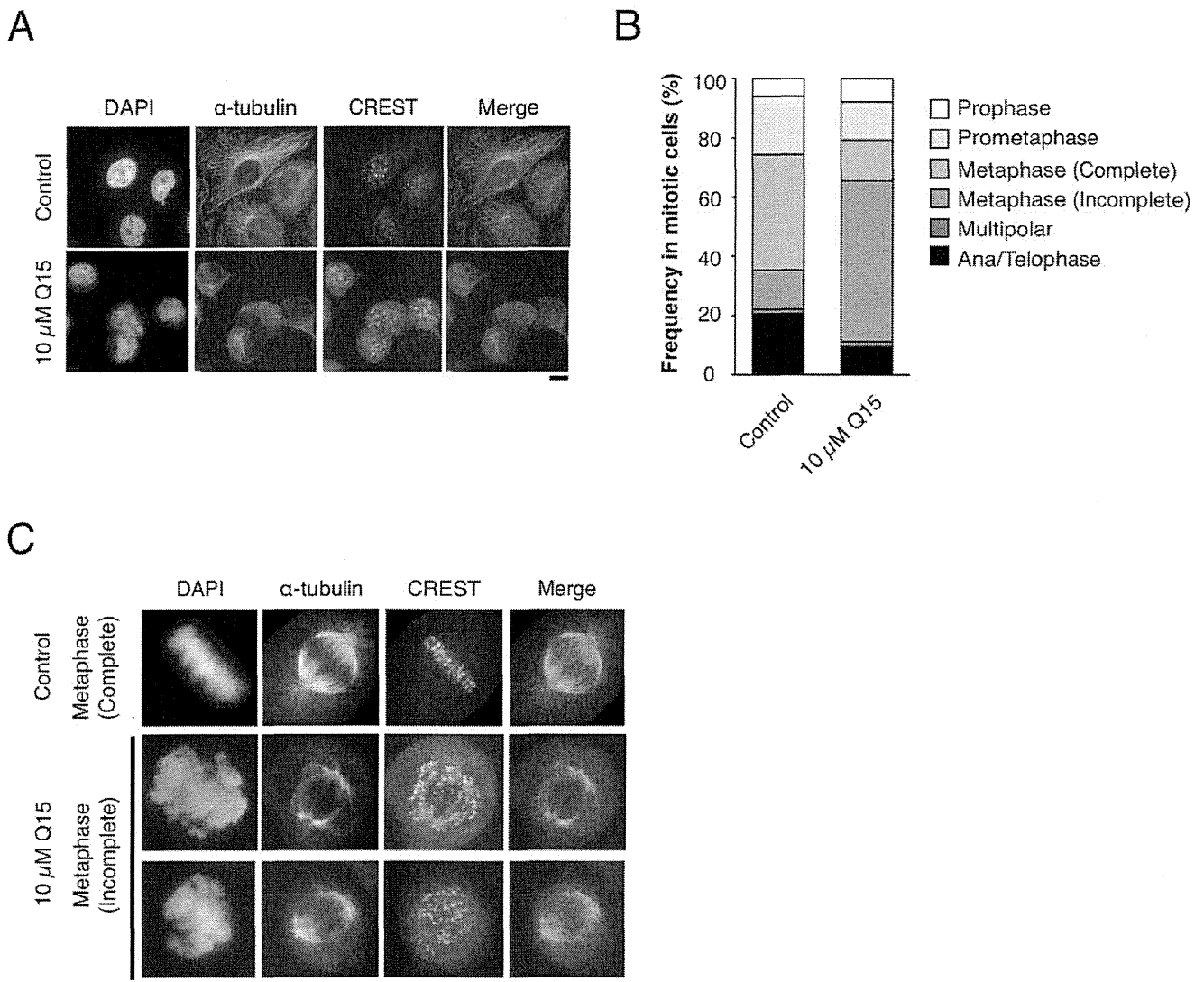


Figure 6. Q15 induces abnormal chromosome segregation. HeLa cells were treated with DMSO (Control) or 10 μ M Q15 for 24 h, and immunofluorescence labeling with an antibody against α -tubulin (green) and CREST (red) was performed. DNA was stained with DAPI. (A) Representative images of interphase cells are shown. Bar, 10 μ m. (B) Percentages of cells at different stages in mitotic populations are shown. The mitotic stages and alignment defects were judged by DAPI staining. (C) Representative metaphase cells are shown. Q15-treated cells display a dramatically high frequency of metaphase cells with incompletely aligned chromosomes. Bar, 10 μ m. doi:10.1371/journal.pone.0044889.g006

72 h treatment, 20 μ L of the CellTiter 96 AQueous One Solution reagent (Promega) was directly added to each well and the plates were incubated at 37°C for 2 h in 5% CO₂ in air. The absorbance at 490 nm was read using a SafireTM microplate reader (Tecan, Mannedorf, Switzerland).

Immunoblot Analysis

Cells were treated with 20 μ M Q15 for 48 h followed by lysis in RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.05% SDS, 1% NP-40) containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Protein concentrations were determined with a BCA protein assay kit (Thermo Scientific, Waltham, MA). Protein (20 μ g) was run on 10 or 15% SDS-PAGE gel and analyzed with antibodies against caspase-9, caspase-3, PARP, p44/42 MAP kinase or phospho-p44/42 MAP kinase (all from Cell Signaling Technology, Beverly,

MA). The blots were visualized with ECL chemiluminescence reagents (GE Healthcare, Waukesha, WI).

In vivo Tumor Growth Assay

Animal experiments were approved by the Ethics Committee for Animal Experiments at Keio University (no. 09118-0). *In vivo* tumor inhibition assay was performed as previously described [33] with several modifications. Briefly, 3×10^7 KMS34 cells were subcutaneously inoculated into 5-week-old male Icr/scid mice (CLEA, Tokyo, Japan). Plasmacytoma developed in 6 to 7 weeks, and when the size of the tumor had reached 50 mm³ (day 1), vehicle (saline, 10% DMSO and 1% Tween 80) or 20 mg/kg of Q15 was injected intraperitoneally twice every 3 days. Tumor volumes was calculated according to the following formula: width \times length² \times 0.52 [33]. Differences in tumor size on day 14 were evaluated by means of Student's *t* test. *P* < 0.05 was considered to indicate statistical significance.

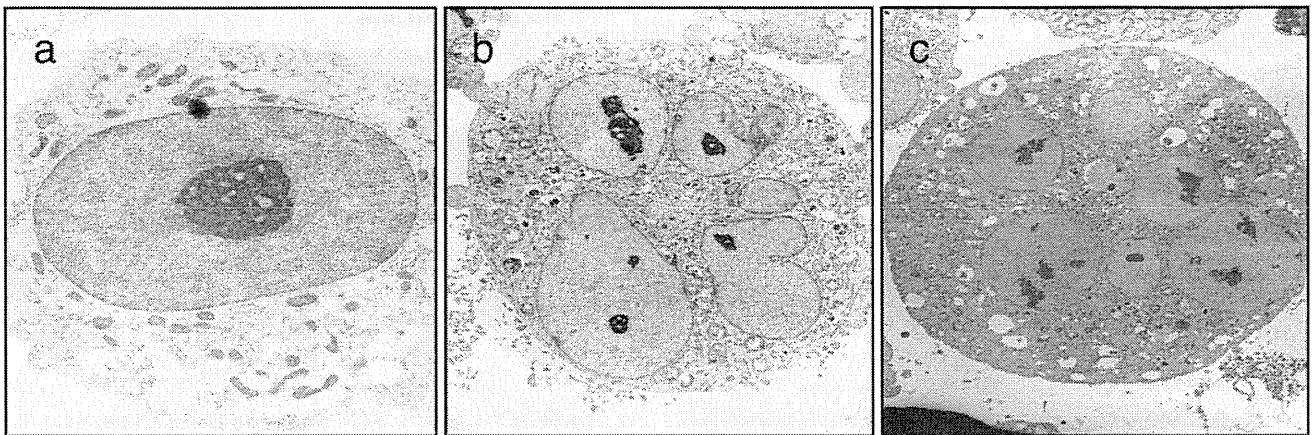


Figure 7. Q15 induces abnormal cell division. KMS34 cells were treated with DMSO (a) or 5 μM Q15 (b and c) for 24 h, then observed with an electron microscope. Micronuclei can be seen in Q15-treated cells. doi:10.1371/journal.pone.0044889.g007

Histopathologic Examination

Histopathologic analysis was performed as previously described [33] with several modifications. When the size of subcutaneous tumors reached 50 mm^3 , vehicle or 20 mg/kg Q15 was injected intraperitoneally twice every 3 days. After 14 days, the mice were killed and the tumors were isolated. Tumor samples were fixed with 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin.

mRNA Display Selection

Total RNA from SW480 cells was extracted with an RNeasy mini kit (Qiagen, Valencia, CA) and purified with a mTRAP mRNA isolation kit (Active Motif, Carlsbad, CA). A cDNA library was prepared as previously described [15]. The resulting cDNA library derived from KMS34 cells was transcribed using a RiboMAX large-scale RNA production system-SP6 (Promega) in a total volume of 20 μL containing 80 mM HEPES-KOH, pH 7.5, 2 mM spermidine, 40 mM DTT, 32 mM MgCl_2 , 5 mM each of ATP, CTP, and UTP, 1 mM GTP, 5 mM $\text{m}^7\text{G}(5')\text{ppp}(5')\text{G}$ RNA capping analog (Invitrogen, Carlsbad, CA), 1 pmol of KMS34 cDNA library and SP6 RNA polymerase. The resulting RNA was purified with an RNeasy mini kit and ligated with a PEG-puromycin spacer [p(dCp)₂-T(fluorescent)p-PEGp-(dCp)₂-puromycin] [15] using T4 RNA ligase (Takara) for 15 h at 15°C in a total volume of 50 μL containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , 12 mM DTT, 1.4 mM ATP, 5% DMSO, 0.002% BSA, 40 U of RNase inhibitor (Toyobo, Osaka, Japan), 0.2 mM PEG-puromycin spacer, 0.6 mM PEG2000 (Nacalai Tesque), 50 pmol of RNA, and 200 U of T4 RNA ligase. The resulting RNA-PEG-puromycin library was purified with the RNeasy mini kit. *In vitro* translation was performed in a total volume of 250 μL containing 25 pmol of the RNA-PEG-puromycin library, 50 μL of wheat germ cell-free extract (Zoegene, Kanagawa, Japan), 100 μg of creatine kinase, and 50 μL of a translation buffer (Zoegene) for 1 h at 26°C. The reaction mixture was added to Q15-Bio immobilized on Magnetex SA beads (Takara, Otsu, Japan) pre-equilibrated with IPP150 (10 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.1% NP-40), and mixed on a rotator for 1 h at 4°C. The beads were washed five times with IPP150 and used as the template for RT-PCR with a OneStep RT-PCR kit (Qiagen) using primers (5'- GGAA-GATCTATTTAGGTGACACTATAGAACAACAACAACA-CAAACAACAACAATAAT-G-3') and (5'-

TTTTTCTTGTGTCGTCATCGTCCTTGTAGTC-3'). The RT-PCR product was used for the next round of selection as described above. After five rounds of selection, the RT-PCR product was cloned using a PCR cloning kit (Qiagen) and sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

In vitro Binding Assay

SW480 or KMS34 cells were lysed with lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40 and 0.1% DOC) containing protease inhibitor cocktail (Nacalai tesque). The whole cell lysates were added to Q15-immobilized beads and mixed on a rotator for 1 h at 4°C in the absence or presence of 100 μM free Q15. The beads were washed five times with lysis buffer, followed by vortexing to elute the bound molecules. The resulting eluate was loaded on a 10% SDS-PAGE gel, and analyzed by immunoblot analysis with antibodies against LUZP5 (Bethyl Laboratories, Montgomery, TX) and SMC2 (Bethyl Laboratories).

Immunofluorescence Assay

HeLa cells on coverslips were treated with 5 μM Q15 for 24 h. Colcemid was then added at a final concentration of 0.02 $\mu\text{g}/\text{mL}$, and incubation was continued for another 3 h. Mitotic cells were collected by tapping the coverslips, followed by centrifugation at 1,000g for 5 min (Thermo/Shandon, Cyospin 4). The collected cells were fixed with 2% PFA in PBS at room temperature for 15 min, and treated with 0.5% Triton X-100 in PBS at room temperature for 5 min. The cells on coverslips were stained with an antibody against α -tubulin (Sigma, T9026/DM1A) and CREST [34], followed by Alexa488-labelled goat anti-mouse IgG and Alexa594-labelled goat anti-human IgG (Invitrogen, Carlsbad, CA). The mitotic spread cells were stained with antibodies against hCAP-G conjugated with biotin [30,35] and hCAP-H2 [9], followed by Alexa488-labelled streptavidin and Alexa594-labelled donkey anti-rabbit IgG (Invitrogen).

Electron Microscopic Observation

KMS34 cells were treated with 5 μM Q15 for 24 h. The cells were fixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate (pH 7.2) for 1 h, washed, postfixed in 1% osmium tetroxide for 2 h, stained en bloc with uranyl acetate, dehydrated in a series of graded ethanol solutions, and embedded in an Epon/Araldite mixture. Ultrathin sections were stained with lead citrate and

examined under an electron microscope (Nippon Denshi EX-200, Tokyo, Japan).

Supporting Information

Methods S1 Synthesis of Q15 and biotinylated Q15.
(PDF)

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Author Contributions

Conceived and designed the experiments: HS YN HK HT TO TH WD TY SI YH HY. Performed the experiments: HS YN HK HG HT MT TO WD SI. Analyzed the data: HS YN HK HT MT TO TH WD TY ND SI YH HY. Contributed reagents/materials/analysis tools: HT TH TY ND SI YH HY. Wrote the paper: HS YN TO TY ND YH HY.

A Phthalimide Derivative That Inhibits Centrosomal Clustering Is Effective on Multiple Myeloma

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Abstract

Despite the introduction of newly developed drugs such as lenalidomide and bortezomib, patients with multiple myeloma are still difficult to treat and have a poor prognosis. In order to find novel drugs that are effective for multiple myeloma, we tested the antitumor activity of 29 phthalimide derivatives against several multiple myeloma cell lines. Among these derivatives, 2-(2,6-diisopropylphenyl)-5-amino-1*H*-isoindole-1,3-dione (TC11) was found to be a potent inhibitor of tumor cell proliferation and an inducer of apoptosis *via* activation of caspase-3, 8 and 9. This compound also showed *in vivo* activity against multiple myeloma cell line KMS34 tumor xenografts in ICR/SCID mice. By means of mRNA display selection on a microfluidic chip, the target protein of TC11 was identified as nucleophosmin 1 (NPM). Binding of TC11 and NPM monomer was confirmed by surface plasmon resonance. Immunofluorescence and NPM knockdown studies in HeLa cells suggested that TC11 inhibits centrosomal clustering by inhibiting the centrosomal-regulatory function of NPM, thereby inducing multipolar mitotic cells, which undergo apoptosis. NPM may become a novel target for development of antitumor drugs active against multiple myeloma.

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Introduction

Multiple myeloma is one of the hematopoietic organ tumors which is characterized by the monoclonal proliferation of malignant plasma cells, resulting in appearance of serum or urinary monoclonal protein [1–3]. Although treatments include chemotherapy with melphalan, prednisolone or cyclophosphamide, as well as hematopoietic stem cell transplantation [4–8], most patients become refractory to the therapy and this leads to a fatal outcome. A group of high-risk patients is exclusively poorly responsive, with short survival. Tumor cells derived from high-risk patients have deletion of chromosome 17 (del 17), on which the p53 tumor suppressor gene is located, deletion of chromosome 13 or chromosomal translocation t(4;14) accompanied with constitutive activation of FGFR-3 on chromosome 4 [9–11]. Therefore, development of novel drugs which are active against multiple myeloma cells with these high-risk chromosomal or genetic alterations is necessary to improve the prognosis. Moreover, elucidation of the mechanisms of growth suppression of multiple myeloma cells is expected to improve our understanding of the molecular pathogenesis of multiple myeloma. Hence, our aim in this study was to find a

novel anti-tumor drug for multiple myeloma and to elucidate its molecular mechanism of action.

Drugs such as thalidomide, lenalidomide, and bortezomib show anti-tumor effect on multiple myeloma and have received much attention in recent years [12–14]; however, even these potent drugs are of limited value in high-risk cases [15]. Here, we tested the growth-inhibitory effect of 29 phthalimide derivatives, which are similar in structure to thalidomide, against several multiple myeloma cell lines including those with del 17 or t(4;14). We found that one of these derivatives, 2-(2,6-diisopropylphenyl)-5-amino-1*H*-isoindole-1,3-dione (TC11), showed potent inhibition of tumor cell proliferation both *in vitro* and *in vivo*, and induced apoptosis. Furthermore, we utilized mRNA display [16] to identify nucleophosmin 1 (NPM/B23), a nucleolar phosphoprotein [17,18], as a target of TC11 for inducing apoptosis of tumor cells. Inhibiting the function of NPM led to induction of multipolar mitoses by blocking centrosomal clustering, and this leads to cell death. Our results thus indicate that NPM could be a novel target for development of anticancer drugs to overcome the poor prognosis of high-risk multiple myeloma patients.

Results

Screening of Synthetic Phthalimide Derivatives to Identify Compounds Inhibiting Multiple Myeloma Cell Proliferation

Initially, we tested 29 phthalimide derivatives, TC1-29, each of which has various functional groups (Figure S1), for activity to inhibit proliferation of multiple myeloma KMS34 cells with t(4;14) and del 17. In the first screening, we performed cell proliferation assay to examine inhibitory activity. KMS34 cells were incubated with 50 μM of each compound for 0, 24, 48 or 72 h and the cell viability was determined with WST-1 assay. Nine compounds, TC8, TC9, TC10, TC11, TC12, TC13, TC14, TC15, and TC16, showed growth-inhibitory activity, while the others were inactive (Figure S2A). Therefore, we further examined the active compounds.

To identify the most potent compound, we investigated the ability of the hit compounds from the first screening to inhibit proliferation or to induce apoptosis of several multiple myeloma cell lines. In the second screening, KMM1, KMS11, KMS27, KMS34 and RPMI8226 cells were incubated with 0–50 μM TC8, TC9, TC10, TC11, TC12, TC13, TC14, TC15, and TC16. One arm of chromosome 17 is deleted in KMM1, KMS11 and KMS34 cells, and KMS11 and KMS34 also show t(4;14) (our unpublished data). In this screening, TC11 and TC13 showed potent activity against all cell lines tested, with IC_{50} values of 4–8 μM and 4–11 μM , respectively (Table 1). Furthermore, we tested the apoptosis-inducing activity of TC11 and TC13. The results indicated that TC11 required a lower concentration or a shorter treatment time to induce apoptosis of several multiple myeloma cell lines (Figure S2B). Thus, we identified TC11 as the most potent compound, capable of inhibiting multiple myeloma cell lines with high-risk chromosomal changes, t(4;14) and del 17.

To optimize the potency of TC11, we further synthesized TC11 derivatives TC30–42 (Figure 1) and tested them. KMS34 cells were incubated with 0–50 μM TC11 derivatives for 72 h and the cell viability was assayed. First of all, to examine the importance of the position of the amino group in the phthalimide moiety, we synthesized 7 derivatives with a 4-amino group (Type 2 in Figure 1) instead of a 5-amino group (Type 1 in Figure 1). Almost all derivatives with a 4-amino group lacked anti-tumor activity, suggesting that the 5-amino group of TC11 is important for anti-tumor activity. We next replaced functional groups of the phenyl ring of TC11 [R1–R4] with others [H, CH_3 , C_2H_5 , $\text{CH}(\text{CH}_3)_2$, $\text{C}(\text{CH}_3)_3$, CF_3 , F or Cl]. Substitution of R1 and R4 with a hydrogen atom (TC1) dramatically decreased the anti-tumor activity. Furthermore, 5-amino phthalimide derivatives with methyl groups (TC30), ethyl groups (TC31), fluorine atoms (TC41) or chlorine (TC42) atoms at the R1 and R4 positions

showed decreased anti-tumor activity. A 5-amino phthalimide derivative (TC32) with sterically bulky tertiary butyl groups at the R1 and R3 positions of the phenyl ring showed much the same anti-tumor activity as TC31 with ethyl groups at the R1 and R4 positions of the phenyl ring. These results indicate that the phenyl ring requires relatively large functional groups, such as isopropyl groups, at both R1 and R4. These findings suggested that TC11 with an amino group at the 5-position of phthalimide moiety and isopropyl groups at both *ortho* positions of the phenyl ring is the most potent compound, in terms of growth inhibition of multiple myeloma cells.

TC11 Induced Apoptosis of MM Cell Lines *in vitro* and *in vivo*

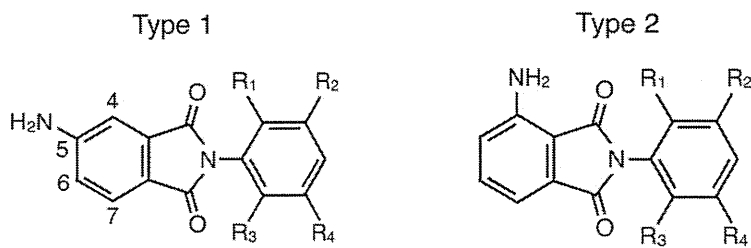
To examine whether TC11-induced apoptosis is dependent on the caspase pathway, we performed immunoblot analysis of lysates from TC11-treated KMS34 cells. After treatment with TC11 for 24 h, cleavage of PARP in both cell lines was detected, while treatment with thalidomide showed no effect (Figure 2A). Likewise, cleavage of procaspase-3, 8 and 9 (activated forms of caspase) was detected following treatment with TC11 (Figure 2B). DNA fragmentation was also observed in KMS34 cells treated with TC11 or staurosporine (Figure 2C). Besides these early-phase events (Fig. 2A–C), we also examined structural change of the cell membrane during apoptosis in the late phase (Fig. 2D). KMS34 cells were treated with 50 μM TC11 for 96 h. Annexin V-FITC and propidium iodide (PI) staining, followed by flow cytometric analyses, showed that TC11 treatment increased both Annexin V-positive/PI-negative and Annexin V-positive/PI-positive fractions (early and later apoptotic cells, respectively) of KMS34 cells, while thalidomide treatment did not. These results suggested that TC11 induced apoptosis depending on activation of caspase-3, 8 and 9.

We next tested the anti-tumor activity of TC11 *in vivo*. KMS34 tumor xenografts ($\sim 50 \text{ mm}^3$) were treated with intraperitoneal injection of 20 mg/kg TC11 twice with a 3-day interval, followed by time-course analysis of tumor volume for 15 days (Figure 3A). After 7 and 14 days, TC11 showed significant tumor suppression ($P < 0.05$). In the animal experiments, no mouse died and no macroscopic indications of TC11 toxicity were observed at autopsy. Hematoxylin-eosin staining of tumor tissue slices showed that cells with aggregated chromatin were increased in TC11-treated mice. Cytoplasm of these cells appeared round and little shrunken (Figure 3B). To examine whether these structural changes were caused by apoptosis, immunohistochemistry using anti-ssDNA antibody, which specifically detects fragmented single-strand DNA, was carried out. As shown in Figure 3C, DNA-fragmented cells detected by anti-ssDNA antibody were increased in the tumor tissue treated with TC11 (Figure 3C). These results

Table 1. IC_{50} values (μM) of thalidomide derivatives for inhibiting proliferation of multiple myeloma cell lines.

| Cell line | Compound | | | | | | | | | |
|-----------|----------|-----|------|------|------|------|------|------|------|-------------|
| | TC8 | TC9 | TC10 | TC11 | TC12 | TC13 | TC14 | TC15 | TC16 | Thalidomide |
| KMM1 | >50 | >50 | >50 | 7 | 18 | 5 | 32 | >50 | >50 | >50 |
| KMM11 | >50 | >50 | >50 | 6 | 14 | 4 | 7 | >50 | >50 | >50 |
| KMS27 | >50 | >50 | >50 | 8 | 25 | 8 | 34 | >50 | >50 | >50 |
| KMS34 | >50 | >50 | 25 | 4 | 16 | 5 | 14 | >50 | >50 | >50 |
| RPMI8226 | >50 | >50 | >50 | 7 | 26 | 11 | 24 | >50 | >50 | >50 |

doi:10.1371/journal.pone.0038878.t001



IC₅₀ value of TC11 derivatives for inhibiting proliferation of KMS34 cells

| Compound | Type | R ₁ | R ₂ | R ₃ | R ₄ | IC ₅₀ (μM) |
|----------|------|-----------------------------------|-----------------|-----------------------------------|----------------------------------|-----------------------|
| TC1 | 1 | H | H | H | H | >50 |
| TC11 | 1 | CH(CH ₃) ₂ | H | CH(CH ₃) ₂ | H | 4 |
| TC30 | 1 | CH ₃ | H | CH ₃ | H | >50 |
| TC31 | 1 | C ₂ H ₅ | H | C ₂ H ₅ | H | 13 |
| TC32 | 1 | C(CH ₃) ₃ | H | H | C(CH ₃) ₃ | 12 |
| TC33 | 1 | H | CF ₃ | H | CF ₃ | 32 |
| TC34 | 2 | C(CH ₃) ₃ | H | H | C(CH ₃) ₃ | >50 |
| TC35 | 2 | H | CF ₃ | H | CF ₃ | 36 |
| TC36 | 2 | CH(CH ₃) ₂ | H | CH(CH ₃) ₂ | H | >50 |
| TC37 | 2 | C ₂ H ₅ | H | C ₂ H ₅ | H | >50 |
| TC38 | 2 | CH ₃ | H | CH ₃ | H | >50 |
| TC39 | 2 | F | H | F | H | >50 |
| TC40 | 2 | Cl | H | Cl | H | >50 |
| TC41 | 1 | F | H | F | H | 29 |
| TC42 | 1 | Cl | H | Cl | H | 16 |

Figure 1. IC₅₀ values of TC11 derivatives for inhibiting proliferation of KMS34 cells. KMS34 cells (1×10^4 cells/well) in 96-well plate were incubated with 0–50 μM of indicated compound for 72 h. Then cell viability was determined with WST-1 assay. doi:10.1371/journal.pone.0038878.g001

suggested that TC11 likely induced apoptosis of KMS34 cell line tumor xenograft and exhibited anti-tumor activity *in vivo*.

Identification of TC11-binding Proteins by mRNA Display

To identify TC11-binding proteins, we used mRNA display [16], which is a powerful tool for *in vitro* selection of proteins that bind to various targets including small-molecular compounds. We first prepared a cDNA library derived from KMS34 cells, because our data suggested that KMS34 cells were the most sensitive to TC11. As a bait, biotinylated TC11 (Figure 4A) was immobilized on a microfluidic chip and selection of TC11-binding proteins were performed (Figure 4B). Although the 4-amino group of TC11, which was experimentally inferred to be critical for the activity, was biotinylated via a linker, the biotinylation hardly affect the antitumor activity (data not shown).

Among 11 candidate TC11-binding proteins identified by mRNA display after 4 rounds of selection, we focused on nucleolar phosphoprotein nucleophosmin (NPM). Sequencing revealed that

three selected NPM clones, designated 1–183 NPM, encoded the 183 NH₂-terminal amino acids of NPM, which contains the oligomerization domain and a part of the histone binding domain (Figure 5A). The enrichment efficiency of the NPM clones was confirmed to be 10^4 -fold after 4 rounds of selection by RT-PCR. NPM is known to be a multifunctional protein involved in both tumorigenesis and tumor suppression [19], for example, it regulates cell proliferation and centrosome duplication [20,21] and stabilizes oncoprotein Myc [22] and tumor-suppressor protein p53 [23,24]. Therefore, we hypothesized that NPM is involved in TC11-induced apoptosis of tumor cells.

To determine whether NPM interacts with TC11 directly, we examined the interaction between recombinant NPM and TC11. The initial *in vitro* binding assay between NPM and TC11 immobilized on beads revealed no interaction (data not shown). However, as NPM oligomerizes under native conditions [22] and its oligomerization domain may bind to TC11, we next performed gel filtration to separate oligomeric and monomeric forms of

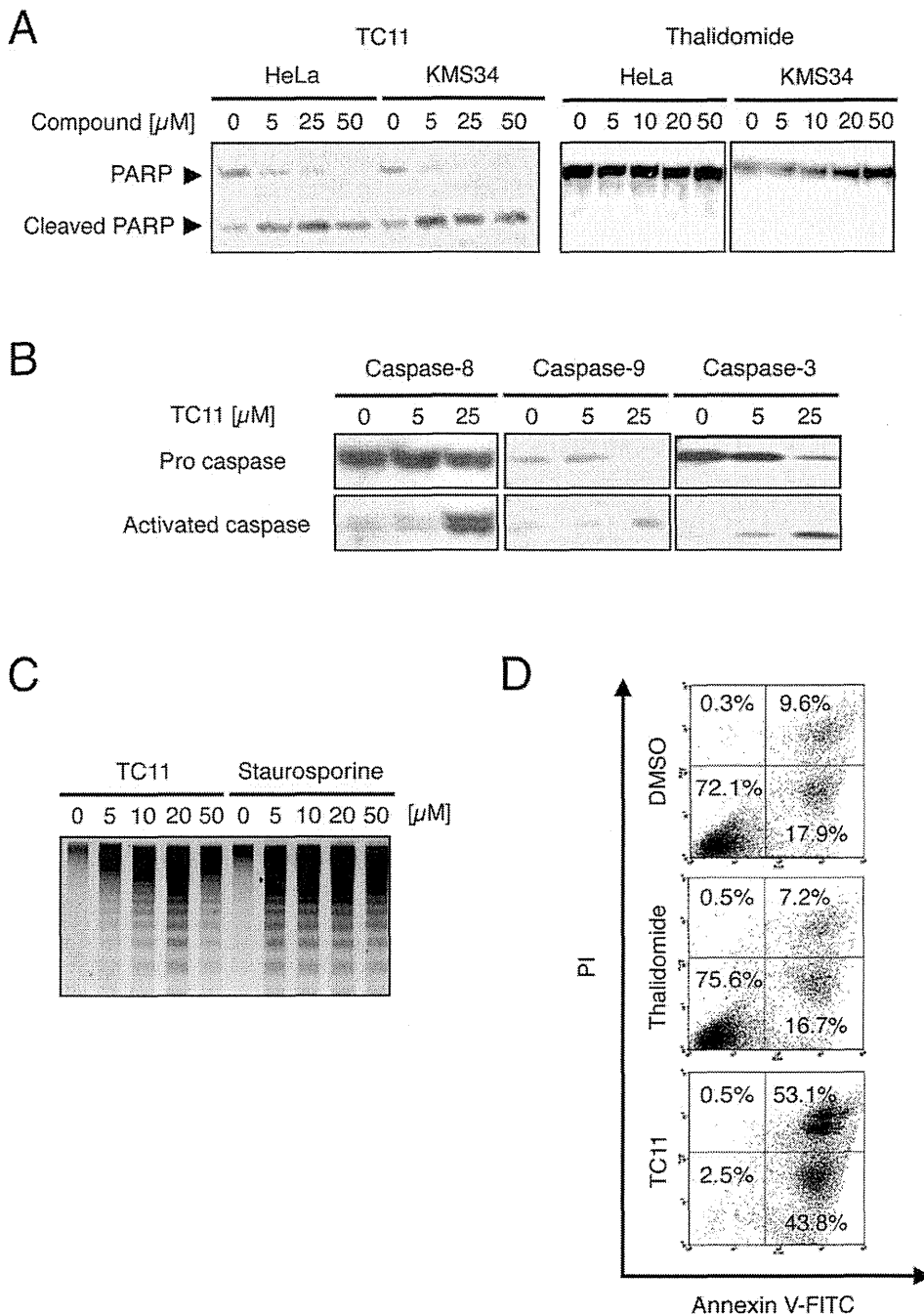


Figure 2. TC11 induces apoptosis of multiple myeloma cells in a caspase-dependent manner. (A) HeLa or KMS34 cells were treated with 0–50 μ M TC11 or thalidomide for 24 h. The whole cell lysates were analyzed by Western blotting with anti-PARP antibody. (B) KMS34 cells were treated with 0, 5 or 25 μ M TC11 for 6 h. The whole cell lysates were analyzed by Western blotting with anti-caspase-3, 8 or 9 antibody, respectively. (C) KMS34 cells were treated with 0–50 μ M TC11 or staurosporin A for 6 h. After DNA extraction, 1% agarose gel electrophoresis was performed. (D) KMS34 cells were treated with 50 μ M thalidomide or TC11. After 96 h, cells were stained with FITC-coupled annexin V and propidium iodide, and induction of apoptosis was evaluated by flow cytometry. doi:10.1371/journal.pone.0038878.g002

NPM, followed by surface plasmon resonance analysis of their affinity for TC11 (Figure 5B). These results indicate that the monomeric form of NPM binds to TC11 with a K_D value of 6.6×10^{-8} M, while the oligomer binds with a K_D value of 1.3×10^{-4} M, so that the monomeric form of NPM is the interactor with TC11.

TC11 Inhibited Centrosomal Clustering and Thereby Induced Apoptosis

Although the oligomeric form of NPM regulates tumor-suppressor protein p53, and inhibition of NPM oligomerization results in activation of p53 leading to apoptosis of several tumor cell lines [23,25], we used multiple myeloma cell line KMS34 in which p53 was inactivated in this study. Moreover, inhibition of

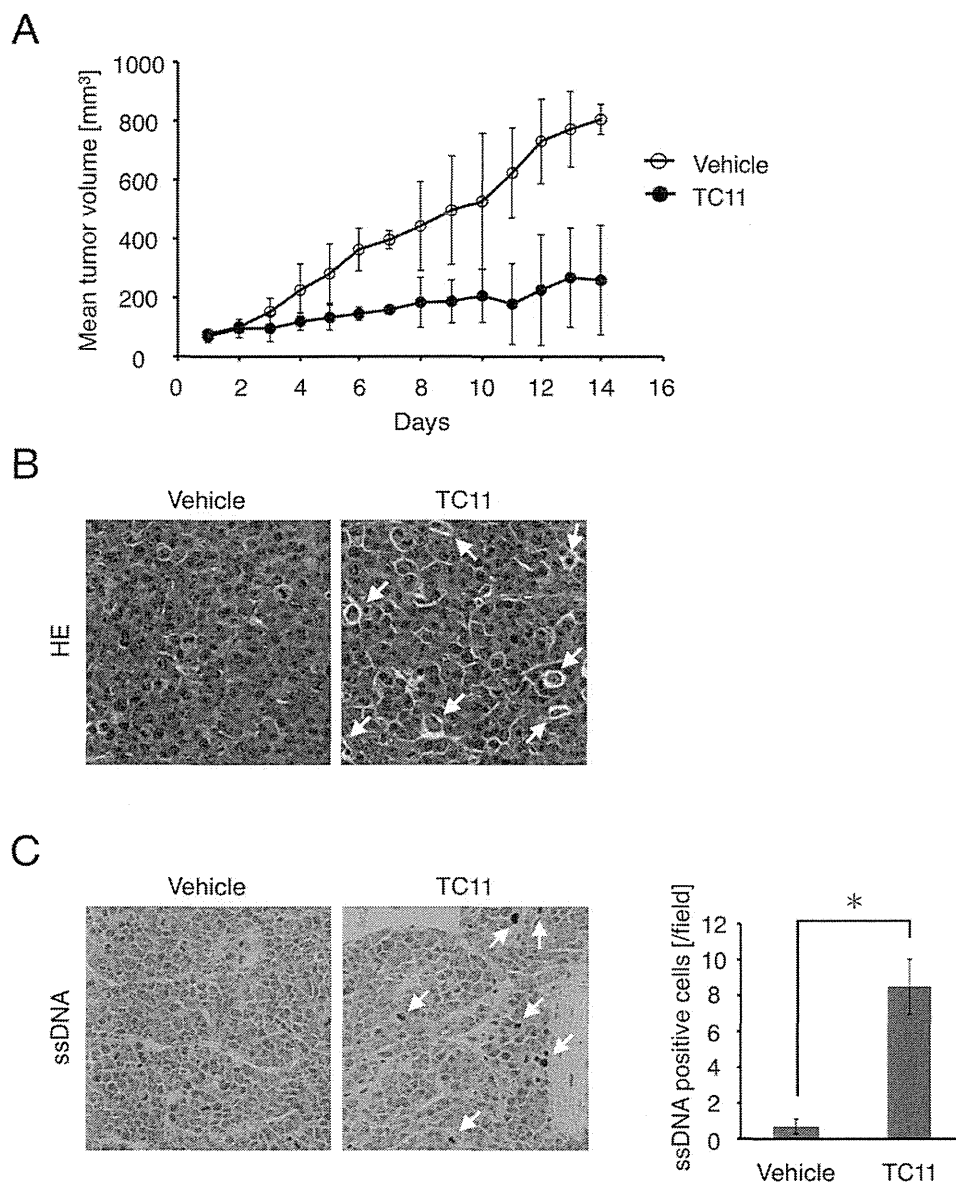


Figure 3. TC11 inhibits tumor cell growth and induces apoptosis *in vivo*. KMS34 (1.2×10^7 cells) were injected intraperitoneally into Icr/scid mice and plasmacytoma was established. When the size of the tumor had reached 50 mm^3 (day 1), $100 \mu\text{L}$ of TC11 (20 mg/kg) or vehicle alone (10% DMSO, 1% Tween 80-saline) was injected intraperitoneally into a mouse twice with a 3-day interval (i.e., injections were done on days 1, 2, 4, 5, 7, 8, 10, 11, 13 and 14). (A) The width and length of the plasmacytoma were measured and tumor volume was calculated ($n=7$). (B) Sections were stained with hematoxylin and eosin (HE). Cells with aggregated chromatin are indicated by white arrows. (C) Apoptotic cell death was detected by immunohistochemical staining with anti-single-stranded DNA antibody. Apoptotic cells are indicated by white arrows. The plot on the right side shows that the difference in the number of ssDNA-positive cells between the vehicle and TC11 groups was statistically significant (Student's *t*-test, $P<0.002$). For this plot, the number of ssDNA-positive cells was counted in two fields per tumor in five tumors (10 fields in all) in each group. doi:10.1371/journal.pone.0038878.g003

NPM oligomerization and increase of p53 protein level were not observed in cell lines with active p53, such as HeLa cells (data not shown). Therefore, we could rule out the possibility that TC11-induced apoptosis of tumor cells is p53-pathway-dependent. It has been reported that NPM is localized on centrosomes during the mitotic phase of cells and regulates centrosomal duplication [17,19–21]. Therefore, to examine the effect of TC11 on centrosomes, we performed immunofluorescence staining and fluorescence microscopic observation. In order to carefully observe intra-nuclear structures of TC11-treated cells, and due to difficulty of gene transfer to multiple myeloma cell lines, we chose HeLa cells. HeLa cells were incubated with 0–20 μM TC11 for 6 h,

followed by staining with anti- γ -tubulin antibody. Many mitotic-phase cells with multipolar spindles (>2) were observed in the case of treatment with TC11, while many mitotic cells with bipolar spindles were observed in the case of treatment with DMSO (Figure 6A). Quantification of multipolarity indicated that induction of multipolar mitoses was TC11 concentration-dependent (Figure 6B). Several studies have shown that multipolarity occurs due to inhibition of centrosomal clustering [26,27]. Thus, TC11 may inhibit centrosomal clustering and induce multipolar spindles in mitotic cells.

Additionally, we found that mitosis of cells with multipolar spindles resulted in multinucleated cells. After 24 h treatment of

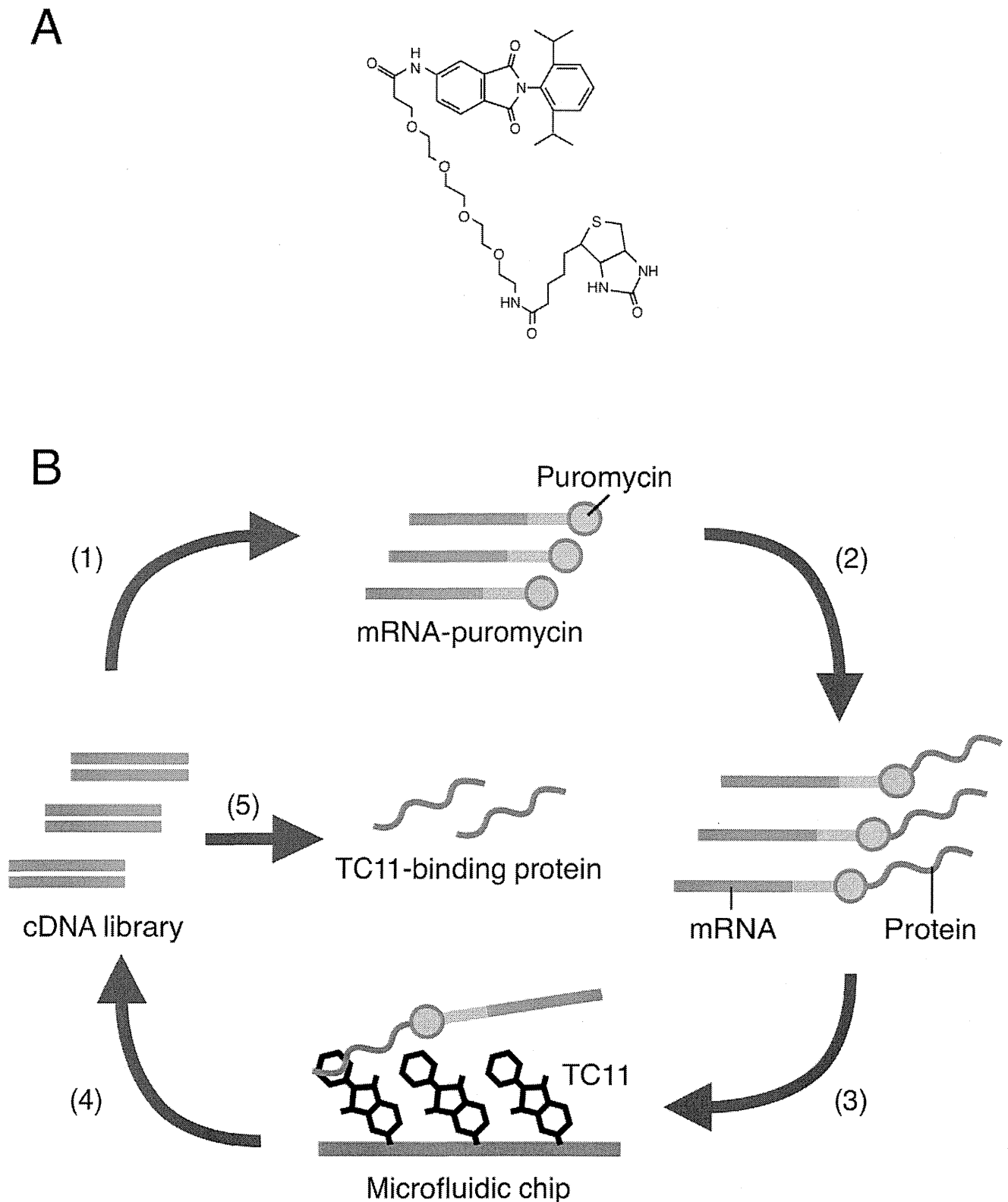
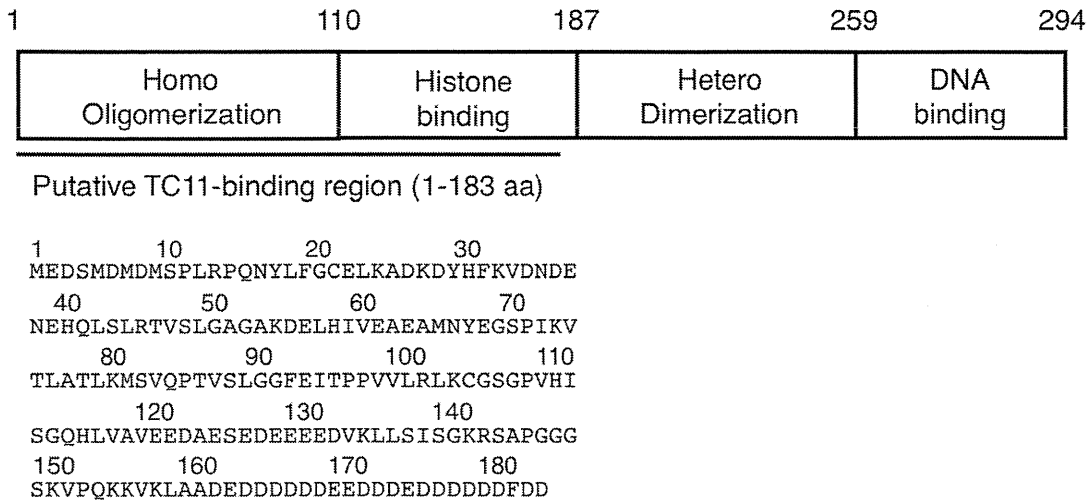


Figure 4. Schematic representation of *in vitro* selection of TC11-binding protein using mRNA display. (A) The chemical structure of biotinylated TC11. (B) A cDNA library derived from KMS34 cells was transcribed, ligated with PEG-Puro spacer (1) and *in vitro* translated (2) to form a protein-mRNA conjugates library. The library is injected into micro fluidic chip on which TC11 is immobilized (3) and unbound molecules were washed away. The bound molecules were eluted and their mRNA portion is amplified by RT-PCR (4). The resulted DNA can be used for the next of round and analyzed by cloning and sequencing. (See also Materials and Methods.)
doi:10.1371/journal.pone.0038878.g004

A



B

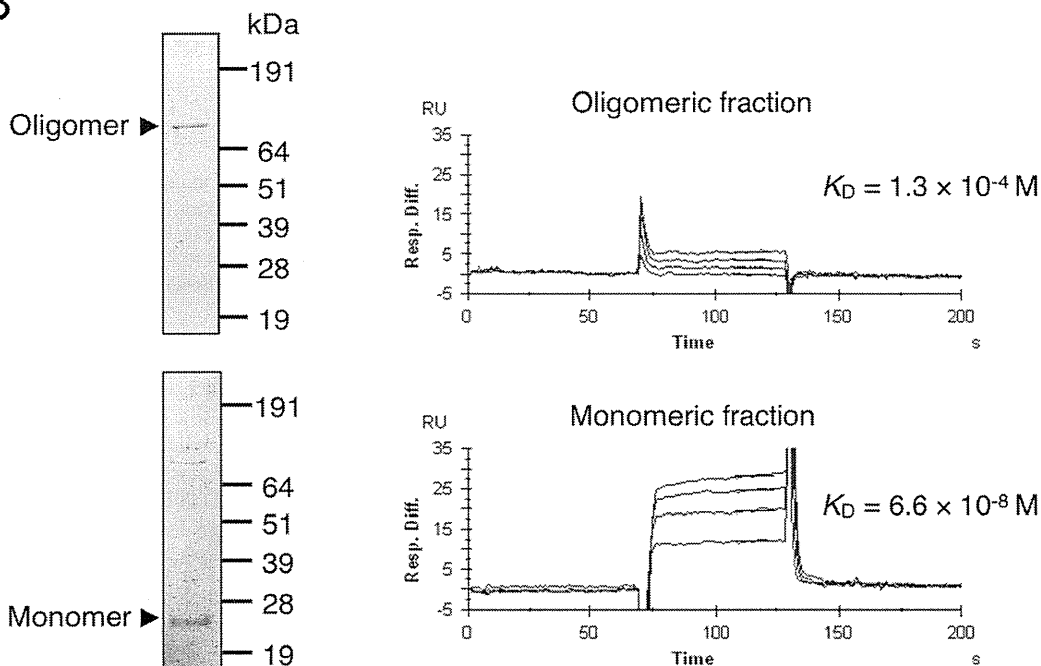


Figure 5. Monomeric NPM interacts with TC11. (A) The domain structure of NPM and the amino acid sequence predicted by NPM clone 1–183, which was identified as encoding a binding protein of TC11 by mRNA display using a cDNA library prepared from KMS34 cells. The underlined part indicates the region (1–183 a. a.) identified by mRNA display selection. The sequence indicated by the solid line corresponds to that encoded by the ORF of NPM cDNA. (B) Recombinant NPM_{1–183} was expressed in *E. coli* and fractionated into monomeric and oligomeric forms. Then, each fraction was subjected to 10% SDS-PAGE followed by CBB staining (left). A representative biosensorgram of NPM binding on SA sensor chips with immobilized biotinylated-TC11 is shown. The K_D values were determined (right).
doi:10.1371/journal.pone.0038878.g005

HeLa cells with TC11, most interphase cells had multiple nuclei, while control cells had a single nucleus (Figure 6C). The former cells may occur as a result of division of cells with multipolar spindles. It was recently reported that cells with multiple nuclei have poor viability and undergo apoptosis [28]. Therefore, we considered that TC11-induced apoptosis would occur through this mechanism.

NPM Knockdown Induced Multipolar Spindles and Apoptosis

To investigate whether NPM is related to the multipolarity of mitotic cells, we performed a NPM knockdown experiment. HeLa cells were transfected with siRNA for NPM and after 48 h, NPM protein levels were confirmed to be repressed to <20% (Figure 7A).

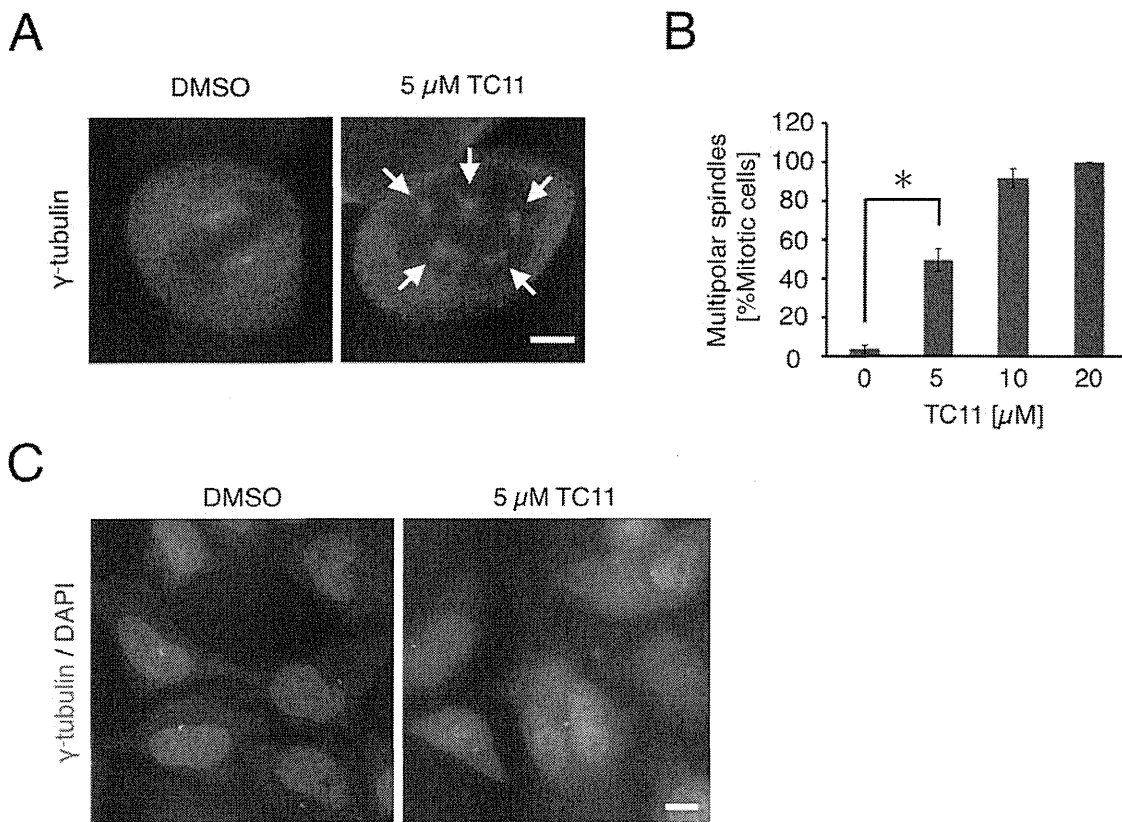


Figure 6. TC11 increases spindle multipolarity, resulting in multinucleation. HeLa cells were treated with 0–20 μ M TC11 for 6 h. Then, immunofluorescence staining of γ -tubulin was performed. (A) Representative mitotic cell under the indicated conditions. (B) Mitotic cells with multipolar spindles were counted under the indicated conditions. At least 100 mitotic cells were counted per sample in three independent experiments. An asterisk denotes a statistically significant difference according to Student's *t*-test ($P < 0.05$). White arrows indicate centrosomes. (C) HeLa cells were treated with 5 μ M TC11 for 24 h, followed by immunofluorescence staining with anti- γ -tubulin antibody (green). Nucleus was stained with DAPI (blue). Bar; 10 μ m.

doi:10.1371/journal.pone.0038878.g006

After siRNA transfection, we performed immunofluorescence staining with anti- γ -tubulin antibody. We found that mitotic cells with multipolar spindles amounted to 16% of NPM-depleted cells, but only 2% of control cells (Figure 7B). Additionally, multinucleated cells were observed only among cells transfected with siRNA for NPM (Figure 7C). To examine whether knockdown of NPM results in apoptosis, we also performed caspase-9 activity assay. Caspase-9 activity was 6-fold higher in the case of NPM-depleted cells than control cells (Figure 7D). We finally examined whether the inhibitory activity of TC11 on cell proliferation involves NPM. HeLa cells were treated with TC11 after knockdown of NPM and the cell viability was determined by means of WST-1 assay (Figure 7E). We found that TC11 showed more potent cytotoxicity against NPM-depleted HeLa cells than against control cells, indicating that down-regulation of NPM increases the sensitivity of HeLa cells to TC11. These results suggested that inhibition of NPM function results in multipolarity of mitotic cells, leading to apoptosis.

Discussion

Although the prognosis of multiple myeloma has been improved by the introduction of drugs such as thalidomide, lenalidomide and bortezomib, high-risk patients tend to show a poor response. We considered that KMS34 cell line would be a good model for high-risk cases with t(4;14) or del 17. Therefore, we used KMS34

cells to screen for compounds able to induce apoptosis of high-risk myeloma cells. Among the 29 phthalimide derivatives examined, TC11 showed significant anti-tumor activity against multiple myeloma KMS34 cells both *in vitro* and *in vivo*, while existing drugs such as thalidomide and lenalidomide had little or no effect *in vitro* (Fig. S2 and unpublished data). These results suggest that TC11 would be effective against multiple myeloma with high-risk chromosomal changes, t(4;14) and del17 (where the p53 tumor suppressor gene is located).

We found that TC11 induced apoptosis of tumor cells through inhibition of centrosomal clustering. Rebacz *et al.* recently reported that griseofulvin also inhibits centrosomal clustering [27]. Both TC11 and griseofulvin induce caspase-3, 8 and 9 activation, followed by cleavage of PARP. Although their action mechanisms for inducing apoptosis seem very similar, TC11 is a more potent inducer of multipolarity and apoptosis. Furthermore, multinucleated cells were observed following treatment with TC11 for 24 h, probably due to aberrant cell division, because it was previously reported that multinucleated cells resulted from aberrant cell division of cells with multipolar spindles [29]. Although the molecular mechanism that triggers apoptosis after aberrant cell division is unknown, another study also found that multinucleated cells show poor survival capability [28]. Thus, inhibition of centrosomal clustering may play a major role in TC11-induced apoptosis.

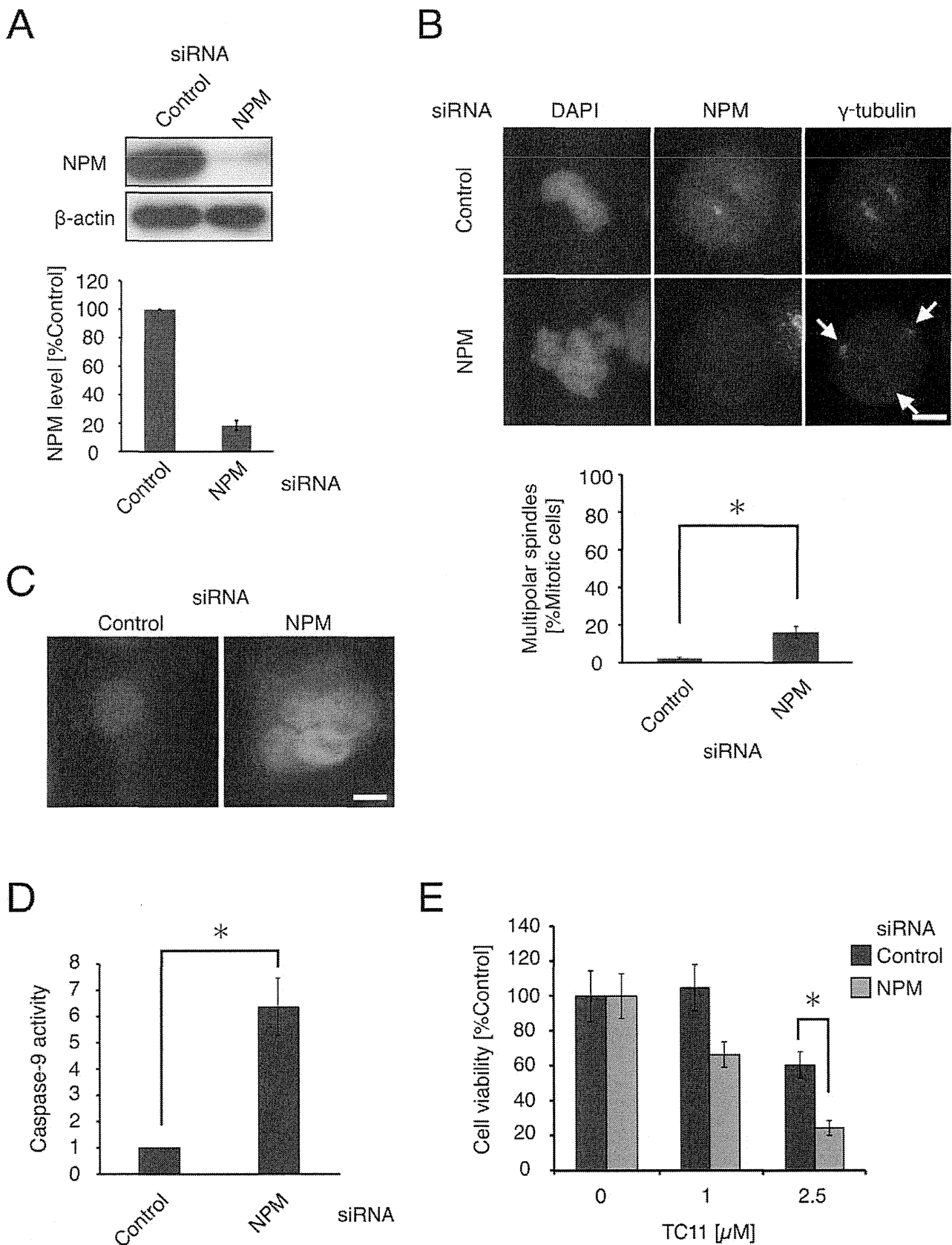


Figure 7. NPM knockdown induces multipolar spindles. HeLa cells were transfected with siRNA for luciferase (control) or NPM. (A) After 48 h, the whole cell lysates were analyzed by western blotting with antibody against NPM (left) and the band intensities were quantified (right). (B) Immunofluorescence with anti-NPM (green) and anti- γ -tubulin (red) antibody was performed (left). White arrows indicate centrosomes. At least 50

mitotic cells were counted in three independent experiments. The ratio of cells with multipolar spindles under the indicated conditions was quantified (below). (C) Representative multinucleated cell under the indicated conditions. (D) After 72 h, caspase-9 activity was determined using luminescence-based assay. (E) At 48 h after siRNA transfection, the cells were treated with 0–2.5 μ M TC11 and then incubated for a further 72 h. Cell viability was determined by means of WST-1 assay in three independent experiments. An asterisk denotes a statistically significant difference according to Student's *t*-test ($P < 0.05$). Bar; 10 μ m. doi:10.1371/journal.pone.0038878.g007

We identified NPM as a candidate target protein of TC11 for inducing apoptosis and found that the monomeric form of NPM, but not oligomeric NPM, bound tightly to TC11. Since the putative TC11-binding region of NPM includes its oligomerization domain, and it has been reported that inhibiting oligomerization of NPM leads to apoptosis [30,31], we hypothesized that TC11 inhibits oligomerization of NPM. However, inhibition of the oligomerization could not be confirmed with native-PAGE (data not shown).

Our data suggested that NPM may be involved in the appearance of multipolar spindles in mitotic cells. NPM is known to regulate centrosomal duplication during the mitotic phase of cell division. Although a recent study did not identify NPM as a protein that was required for centrosomal clustering [32], siRNA-induced knockdown of the NPM gene has been reported to induce multipolar spindles in HeLa cells [33], and we also confirmed this result in the present work. Wang *et al.* previously reported that preventing export of NPM to cytoplasm from nucleus through disruption of the NPM-CRM1 complex with the CRM1 inhibitor leptomycin B (LMB) resulted in dissociation of NPM from centrosomes, and thereby led to multipolar spindle formation [34]. In conflict with that report, our immunofluorescence experiments indicated that NPM in both TC11 and LMB-treated cells is localized on centrosomes (Figure S3). In support of our finding, a recent study by Rousselet indicated that LMB does not disturb localization of NPM to centrosomes or affect centrosomes numbers [35]. Therefore, it appears that interaction of TC11 with NPM may inhibit its centrosomal-regulatory function without affecting its localization on centrosomes. Little is known about the region of NPM that is required to regulate centrosomal duplication or whether NPM exists as oligomer when it localizes to centrosomes. Moreover, NPM interacts with many kinds of proteins such as Myc, p53, MDM2 and so on [22–24,36]. It is possible that other NPM-interacting protein(s) are involved in centrosomal clustering. Further study is needed to establish the significance of TC11-NPM interaction for the anticancer effect of TC11.

We also found that thalidomide did not inhibit centrosomal clustering (Figure S4). It was previously reported that thalidomide interferes with tumor angiogenesis. The difference in action mechanism between thalidomide and TC11 is presumably related to the difference in their chemical structure. It is noteworthy that TC11 showed anti-tumor activity against multiple myeloma KMS34 cells that were resistant to thalidomide (Figure S2) and lenalidomide (unpublished data).

Inatsuki *et al.* previously reported that a compound with the same structure as TC11 inhibited tubulin polymerization [37]. In our mRNA display experiments, α -tubulin was identified as TC11-binding protein, as well as NPM (data not shown). Thus, there may be a possibility that TC11-induced apoptosis is triggered by inhibition of tubulin polymerization. However, colchicine, which inhibits tubulin polymerization and induces apoptosis, did not induce multipolarity of mitotic cells (data not shown) [38]. Moreover, our data showed that repression of NPM leads to apoptosis and increased sensitivity to TC11. Further experiments, for example with NPM mutants that do not bind to TC11, might be helpful to establish the relationship between NPM and TC11-

induced apoptosis. Therefore, if binding of TC11 to α -tubulin does induce apoptosis by inhibition of tubulin polymerization, we consider that this occurs independently of the pathway that relates to inhibition of centrosomal clustering.

In conclusion, we have identified TC11 as a potent suppressor of proliferation of multiple myeloma cells with high-risk chromosomal or genetic changes, both *in vitro* and *in vivo*. Our results suggest that TC11 inhibits the centrosomal-regulatory function of NPM, thereby inducing multipolar mitotic cells, which undergo apoptosis. Although further work is required to fully establish the role of NPM in apoptosis, NPM may become a novel target for development of antitumor drugs active against myeloma cells.

Materials and Methods

Cell Lines

Multiple myeloma (MM) cell lines (KMM1, KMS11, KMS26, KMS27, KMS34 and RPMI8226) were established by T Otsuki (Kawasaki Medical College, Kurashiki, Japan) from Japanese patients [39] and were maintained in RPMI1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin. HeLa cells (RIKEN Cell Bank, 2002) were maintained in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin. The identification of cell lines was performed based on an STR Multiplex method that uses 9 different loci: D5S818, D13S317, D7S820, D16S539, vWA, TH01, Amelogenin, TPOX and CSF1PO (Powerplex 1.2 system, Promega Corporation) in 2011.

Compounds

The compounds listed in Figs. S1 and S2 were prepared in 50–95% yields by refluxing a mixture of phthalic acid anhydride derivatives and appropriate amines in acetic acid for several hours. Phthalimide derivatives with an amino group, such as TC1-16 and TC11-42, were synthesized from phthalic acid anhydride with a nitro group and appropriate amines, followed by catalytic hydrogenation under a hydrogen atmosphere. The chemical structures of synthetic compounds were confirmed by ¹H-NMR spectroscopy and mass spectrometry.

Compound Screening

Synthetic compounds were dissolved in DMSO to make 20 mM stock solutions. The stock solutions were diluted to 0.5–50 μ M in medium and distributed into 96-well plates. Then, MM cells (1×10^4 cells/well) were seeded in each well and incubated for 72 h. The number of viable cells was determined with the reagent WST-1 (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Immunoblot Analysis

Cells were treated with various concentrations of TC11, TC13 or thalidomide for 6–48 h, followed by lysis in RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.05% SDS, 1% NP-40) containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Protein concentrations were determined using BCA protein assay kit (Thermo Scientific, Waltham, MA) and 20 μ g of protein was loaded on a 10% or 15% SDS-PAGE gel and analyzed with

antibodies against caspase-3, caspase-8, caspase-9, PARP (Cell Signaling Technology, Beverly, MA), or NPM (Sigma, St. Louis, MO, USA). The blots were developed using ECL chemiluminescence reagents (GE Healthcare, Waukesha, WI).

DNA Fragmentation Assay

KMS34 cells were treated with various concentrations of TC11 for 6 h. The cells were lysed with lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM EDTA, 0.2% Triton X-100), followed by incubation on ice for 15 min. The solution was centrifuged at 10,000g for 20 min and the supernatant was treated with 100 µg/ml RNase A. The resulting solution was purified with MaXtract (Qiagen, Valencia, CA) according to the manufacturer's instructions, followed by isopropanol precipitation. DNA laddering was detected by EtBr staining after agarose gel electrophoresis.

Flow Cytometric Analysis

KMS26, KMS27 and KMS34 cells (2×10^5 cells, respectively) were incubated with 50 µM thalidomide or TC11 for 96 h. The cells were stained with an Annexin V-FITC kit (Bender Medsystems, Vienna, Austria) and Annexin V-positive cells were quantified with a Becton Dickinson FACSCalibur system (Becton Dickinson, San Jose, CA).

In vivo Tumor Growth Assay

All of the animal experiments were approved by the Ethics Committee for Animal Experiments at Keio University School of Medicine (Approval no. 09118-0). *In vivo* tumor-inhibitory activity assay was performed as previously described [40] with several modifications. Briefly, 3×10^7 KMS34 cells were subcutaneously inoculated into 5-week-old male lcr/scid mice (CLEA, Tokyo, Japan) and plasmacytoma developed in 4 to 6 weeks. TC11 was dissolved in DMSO (Sigma) at concentration of 20 mg/mL and then diluted with 5% carboxymethyl cellulose-saline solution to 2.5 mg/mL. Tumor volume was calculated according to the following formula: width \times length² \times 0.52 [40]. Differences in the size of tumors on days 7, 10 and 14 were compared and evaluated by means of Student's *t* test. $P < 0.05$ was considered to indicate statistical significance.

Histopathologic Examination

Histopathologic analysis was performed as previously described [40] with several modifications. When the subcutaneous tumors reached 50 mm³, 5% carboxymethylcellulose or 20 mg/kg TC11 was injected intraperitoneally twice with an interval of 3 days. After 14 days, the mice were killed and the tumors were isolated. Tumor samples were fixed with 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. Apoptotic cell death was determined with anti-single-stranded DNA antibody (DakoCytomation, Carpinteria, CA).

mRNA Display Selection on a Microfluidic Chip

The affinity selection of target proteins of TC11 was performed by combined mRNA display with a microfluidic system [41]. Total RNA from KMS34 cells was extracted with an RNeasy mini kit (Qiagen), followed by purification with a mTRAP mRNA isolation kit (Active Motif, Carlsbad, CA). Thereafter, preparation of a cDNA library was performed as previously described [42]. The resulting cDNA library derived from KMS34 cells was transcribed using a RiboMAX large-scale RNA production system-SP6 (Promega, Madison, WI). The resulting RNA was purified with the RNeasy mini kit and ligated with a PEG-puromycin spacer [p(dCp)₂-T(fluorescein)p-PEGp-(dCp)₂-puromycin] [16] using T4

RNA ligase (Takara, Otsu, Japan) at 15°C for 15 h. The resulting RNA-PEG-puromycin library was purified with the RNeasy mini kit. *In vitro* translation was performed using wheat germ cell-free extract (Zoegene). The reaction mixture was subjected to gel filtration on Sephadex G200 (GE Healthcare) using a 0.8 \times 4 cm column (Bio-Rad, Richmond, CA) with TBS containing 10 mM EDTA, and 2-drop fractions were collected. The fluorescence of mRNA-displayed proteins in eluate fractions was monitored with a Multi-detection microplate reader Powerscan HT (Dainippon Pharmaceutical). The biotinylated TC11 was immobilized on SA sensor chips (GE Healthcare) by passing HBS-EP buffer at a flow rate of 10 µL/min on a Biacore 3000 instrument (GE Healthcare). Amounts of biotinylated TC11 immobilized on the chips in flow cells Fc-1, Fc-2, Fc-3 and Fc-4 were 203, 205, 206 and 207 RU, respectively. The 4th to 7th fractions containing mRNA-displayed proteins were diluted with HBS-EP buffer to 300 µL and injected onto the antigen-immobilized sensor chip. The selection experiments were performed at 25°C with the Biacore 3000 using HBS-EP buffer at a flow rate of 20 µL/min. After association for 250 s and dissociation for 1000 s, the sensor surfaces were washed once with HBS-EP buffer for 600 s. The bound molecules were eluted competitively from the sensor surface with 7 µL of 200 µM free TC11 for 600 s. Then, recovered solution was subjected to RT-PCR in a total volume of 100 µL containing 1 mM dNTPs, 50 mM Tris-HCl, pH 8.0, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 200 U of RNase inhibitor, 500 U of ReverTraAce (Toyobo), and 50 pmol 3RV30 primer (5'-TTTTTTTCTTGTCGTCATCGTCCTTGTA-3'), at 50°C for 30 min and heated at 99°C for 5 min. The RT product was amplified by PCR with KOD-plus DNA polymerase using primers Gsp6Omega F (5'-GGAAGATCTATTTAGGTGACACTATA-GAACAACAACAACAACAACAACAACAATAATG-3') and 3RV30 [24–36 cycles of 30 s at 94°C, 30 s at 58°C, and 2 min at 68°C]. The resulting DNA was purified using a Wizard PCR preps DNA purification kit (Promega). Finally, selected DNAs were cloned using a TOPO TA cloning kit (Invitrogen) and sequenced with a CEQ 2000 DNA analysis system (Beckman Coulter, Brea, CA). Genetyx-mac 13.0.10 sequence analysis software and ClustalX 1.83 were used for alignment and sequence manipulations.

Surface Plasmon Resonance Analysis

Binding kinetics was determined by surface plasmon resonance (SPR) analysis with a Biacore 3000 (GE Healthcare). All experiments were performed at 25°C using HBS-EP buffer. Biotinylated TC11 was immobilized onto the SA sensor chip (GE Healthcare). The measurements were performed under conditions of 276 resonance units of the ligand and at a flow rate of 30 µL/min. To determine dissociation constants, four different concentrations of partially purified monomeric NPM and oligomeric NPM were injected. The injection period for association was 60 s. After each measurement, the chip surface was regenerated with 10 µL of Glycine 2.0 (GE Healthcare). The binding data were analyzed with the steady-state affinity model in the BIAevaluation software ver. 4.1 (GE Healthcare).

Immunofluorescence Assay and TUNEL Staining

HeLa cells on coverslips were treated with 5–20 µM TC11 for 6 h and then fixed with cold methanol and blocked with 1% BSA in PBS for 30 min. The sample was stained with antibody against NPM (Invitrogen) or γ -tubulin followed by Alexa488-conjugated anti-mouse IgG (Invitrogen) or CF568-conjugated anti-rabbit IgG (Biotium, Hayward, CA), respectively.

siRNA Transfection

HeLa cells (5×10^4 cells) were transfected with NPM siRNA oligonucleotides (5'-ATGGAATGTTATGATAGGACA-3') or luciferase siRNA oligonucleotides (5'-CGTACGCGGAA-TACTTTCGA-3') (Invitrogen) using a Neon transfection system (Invitrogen) according to the manufacturer's instructions.

Measurement of Caspase-9 Activity

Caspase-9 activity was measured with a luminescence-based assay kit, Caspase-Glo 9 Assay (Promega). After having been transfected with siRNA, 1×10^4 HeLa cells were cultured in 96-well plates for 72 h. At the end of incubation, 100 μ l of assay reagent was added and incubation was continued for 1 h at room temperature. Luminescence was measured using a Microplate luminometer (Promega).

Supporting Information

Figure S1 Chemical structures of phthalimide derivatives. (TIF)

Figure S2 Screening of compound that inhibit multiple myeloma cell lines. (A) KMS34 cells (1×10^4 cells/well) in 96-well plate were incubated with 50 μ M of each compound from phthalimide derivatives library for 0, 24, 48 or 72 h. Then cell viability was determined with WST-1 assay according to instructions provided by the manufacturer. (B) KMS34 or RPMI8226 cells were treated with 0, 5 or 50 μ M TC11 or TC15 for 6 h, respectively. The whole cell lysates were analyzed by Western blot with anti-PARP antibody. (TIF)

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Figure S3 TC11 or LMB does not affect localization of NPM on centrosome. (A) HeLa cells were treated with 5 μ M TC11 or 100 nM LMB for 6 h. Then, immunofluorescence staining of NPM (green) or γ -tubulin (red) was performed. Representative mitotic cells under the indicated conditions are shown. White arrowheads and arrows indicate NPM and centrosomes, respectively. White arrow indicates centrosome. Bar; 10 μ m. (TIF)

Figure S4 Thalidomide does not induce multipolarity of mitotic cells. HeLa cells were treated with 10 or 20 μ M thalidomide for 6 h. Then, immunofluorescence staining of γ -tubulin was performed. At least 50 mitotic cells were counted in three independent experiments. The ratio of cells with multipolar spindles under the indicated conditions was quantified. (TIF)

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Author Contributions

Conceived and designed the experiments: HT YH HY. Performed the experiments: HS FT NT IH NM YO WD. Analyzed the data: HS FT NT ND SI YH HY. Contributed reagents/materials/analysis tools: YO TY MS TS YH HY. Wrote the paper: HS NT ND YH HY.

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Altered stratum corneum barrier and enhanced percutaneous immune responses in filaggrin-null mice

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Background: Loss-of-function mutations in filaggrin are major predisposing factors for atopic dermatitis. Although various reports suggest a critical role for filaggrin in stratum corneum (SC) barrier formation, the lack of filaggrin-null (*Flg*^{-/-}) mice has hampered detailed *in vivo* analysis of filaggrin's functions. **Objective:** We sought to generate *Flg*^{-/-} mice and to assess the effect of filaggrin loss on SC barrier function and percutaneous immune responses.

Methods: We generated *Flg*^{-/-} mice using gene targeting and assessed the morphology, hydration, mechanical strength, and antigen permeability of their SC. Percutaneous immune responses were evaluated through irritant- and hapten-induced contact hypersensitivity studies and by measuring humoral responses to epicutaneous sensitization with protein antigen.

Results: Newborn *Flg*^{-/-} mice exhibited dry scaly skin. Despite marked decreases in natural moisturizing factor levels, which are filaggrin degradation products, SC hydration and transepidermal water loss were normal. Microscopic analyses suggested premature shedding of SC layers, and indeed, increased desquamation under mechanical stress was demonstrated. Loss of keratin patterns, which are critical for corneocyte stabilization, is likely attributable to fragility in the *Flg*^{-/-} SC. Antigens penetrated the *Flg*^{-/-} SC more efficiently, leading to enhanced responses in hapten-induced contact hypersensitivity and higher serum levels of anti-ovalbumin IgG₁ and IgE.

Conclusion: Complete filaggrin deficiency led to altered barrier integrity and enhanced sensitization, which are important

factors in early-phase atopic dermatitis. *Flg*^{-/-} mice should provide a valuable tool to further explore additional factors the dysfunction of which leads to uncontrolled inflammation in patients with atopic diseases. (J Allergy Clin Immunol 2012;129:1538-46.)

Key words: Filaggrin, filaggrin-null mice, atopic dermatitis, ichthyosis vulgaris, barrier function, flaky tail mice, stratum corneum, percutaneous immune response

The skin is a vital barrier that segregates living organisms from the external environment, thereby protecting them from a variety of physical insults.¹ The stratum corneum (SC) is the outermost layer of the epidermis and acts as the first line of defense. The SC is produced by a highly organized differentiation process in which keratinocytes in the basal layer of the epidermis move to the spinous and granular layers, ultimately forming a tough multilayer of corneocytes rich in intercellular lipids, such as ceramides, cholesterol, and free fatty acids.²

Filaggrin,³ a major structural protein in the SC, is produced as the precursor profilaggrin.⁴ Profilaggrin is the main constituent of keratohyalin granules in the granular layer (stratum granulosum [SG]) of the epidermis.⁴ During the later stages of epidermal terminal differentiation, profilaggrin is dephosphorylated and proteolyzed into filaggrin monomers in the SG.⁴ It has been shown that *in vitro* filaggrin monomers bind to and assemble keratin intermediate filaments, thereby generating macrofibrils.^{3,5} Filaggrin is also postulated to contribute to the mechanical strength and integrity of the SC *in vivo*.⁶ In the upper layers of the SC, filaggrin monomers are further processed to hygroscopic amino acids and their derivatives by proteases.⁷ Together with chloride and sodium ions, lactate, and urea, filaggrin breakdown products form natural moisturizing factors (NMFs), which are believed to play a major role in SC hydration.⁸ Although circumstantial evidence suggests that filaggrin is crucial for maintaining SC integrity, its detailed mechanisms and *in vivo* roles remain elusive.

Recently, loss-of-function mutations in the filaggrin gene (*FLG*) were reported to cause ichthyosis vulgaris (IV),⁹ a common autosomal dominant keratinization disorder characterized by scaly skin, and were further identified as major predisposing factors for atopic dermatitis (AD).^{10,11} AD is a chronic skin disorder characterized by dry skin and eczema and is frequently associated with increased serum IgE levels and a personal or family history of AD, allergic rhinitis, and/or asthma.¹² It has been hypothesized that SC barrier disruption caused by filaggrin deficiency is the primary event in AD, allowing antigen penetration and percutaneous sensitization, followed by allergic responses

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