AWARD NUMBER: W81XWH-13-1-0242

TITLE: Regulation of Mitochondria Function by TRAF3 in B Lymphocytes and B cell Malignancies

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REPORT DATE: October 2015

TYPE OF REPORT: Final report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Final	1 Aug 2013 – 31 Jul 2015

4. TITLE AND SUBTITLE

Regulation of mitochondria function by TRAF3 in B Lymphocytes and B cell malignancies

5a. CONTRACT NUMBER
W81XWH-13-1-0242

5b. GRANT NUMBER
W81XWH-13-1-0242

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Rutgers, the State University of New Jersey
New Brunswick, NJ 08901

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project aims to investigate how a new tumor suppressor gene, TRAF3, regulates mitochondria function in B lymphocytes and B cell malignancies. TRAF3 deletions and mutations occur in a variety of B cell malignancies, including B cell chronic lymphocytic leukemia (B-CLL), non-Hodgkin lymphoma (NHL, such as splenic marginal zone lymphoma and mantle cell lymphoma), multiple myeloma (MM), and Waldenström's macroglobulinemia. We found that specific deletion of TRAF3 from B lymphocytes results in remarkably prolonged survival of mature B cells, which eventually leads to development of splenic marginal zone lymphoma or B1 lymphoma in mice. In this context, understanding how TRAF3 promotes B cell apoptosis is critical for rational design of therapeutic intervention of human B cell neoplasms. In pursuing such underlying mechanisms, we have obtained an unexpected but highly interesting finding. Although it has been widely believed that in the absence of stimulation, TRAF3 protein is evenly distributed in the cytosol, we found that most cellular TRAF3 is localized at mitochondria in resting splenic B cells. This proposal thus aims to test the central hypothesis that TRAF3 directly modulates the physiology of mitochondria to induce apoptosis in B lymphocytes. We will also delineate the profile of proteins assembled in the mitochondrial TRAF3 signaling complex of B cells. Our long-term goal is to gain new insights into the complex mechanisms of B lymphomagenesis, and to identify new therapeutic targets for the treatment of B-CLL, NHL and MM.

15. SUBJECT TERMS

TRAF3, mitochondria, apoptosis, oncogenic B cell survival, B cell malignancies

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	119	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

Annual progress report

1. INTRODUCTION:

This project aims to investigate how a new tumor suppressor gene, TRAF3, regulates mitochondria function in B lymphocytes and B cell malignancies. TRAF3 deletions and mutations occur in a variety of B cell malignancies, including B cell chronic lymphocytic leukemia (B-CLL), non-Hodgkin lymphoma (NHL, such as splenic marginal zone lymphoma and mantle cell lymphoma), multiple myeloma (MM), and Waldenström's macroglobulinemia. We found that specific deletion of TRAF3 from B lymphocytes results in remarkably prolonged survival of mature B cells, which eventually leads to development of splenic marginal zone lymphoma or B1 lymphoma in mice. In this context, understanding how TRAF3 promotes B cell apoptosis is critical for rational design of therapeutic intervention of human B cell neoplasms. In pursuing such underlying mechanisms, we have obtained an unexpected but highly interesting finding. Although it has been widely believed that in the absence of stimulation, TRAF3 protein is evenly distributed in the cytosol, we found that most cellular TRAF3 is localized at mitochondria in resting splenic B cells. This proposal thus aims to test the central hypothesis that TRAF3 directly modulates the physiology of mitochondria to induce apoptosis in B lymphocytes. We will also delineate the profile of proteins assembled in the mitochondrial TRAF3 signaling complex of B cells. Our long-term goal is to gain new insights into the complex mechanisms of B lymphomagenesis, and to identify new therapeutic targets for the treatment of B-CLL, NHL and MM.

2. KEYWORDS:

TRAF3, B lymphocytes, mitochondria, B cell chronic lymphocytic leukemia, non-Hodgkin lymphoma, multiple myeloma, affinity purification, mass spectrometry-based sequencing.

3. ACCOMPLISHMENTS:

(1) What were the major goals of the project?

There are two major goals of this project:

Aim 1. **To elucidate the roles of TRAF3 in modulating mitochondrial functions**

We will perform complementary studies using a new mouse model (B-TRAF3$^{-/-}$ mice) and human patient-derived multiple myeloma cell lines with TRAF3 deletions or mutations. We will analyze a variety of mitochondrial functions, including morphology, number, membrane potential, respiration and energy production, reactive oxygen species production, mitochondria phospholipid levels, and mitochondrial gene expression. We will also employ mutagenesis to determine what structural feature(s) of TRAF3 are required for each of its mitochondria regulatory roles.

Specific tasks of Aim 1:
1a. Mitochondria membrane potential: completed.
1b. Mitochondria morphology and number: completed.
1c. Mitochondria respiration and energy production: in progress.
1d. Mitochondria ROS production and adenine nucleotides: completed.
1e. Mitochondrial phospholipid levels: completed.
1f. Mitochondrial gene expression: in progress.
1g. Reconstitution with TRAF3 mutants: completed.

Aim 2. To identify novel TRAF3-interacting proteins in mitochondria of B lymphocytes

Considering that TRAF3 does not contain any mitochondria targeting motif nor transmembrane domain, we hypothesize that TRAF3 is targeted to mitochondria through interactions with mitochondrial proteins. To test this, we propose to identify novel mitochondrial TRAF3-interacting proteins using biochemical affinity purification followed by mass spectrometry-based sequencing. We will perform extensive proteomic and bioinformative analyses to prioritize the identified proteins. We will next employ genetic means to explore the functions of identified proteins in the survival of normal and malignant B cells.

Specific tasks of Aim 2:
2a. Generation and testing of hTRAF3 vectors for tandem affinity purification: completed.
2b. Tandem affinity purification of mitochondrial TRAF3-interacting proteins: completed.
2c. Mass spectrometry-based sequencing of purified proteins: completed.
2d. Proteomic bioinformatic analyses of identified proteins: completed.
2e. Prioritization of identified proteins: completed.
2f. Verification of interaction with TRAF3 by TAP or co-immunoprecipitation: completed.
2g. Lentiviral shRNA vector-mediated knockdown in TRAF3-/- tumor B cells: in progress.
2h. Lentiviral vector-mediated ectopic overexpression in B cells: in progress.

(2) What was accomplished under these goals?

Aim 1: **To elucidate the roles of TRAF3 in modulating mitochondrial functions**

Our newest data demonstrated that most cellular TRAF3 is not evenly distributed in the cytosol as widely believed, but localized at mitochondria in resting splenic B cells. This led us to test a new hypothesis that TRAF3 directly modulates the physiology of mitochondria to induce apoptosis in B lymphocytes. Such study allowed us to delineate the roles of TRAF3 in regulating mitochondria functions in Aim 1.

1) Mitochondrial membrane potential: Mitochondrial membrane potential change precedes mitochondria-dependent apoptosis. We measured the mitochondrial membrane potential changes in LMC and premalignant TRAF3$^{-/-}$ B cells, and found that TRAF3 deletion drastically inhibited the mitochondrial membrane permeabilization in resting splenic B cells. This supports our hypothesis, and prompts us to thoroughly examine mitochondria morphology and functions.

2) Mitochondrion morphology and number: It is known that mitochondrion morphology and number often reflects its functional state. We have purified LMC and premalignant TRAF3$^{-/-}$ splenic B cells, and fixed the cells in 0.1M cacodylate buffer with 2.5% glutaraldehyde, 4% paraformaldehyde, and 8 mM $CaCl_2$. We have prepared the samples for electron microscopic (EM) examination, and have analyzed on a JOEL 1200EX electron microscope to discern potential changes in mitochondrial morphology or number caused by TRAF3 deficiency. We found that premalignant TRAF3$^{-/-}$ splenic B cells generally have bigger mitochondria than LMC B cells (Fig. 1, top panel). When cultured *ex vivo* in the absence of survival factors for 24 hours, mitochondria of LMC B cells exhibited sick morphology, while mitochondria of TRAF3$^{-/-}$ B cells maintained healthy morphology (Fig. 1, bottom panel). Our findings further support our hypothesis that TRAF3 plays a role in regulating mitochondrial morphology and physiology in B lymphocytes.

3) Mitochondrion respiration and energy production: The primary function of mitochondria includes respiration and energy production. We are in the process of measuring four key parameters of these functions, including basal respiration, ATP turnover, proton leak, and maximal respiration, using a Cell Mito Stress Test Kit (Seahorse). Using this assay, the rate of mitochondrial ATP synthesis in a

defined basal state can be estimated from the decrease in respiration on inhibiting the ATP synthase with oligomycin. Maximal respiratory capacity and total reserve capacity are also measured by injecting the mitochondrial uncoupler FCCP, and Complex III inhibitor antimycin, respectively. We are repeating these experiments.

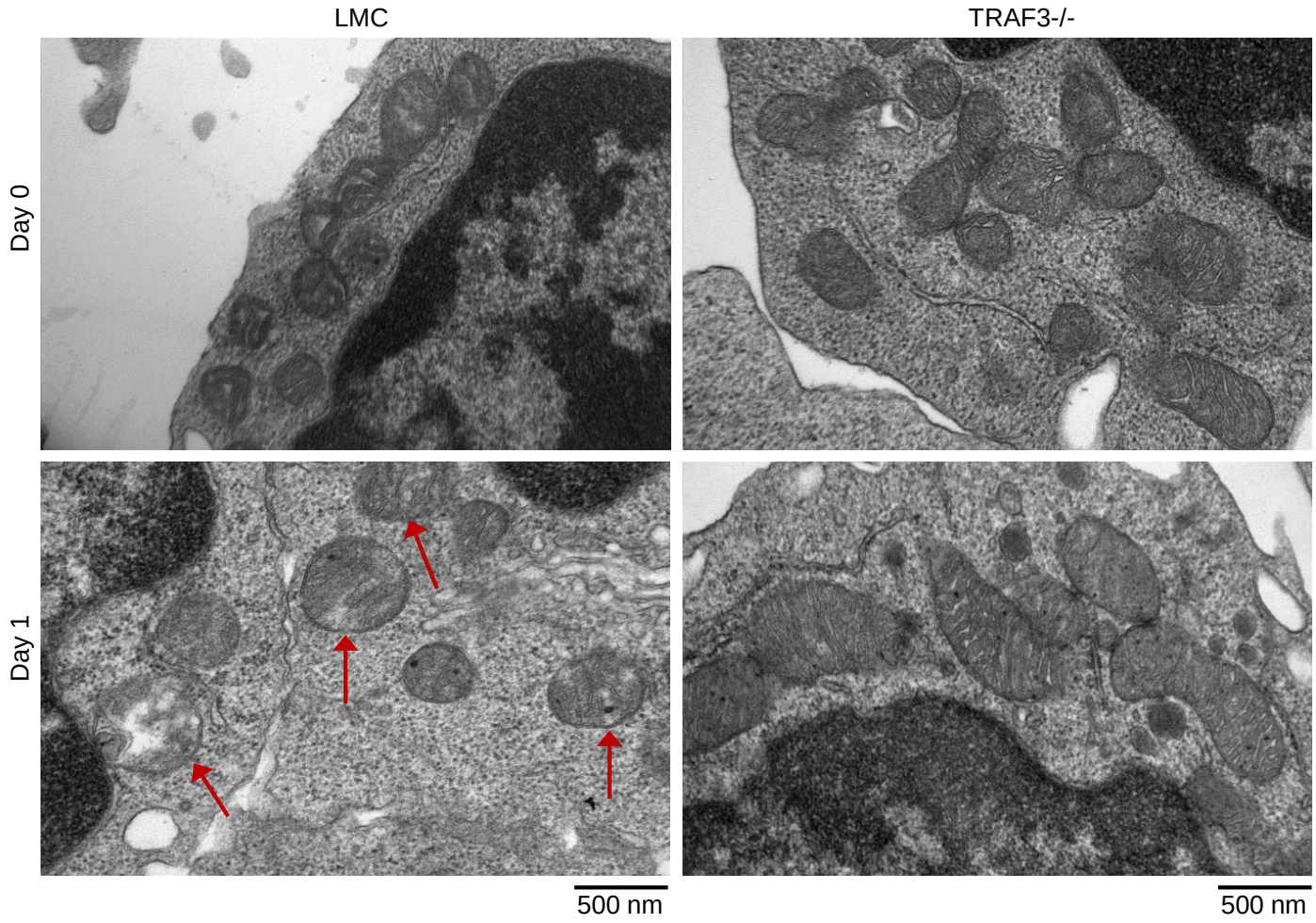

Fig. 1. TRAF3 regulates mitochondrial morphology and physiology in resting B lymphocytes. Resting splenic B cells were purified from young adult B-TRAF3$^{-/-}$ mice (TRAF3-/-) or littermate control mice (LMC). Morphology and healthy states of mitochondria in cells were analyzed by electron microscopic (EM) examination. Top panel images are representative EM micrographs of resting splenic B cells directly purified from mice (Day 0). Bottom panel images are representative EM micrographs of resting splenic B cells after cultured *ex vivo* in the absence of survival factors for 24 hours (Day 1). Sick mitochondria are indicated with red arrows.

4) Mitochondrial reactive oxygen species (ROS) production and adenine nucleotides: Mitochondrial dysfunction may lead to increased ROS production and consequently oxidative stress, and altered TCA cycle intermediates. We have investigated the effects of TRAF3 on ROS production in mouse and human B cells. We determined ROS level using 2'-7'-dichlorodihydro-fluorescene diacetate (DCF-DA, Molecular Probes) staining followed by FACS analyses. We found that there was no significant difference in ROS production between wild type and TRAF3$^{-/-}$ B cells as revealed by DCF-DA staining. We also measured major TCA cycle intermediates, including ATP, ADP, and AMP, by LC-MS. However, there were no significant differences in the levels of these molecules between wild type and TRAF3$^{-/-}$ B cells either.

5) Mitochondrial phospholipid levels: Mitochondrial phospholipids play critical roles in regulating mitochondria functions. We have extracted total cellular lipids and mitochondrial lipids from LMC and premalignant TRAF3$^{-/-}$ splenic B cells, and have analyzed the levels of **169 lipids and phospholipids** in these samples using LC-MS/MS, including phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cardiolipin (CL). We have completed the analyses of the MS data of 169 lipids and phospholipids. We did not detect any significant differences between wild type and TRAF3$^{-/-}$ B cells in the levels of 150 lipids and phospholipids examined in our study. Interestingly, our results revealed that TRAF3$^{-/-}$ B cells contained significantly elevated levels of 11 lipids/phospholipids but decreased levels of 8 lipids than LMC B cells. Representative examples of upregulated phospholipids are phosphatidylcholine [PC; such as PC(32:2) and PC(34:3)], phosphatidylethanolamine [PE; including PE(32:1), PE(34:2), PE(36:3), PE(46:10), and PE(O-36:1)], and phosphatidylinositol [PI; such as PI(36:4), PI(38:4) and PI(38:5)] (Fig. 2). The most strikingly downregulated lipids are diacylglycerol [DG; including DG_NH4(42:1), DG_NH4(40:1), DG_NH4(38:0), DG_NH4(36:0), and DG_NH4(34:0)] and monoacylglycerol [MG; such as MG_Na(16:0) and MG_Na(18:0)] (Fig. 2). Together, our results indicate that TRAF3 specifically regulate the levels of PC, PE, PI, DG and MG in B lymphocytes.

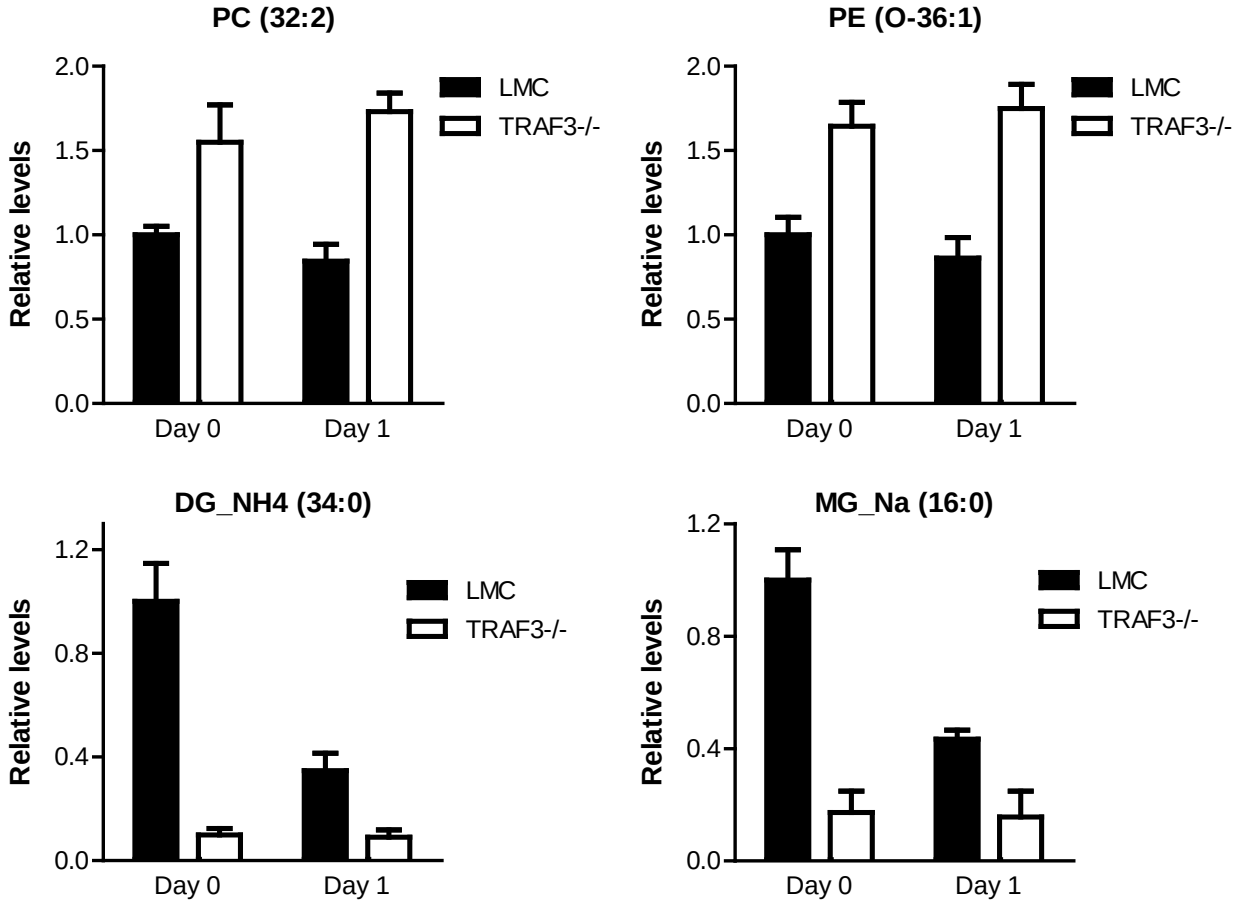

Fig. 2. TRAF3 regulates the levels of several phospholipids in resting B lymphocytes. Resting splenic B cells were purified from young adult B-TRAF3$^{-/-}$ mice (TRAF3-/-) or littermate control mice (LMC). Levels of lipids and phospholipids in resting splenic B cells directly purified from mice (Day 0) or after cultured *ex vivo* in the absence of survival factors for 24 hours (Day 1) were measured using LC-MS/MS. Graphs depict relative levels of representative phosphatidylcholine (PC), phosphatidylethanolamine (PE), diacylglycerol (DG), and monoacylglycerol (MG). Results shown are all $p < 0.01$ (*t* test, significantly different between LMC and TRAF3-/-).

6) Mitochondrial gene expression: Mitochondrion-specific gene expression is essential to proper mitochondrion functions. We have prepared total cellular RNA and cDNA from LMC and premalignant TRAF3$^{-/-}$ splenic B cells. We have also prepared protein lysates from purified mitochondria of LMC and premalignant TRAF3$^{-/-}$ splenic B cells. We are in progress in measuring the transcript levels of key mitochondrial genes by quantitative real-time PCR, including mitofusin 1 and 2, superoxide dismutase 2 (SOD2), mitochondrial uncoupling protein 3 (UCP3), mitochondrial inner membrane protein (IMMT), and ATP synthase. We are also performing Western blot analyses to determine the protein levels of the above mitochondrial genes.

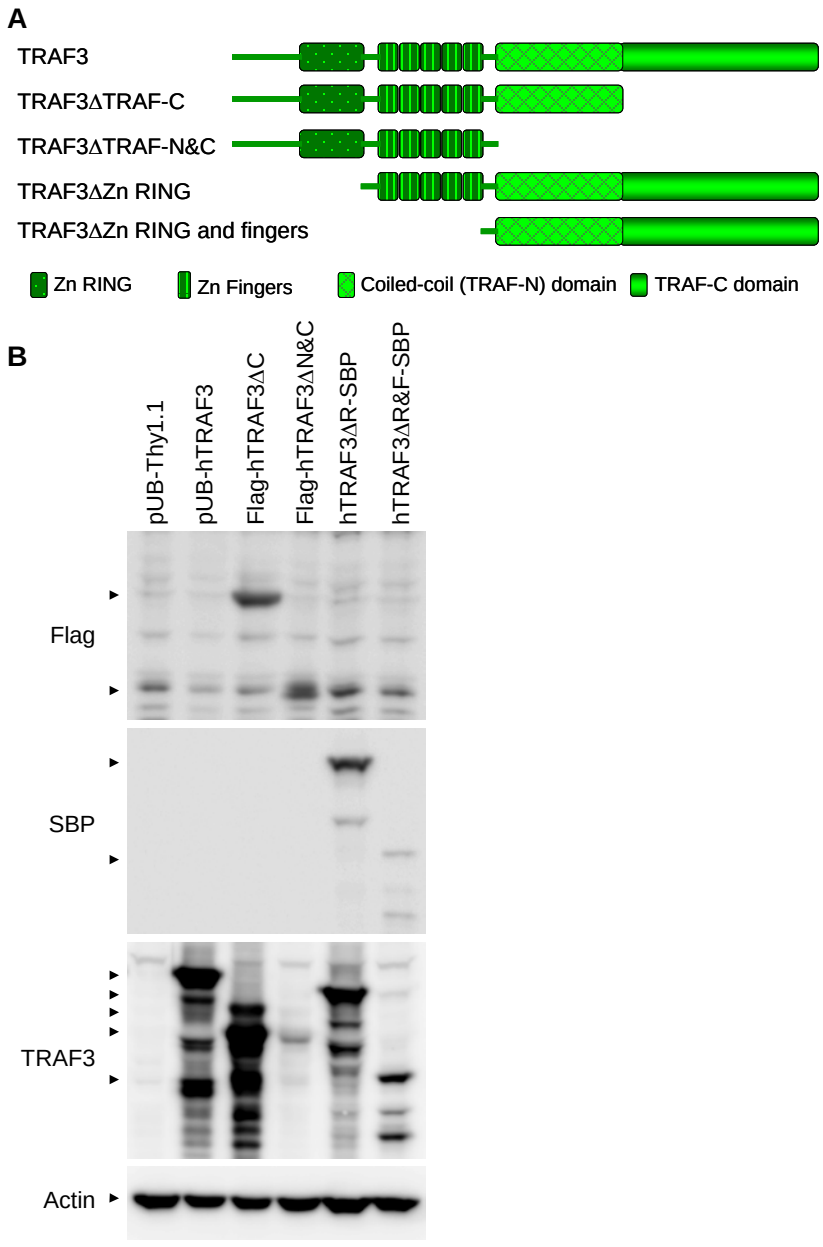

Fig. 3. Lentiviral expression vectors of TRAF3 mutants generated in this study. (**A**) Schematic diagram of the TRAF3 deletion mutants generated in this study. Domains of TRAF3 that are deleted are indicated. (**B**) Verification of expression of TRAF3 mutants by Western blot analysis. Human multiple myeloma 8226 cells (containing biallelic deletions of the TRAF3 gene) were transduced with lentiviral expression vectors of wild type or mutants of human TRAF3. Mutants analyzed include N-terminal Flag-tagged TRAF3ΔTRAF-C (Flag-hTRAF3ΔC), N-terminal Flag-tagged TRAF3ΔTRAF-N&C (Flag-hTRAF3ΔN&C), C-terminal SBP-tagged TRAF3ΔZinc RING (hTRAF3ΔR-SBP), and C-terminal SBP-tagged TRAF3ΔZinc RING and fingers (hTRAF3ΔR&F-SBP). Cells transduced with an empty lentiviral expression vector (pUB-Thy1.1) were used as control in these experiments. Transduction efficiency of each lentiviral vector is > 85%. Total cellular lysates were prepared on day 5 post transduction, and then analyzed by Western blot analyses. Proteins were immunoblotted for Flag, SBP, and TRAF3, followed by actin. Immunoblot of actin was used as a loading control. Please note that the N-terminal Flag-tagged TRAF3ΔTRAF-N&C (Flag-hTRAF3ΔN&C) was not detected by TRAF3 antibody, which recognizes amino acids 322-444 of human TRAF3. We also observed degradation of wild type or mutant TRAF3 in transduced human multiple myeloma 8226 cells.

7) Reconstitution with TRAF3 mutants: We have generated several lentiviral vectors to express deletion mutants of human TRAF3 that lack its different structural domains (Fig. 3A). We have used these lentiviral vectors to transduce TRAF3-deficient human multiple myeloma cell line 8226 cells, and have verified the expression of TRAF3 mutants using Western blot analysis (Fig. 3B). We next compared the cell cycle distribution among three cell types: (1) 8226 cells transduced with a control lentiviral vector; (2) 8226 cells transduced with wild type TRAF3; (3) 8226 cells transduced with each deletion mutant. Our results demonstrate that reconstitution with wild type TRAF3 induced apoptosis and inhibited proliferation of human multiple myeloma 8226 cells. Interestingly, we found that the four TRAF3 mutants examined have completely or partially lost the apoptotic and anti-proliferative functions of TRAF3 (Fig. 4). Our results suggest that the **TRAF-C**, **TRAF-N**, **Zinc RING**, and **Zing fingers** all contribute to the tumor suppressive functions of TRAF3 in B cells.

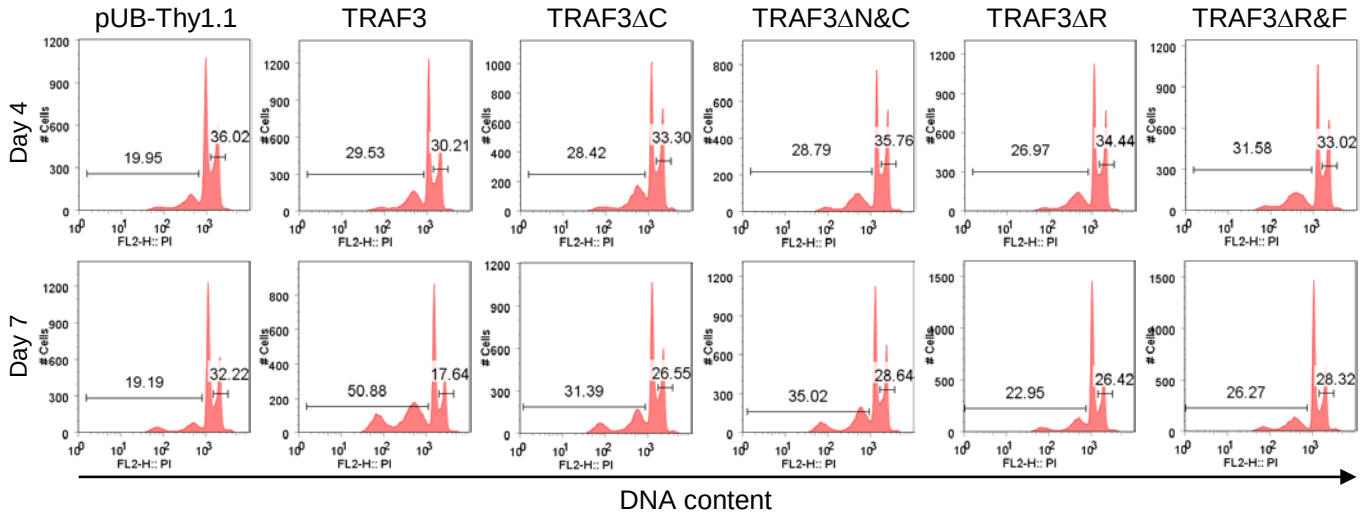

Fig. 4. **Tumor suppressive function of TRAF3 requires its TRAF-C, TRAF-N, Zinc Ring, and Zing finger domains.** Human multiple myeloma 8226 cells (containing biallelic deletions of the TRAF3 gene) were transduced with lentiviral expression vectors of wild type or mutants of human TRAF3. TRAF3 mutants analyzed include TRAF3ΔTRAF-C (TRAF3ΔC), TRAF3ΔTRAF-N&C (TRAF3ΔN&C), TRAF3ΔZinc RING (TRAF3ΔR), and TRAF3ΔZinc RING and fingers (TRAF3ΔR&F). Cells transduced with an empty lentiviral expression vector (pUB-Thy1.1) were used as control in these experiments. Transduction efficiency of each lentiviral vector is > 85%. Cell cycle distribution of transduced cells was determined on day 4 and day 7 post transduction. Cells were fixed, and then stained with propidium iodide (PI). Stained cells were subsequently analyzed on a flow cytometer. Representative FACS histograms of PI staining are shown, and percentages of apoptotic cells (DNA content < 2n; sub-G1) and proliferating cells (2n < DNA content ≤ 4n; S/G2/M) are indicated.

Aim 2: To identify novel TRAF3-interacting proteins in mitochondria of B lymphocytes

Given that TRAF3 does not contain any mitochondria targeting motif nor transmembrane domain, we hypothesize that TRAF3 is localized at mitochondria in resting splenic B cells through interaction with other mitochondrial proteins. We are testing this hypothesis in Aim 2.

1) Generation and testing of tagged hTRAF3 vectors for tandem affinity purification: We employed a newly developed TAP strategy that uses streptavidin-binding peptide (SBP, Stratagene) and 6xHis as the tandem tag. This new TAP strategy offers several advantages as compared to the conventional TAP systems. (1) The overall recovery of the dual affinity purification (>50%) is the highest of existing TAP tags. (2) The protease cleavage step in the original TAP protocol that caused yield loss is avoided, as both tags can be eluted from their respective resins under mild conditions. (3)

Both streptavidin and nickel resins are relatively inexpensive and have a high capacity. (4) The sequential purification can be performed in a single buffer system. (5) The immobilized metal affinity chromatography (IMAC) supports purification under denaturing conditions. We have generated lentiviral expression vectors of an N-terminal FLAG tag or a C-terminal SBP-6xHis tag in frame with the human TRAF3 coding sequence (pUB-Flag-hTRAF3 or pUB-hTRAF3-SBP-6xHis), respectively. We have used these vectors to transduce TRAF3-deficient human patient-derived MM cell lines (HMCLs) 8226 and LP1 cells, and our results verified that both tagged TRAF3 proteins maintain the mitochondrial localization of native TRAF3. We also tested the tandem affinity purification efficiency of pUB-hTRAF3-SBP-6xHis using transfected 293T cells, and confirmed that purification of transfected cell lysates using both streptavidin resin and nickel beads allowed high recovery rate of the SBP-6xHis-tagged TRAF3 (Fig. 5). Interestingly, however, we noticed that nickel beads also pulled down Flag-tagged TRAF3 as well as endogenous TRAF2 and TRAF6 independent of the 6xHis tag (Fig. 5). This reminds us that TRAF proteins contain Zinc binding motifs, and can therefore bind to nickel beads in the absence of histidine tag. Based on these results, we decide to discontinue the use of nickel beads, and isolate TRAF3-interacting proteins using one-step affinity purification by streptavidin resin.

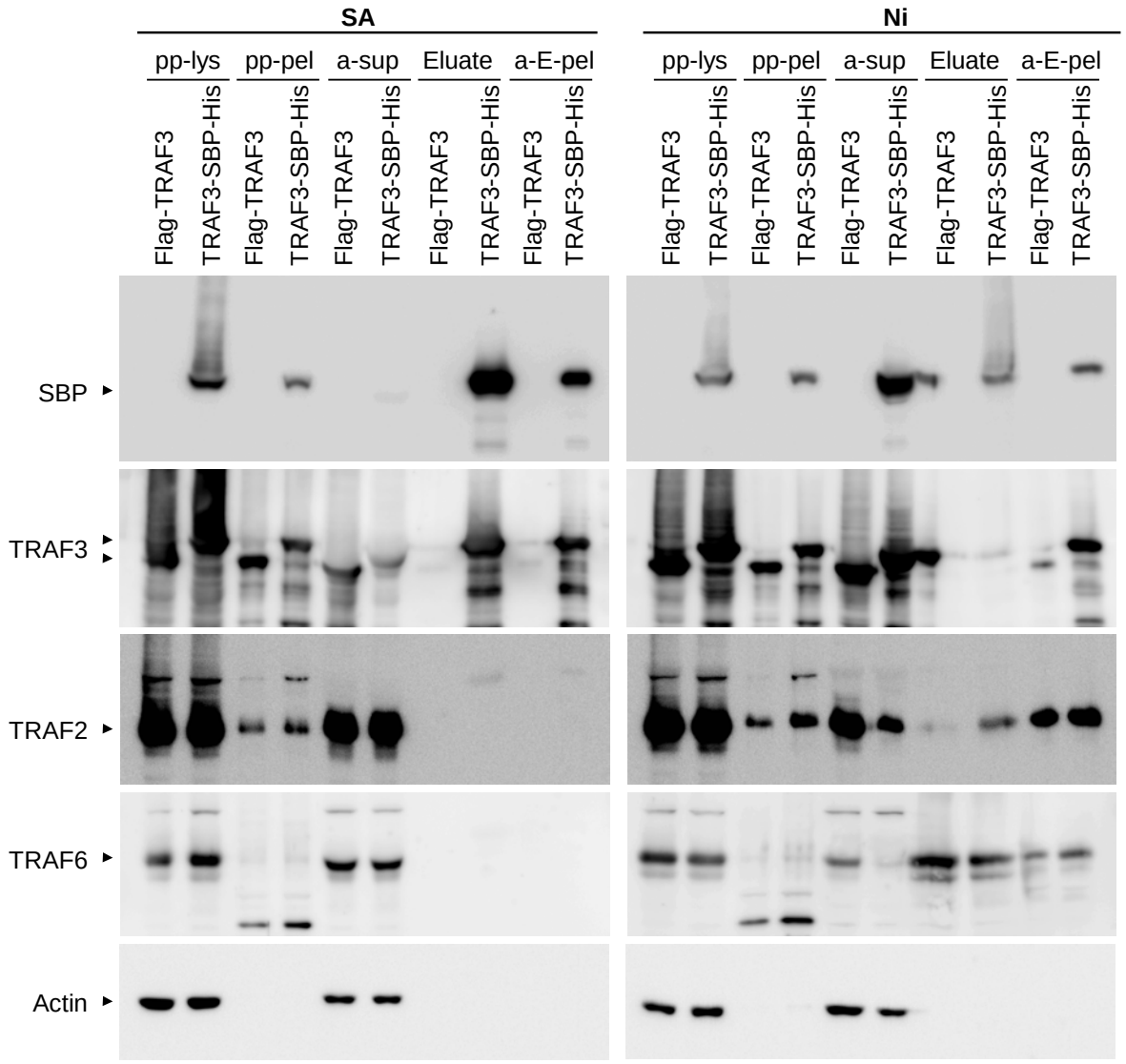

Fig. 5. **Testing of tandem affinity purification of tagged TRAF3.** 293T cells were transfected with lentiviral expression vectors of an N-terminal Flag-tagged TRAF3 (FLAG-TRAF3) or a C-terminal SBP-6xHis tagged TRAF3 (TRAF3-SBP-His). Transduction efficiency of each lentiviral vector is > 85%. Cells were harvested on day 3 post transfection, and total cellular proteins were lysed in 1% CHAPS lysis buffer. Transfected TRAF3-SBP-His were affinity purified by streptavidin-sepharose resin (SA) or nickel beads (Ni), respectively. Lysates of cells transfected with FLAG-TRAF3 that were purified by the same procedures were used as negative control in these experiments. Proteins were immunoblotted for SBP tag, TRAF3, TRAF2, and TRAF6, followed by actin. Blots of whole cell lysates before affinity purification (pp-lys) were used as the input control. Other samples examined include insoluble pellets of 1% CHAPS lysates (pp-pel), supernatant after affinity purification (immunoprecipitation) by SA or Ni (a-sup), eluates of immunoprecipitates (Eluate), and proteins that cannot be eluted and are still associated with beads after elution (a-E-pel).

2) Affinity purification of mitochondrial TRAF3-interacting proteins: We have used the validated lentiviral expression vector pUB-hTRAF3-SBP-6xHis to transduce human multiple myeloma 8226 cells (containing biallelic deletions of the TRAF3 gene). Use of these cells eliminates the affects of endogenous TRAF3. We purified mitochondria from transduced 8226 cells by biochemical fractionation, and solubilized mitochondrial proteins in 1% CHAPS lysis buffer. We then purified the SBP-6xHis-tagged TRAF3 and associated mitochondrial proteins using streptavidin resin. A small aliquot (5%) of the immunoprecipitates were used for Western blot analysis, and our results confirmed the high recovery rate of SBP-6xHis-tagged TRAF3 (Fig. 6). Cells transduced with Flag-tagged TRAF3 were subjected to the same purification procedure and used as negative control (Fig. 6). All residual streptavidin immunoprecipitates were separated on 4-16% gradient SDS-PAGE (Invitrogen) and visualized using Gel Code Blue (Pierce) staining. The gel lanes for TRAF3 complex (pUB-hTRAF3-SBP-6xHis) and negative control (pUB-Flag-hTRAF3) were each sectioned into 15 continuous slices, which were delivered to our collaborator Drs. David Perlman and Saw Kyin at Princeton University for further processing and sequencing by LC-MS/MS.

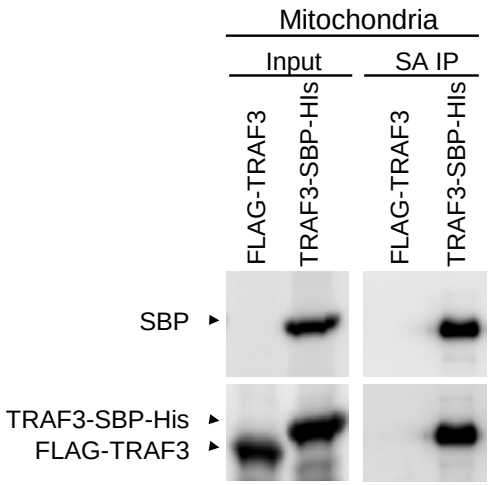

Fig. 6. Large scale affinity purification of SBP-6xHis-tagged TRAF3 from purified mitochondria of transduced human multiple myeloma cells. Human multiple myeloma 8226 cells (150×10^6 cells) were transduced with lentiviral expression vectors of N-terminal Flag-tagged TRAF3 (FLAG-TRAF3) or C-terminal SBP-6xHis tagged TRAF3 (TRAF3-SBP-His). Transduction efficiency of each lentiviral vector is > 85%. Tranduced cells were harvested on day 4 post transduction, and were fractionated to isolate mitochondria. Mitochondrial proteins were solubilized in 1% CHAPS lysis buffer, and then immunoprecipitated with streptavidin-sepharose beads. Immunoprecipitates of TRAF3-SBP-6xHis by streptavidin-sepharose beads (SA IP) from purified mitochondria were used to identify TRAF3-interacting proteins. Immunoprecipitates of FLAG-TRAF3 by streptavidin-sepharose beads were used as negative control in these experiments. Proteins were immunoblotted for SBP tag, and followed by TRAF3. Blots of mitochondrial lysates before immunoprecipitation were used as the input control.

3) Mass spectrometry-based sequencing of purified proteins: The gel slice samples were subjected to thiol reduction by TCEP, alkylation with iodoacetamide, and digestion with sequencing-grade modified trypsin. Peptides were eluted from the gel slices, desalted, and then subjected to reversed-phase nano-flow ultra high performance capillary liquid chromatography (uPLC) followed by high-resolution/ high-mass accuracy MS/MS analysis using an LC-MS platform consisting of an Eksigent Nano Ultra 2D Plus uPLC system hyphenated to a Thermo Orbi Velos mass spectrometer. The MS/MS

were set to operate in data dependent acquisition mode using a duty cycle in which the top 15 most abundant peptide ions in the full scan MS were targeted for MS/MS sequencing. Full scan MS1 spectra were acquired at 100,000 resolving power and can be expected to maintain mass calibration to within 2-3 ppm mass accuracy. Drs. David Perlman and Saw Kyin searched the LC-MS/MS data against the human IPI and UniProt databases using the Mascot and Proteome Discoverer search engines. Protein assignments were considered highly confident using a stringent false discovery rate threshold of <1%, as estimated by reversed database searching, and requiring that ≥2 peptide spectral counts per protein be unambiguously identified. We estimated rough relative protein amounts using spectra counting values. Through these studies, we **have successfully identified 156 TRAF3 interacting proteins at mitochondria of B cells**. Among these, 5 proteins are previously known TRAF3-interacting proteins, including TRAF2, TANK, NUP62, PYCARD, and UBE2N. Thus, 151 identified in our study represent novel TRAF3 interactors.

4) Proteomic bioinformatic analyses of identified proteins: We have performed the following proteomic bioinformatic analyses of our LC-MS/MS sequencing results. **(a)** Expression levels in mature B lymphocytes: We attempted to correlate our protein detection with the reported transcript expression levels of identified proteins in different B cell populations by surveying the public gene expression database of Immune Genome (http://www.immgen.org), including pro-B, pre-B, B1, marginal zone B, and follicular B cells. **(b)** Protein domain and motif analysis: We have analyzed the domain structure and known motifs of each identified protein using the following programs: UCSC Genome Browser (http://genome.ucsc.edu/), ExPASy PROSITE (http://prosite.expasy.org/), and MOTIF (http://www.genome.jp/tools/motif/). **(c)** Subcellular localization analysis: We have also searched the potential mitochondrial localization of identified proteins in the published literature, or predicted using the UCSC Genome Browser, Ingenuity Pathways Analysis, or Mitominer (http://mitominer.mrc-mbu.cam.ac.uk/release-3.1/begin.do). **(d)** Survey public cancer genome and gene expression databases: We have surveyed available cancer genome databases to determine whether the identified genes exhibit alterations in mRNA expression or DNA copy number, or mutations in primary human B lymphomas and other cancers, and whether alterations in these genes correlate with poor prognosis in human cancer patients (http://www.oncomine.org). **(e)** Functional clustering and pathway analysis: We have conducted allocation of identified genes in functional clusters and signaling pathways using Ingenuity Pathways Analysis software (CINJ).

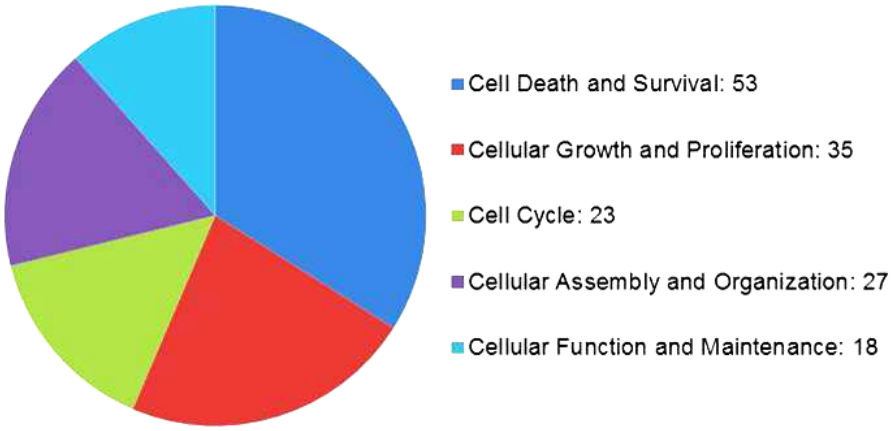

Fig. 7. The TRAF3-interactome in purified mitochondria of human MM cells identified by affinity purification followed by LC-MS/MS. Functional clustering and annotation of mitochondrial TRAF3-interactors identified in human MM cells was performed using Ingenuity Pathways Analysis software. The number of proteins of each functional category is shown.

Our results of functional annotation analyses by Ingenuity showed that mitochondrial TRAF3 interactors are mainly regulators of cell death and survival (53 of 156, 33.9%) (Fig. 7). Other TRAF3 interactors include regulators of cell growth and proliferation, cell cycle, cellular assembly and

organization, and cellular function and maintenance (Fig. 7). Notably, disease association analyses by Ingenuity revealed that cancer is the top disease associated with the TRAF3-interactome identified in our study, as **122 of the 156** (78.2%) TRAF3 interactors in mitochondria of B cells are **associated with cancer**. Together, results of these proteomic and bioinformatic analyses provided additional information to allow us to prioritize the list of mitochondrial TRAF3-interacting proteins identified in our study. We decided to further pursue those most likely to be relevant to human cancer and lymphomagenesis.

5) Prioritization of identified proteins: To select the most critical TRAF3-interacting proteins for further study, we have used the following prioritization scheme: (**a**) expression of the identified gene is clearly detected or high in mature B cells, and is **not** B cell subset-specific, as prolonged survival is observed in all mature B cell subsets, including follicular, marginal zone and B1 B cells of B-TRAF3$^{-/-}$ mice[1]; (**b**) the identified protein contains domain structure or motifs that may mediate direct interaction with TRAF3, such as the coiled-coiled domain or the "PXQXT/S" motif; (**c**) the identified protein is a known mitochondrial protein or contains a mitochondrial targeting motif; (**d**) alterations of the identified gene have been documented in human B-CLL, NHL and multiple myeloma in the published literature or in the public gene expression database of cancers (http://www.oncomine.org), including changes in mRNA expression, DNA copy number, or mutations. (**e**) for proteins within the same signaling pathway, ones that have been most consistently detected across replicates, have the highest number of unique identified peptides or LC-MS/MS spectral counting values, and have not been detected in the negative control sample were given the highest priority. Using this prioritization scheme, we have selected **8 novel TRAF3-interacting proteins** identified in our study for further verification and investigation.

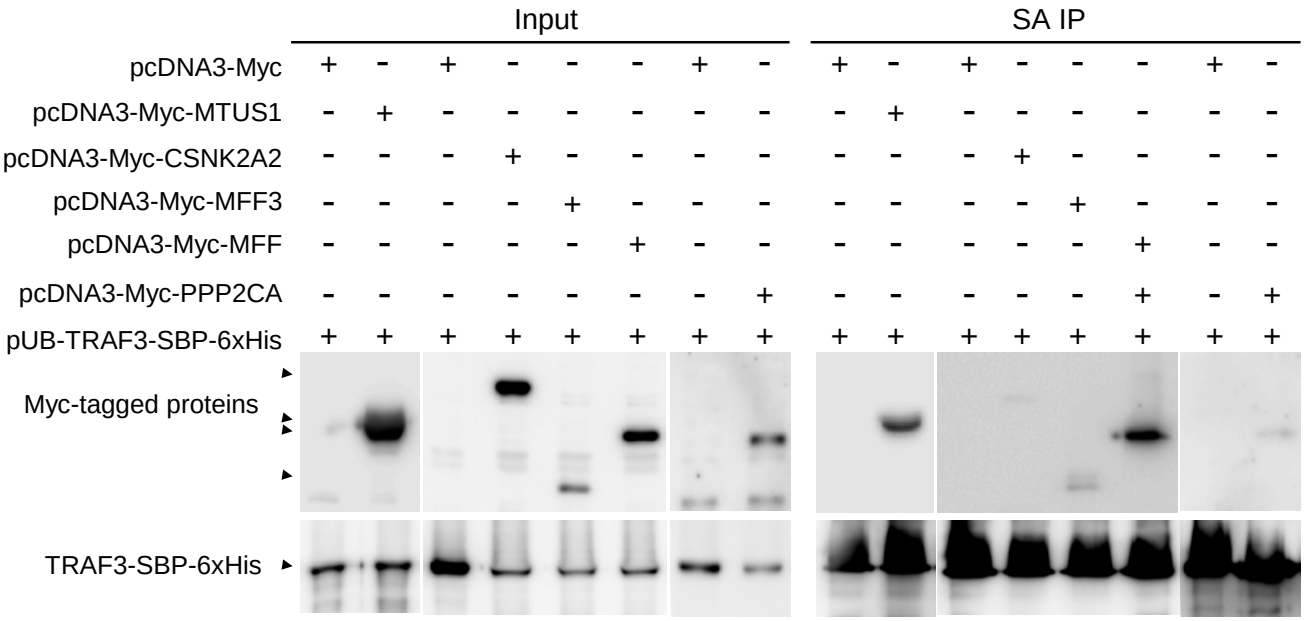

Fig. 8. **Verification of identified novel TRAF3-interacting proteins by co-immunoprecipitation.** 293T cells were transfected with expression vectors of a C-terminal SBP-6xHis tagged TRAF3 (TRAF3-SBP-His) and a novel TRAF3-interacting protein identified in our study tagged with Myc or an empty vector (pcDNA3-Myc). Myc-tagged TRAF3-interacting proteins shown in this figure include MTUS1, CSNK2A2, MFF3, MFF, and PPP2CA. Cells were harvested on day 2 post transfection, and total cellular proteins were lysed in 1% CHAPS lysis buffer. Transfected TRAF3-SBP-6xHis were immunoprecipitated (IP) by streptavidin-sepharose resin (SA). Lysates of cells transfected with TRAF3-SBP-6xHis and the empty vector that were purified by the same procedures were used as negative control in these experiments. Proteins were immunoblotted for Myc tag, followed by TRAF3. Blots of whole cell lysates before immunoprecipitation were used as the input control.

Blots of immunoprecipitated proteins are labeled as SA IP. These results showed that Myc tagged MTUS1, CSNK2A2, MFF3, MFF, and PPP2CA are co-immunoprecipitated with TRAF3-SBP-6xHis.

6) Verification of interaction with TRAF3 by co-immunoprecipitation: Among the 8 proteins that we selected to verify, we have first verified the association of TRAF3 with **6** identified proteins using the co-immunoprecipitation approach, including MFF, MFF3, MTUS1, PPP2CA, CSNK2A2, and NME (Fig. 8). Based on their priority scores and affinity for TRAF3, we next selected 3 proteins (MFF, MTUS1 and PPP2CA) for investigation of their direct binding to TRAF3 using GST-TRAF3 pull-down experiments. Our results demonstrated that MFF, MTUS1, and PPP2CA proteins were easily pulled down by GST-TRAF3 (Fig. 9).

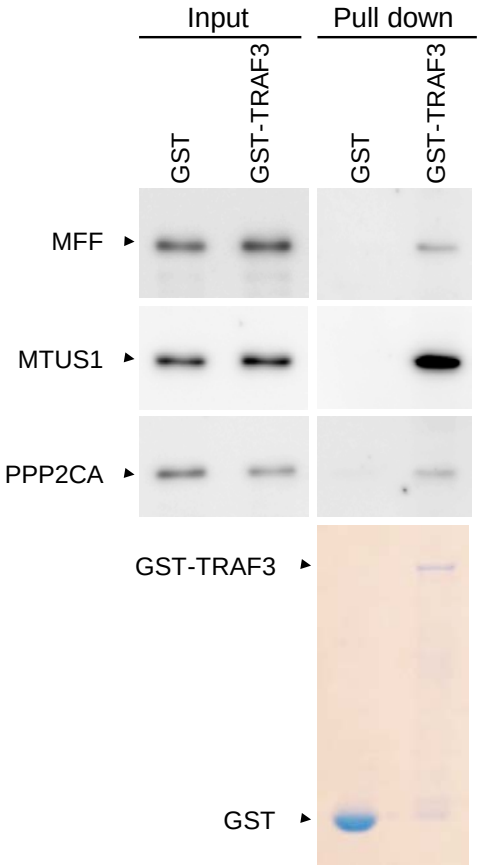

Fig. 9. **Direct binding of identified novel TRAF3-interacting proteins demonstrated by GST pull-down experiments.** Whole cell lysates of 293T cells were used in pull-down experiments by GST-TRAF3 fusion protein or GST. GST and GST-TRAF3 used for pull-down were analyzed by SDS-PAGE and visualized by GelCode blue staining (the bottom panel). Lysates that were purified by the same pull-down procedures with GST were used as negative control in these experiments. Proteins were immunoblotted for MFF, MTUS1 and PPP2CA. Blots of whole cell lysates before pull-down were used as the input control. Blots of proteins after purification by glutathione sepharose beads are labeled as "Pull down". These results showed that MFF, MTUS1, and PPP2CA can directly bind to TRAF3.

We subsequently mapped the structural domains of TRAF3 required for the direct binding with MFF or MTUS1 using GST fusion proteins of four deletion mutants of TRAF3 in comparison to wild type GST-TRAF3. Our data revealed that different domains of TRAF3 are required for interacting with MFF and MTUS1, respectively (Fig. 10). The TRAF-C and coiled-coil (TRAF-N) domains are most important for binding to MFF, while the Zinc fingers and Zing ring domains are required for mediating the interactions with MTUS1 (Fig. 10). Our findings warrants further functional investigation of MFF and MTUS1 in TRAF3-mediated apoptosis in B lymphocytes and TRAF3 inactivation-induced oncogenic survival in B cell malignancies. Together, the above results demonstrated the direct association of TRAF3 and the identified novel interacting proteins.

7) Lentiviral shRNA vector-mediated knockdown in B cells: To knockdown the specific genes to determine their functions in cell survival and apoptosis, we are in the process of generating lentiviral shRNA vectors specific for human MFF and MTUS1, respectively, to allow transduction in tumor B cells. We will use these lentiviral vectors to transduce TRAF3$^{-/-}$ B lymphoma cell lines or the human

MM cell line 8226 cells. Cells transduced with a scrambled shRNA control vector will be used as control. Specific knockdown of the protein will be verified by Western blot analysis. We will determine the functional consequence of each protein knockdown on the survival, proliferation, and tumorigenicity. (**a**) Survival and apoptosis: We will monitor the survival, apoptosis, and cell cycle distribution of transduced cells using annexin V staining and PI staining, followed by FACS analysis. (**b**) Proliferation of tumor B cells: Proliferation of transduced B lymphoma cells and HMCLs will be determined by CFSE labeling, MTT assay, and growth curves. (**c**) *In vivo* tumorigenicity test: For TRAF3$^{-/-}$ B lymphoma cell lines, we will *i.p.* inject cells transduced with the shRNA vector or a scrambled shRNA control into NOD SCID recipient mice. For HMCLs, we will *s.c.* inject transduced cells into the right flank of immunodeficient NSG recipient mice (Jackson Laboratory, Stock Number 005557). Recipient mice with transplanted B lymphomas or HMCL xenografts will be monitored daily, and will be examined for the presence and severity of B lymphomas or for the size of xenografts. Together, the knockdown studies will determine whether each identified protein is necessary for the survival of TRAF3$^{-/-}$ tumor B cells, and whether it can serve as a therapeutic target in B cell neoplasms.

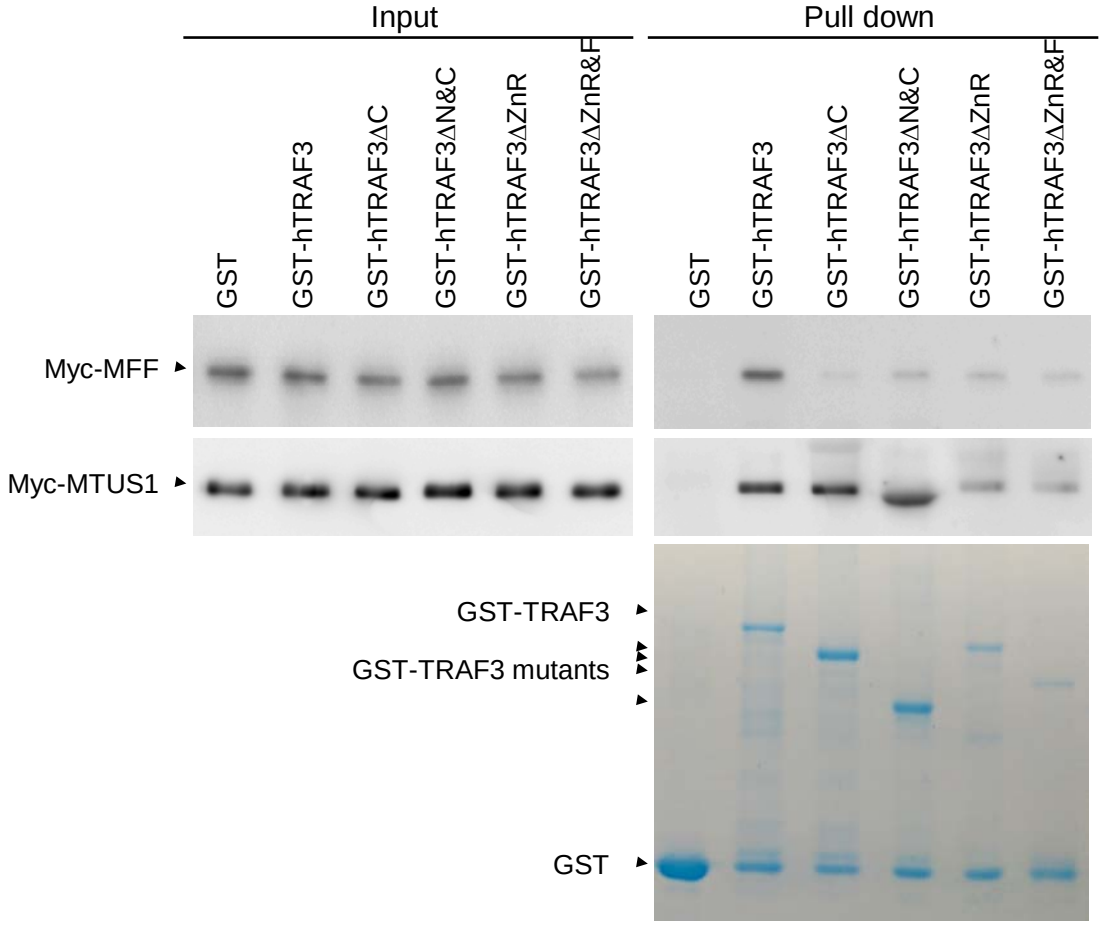

Fig. 10. **Mapping of the domains of TRAF3 required for the interaction with MFF and MTUS1.** Whole cell lysates of 293T cells transfected with expression vectors of Myc-tagged MFF or MTUS1 were used in pull-down experiments by GST, or GST fusion proteins of wild type or deletion mutants of TRAF3. GST, GST-TRAF3, and GST-TRAF3 deletion mutants used for pull-down were analyzed by SDS-PAGE and visualized by GelCode blue staining (the bottom panel). GST-TRAF3 deletion mutants studied include GST-hTRAF3ΔC, GST-hTRAF3ΔN&C, GST-TRAF3ΔZnR, and GST-TRAF3ΔZnR&F. Lysates that were purified by the same pull-down procedures with GST were used as negative control in these experiments. Proteins were

immunoblotted for MFF and MTUS1, respectively. Blots of whole cell lysates before pull-down were used as the input control. Blots of proteins after purification by glutathione sepharose beads are labeled as "Pull down". These results showed that MFF, MTUS1, and PPP2CA can directly bind to TRAF3.

8) Lentiviral vector-mediated ectopic overexpression in B cells: We have generated lentiviral expression vectors to ectopically overexpress MFF or MTUS1 in LMC and TRAF3$^{-/-}$ splenic B cells. Cells transduced with an empty lentiviral expression vector (pUB-Thy1.1) are used as control in these experiments. We have determined the transduction efficiency and verified the protein expression of MFF and MTUS1 by FACS and Western blot analyses (Fig. 11). We are now investigating the functional consequences of ectopic overexpression of MFF and MTUS1, respectively. Survival and apoptosis of transduced cells are being determined. Transduced cells are also being analyzed in the absence or presence of B cell stimuli (BAFF, CD40 ligand, LPS or CpG). We expect to unravel the roles of these novel TRAF3-interacting protein in regulating the survival of B lymphocytes.

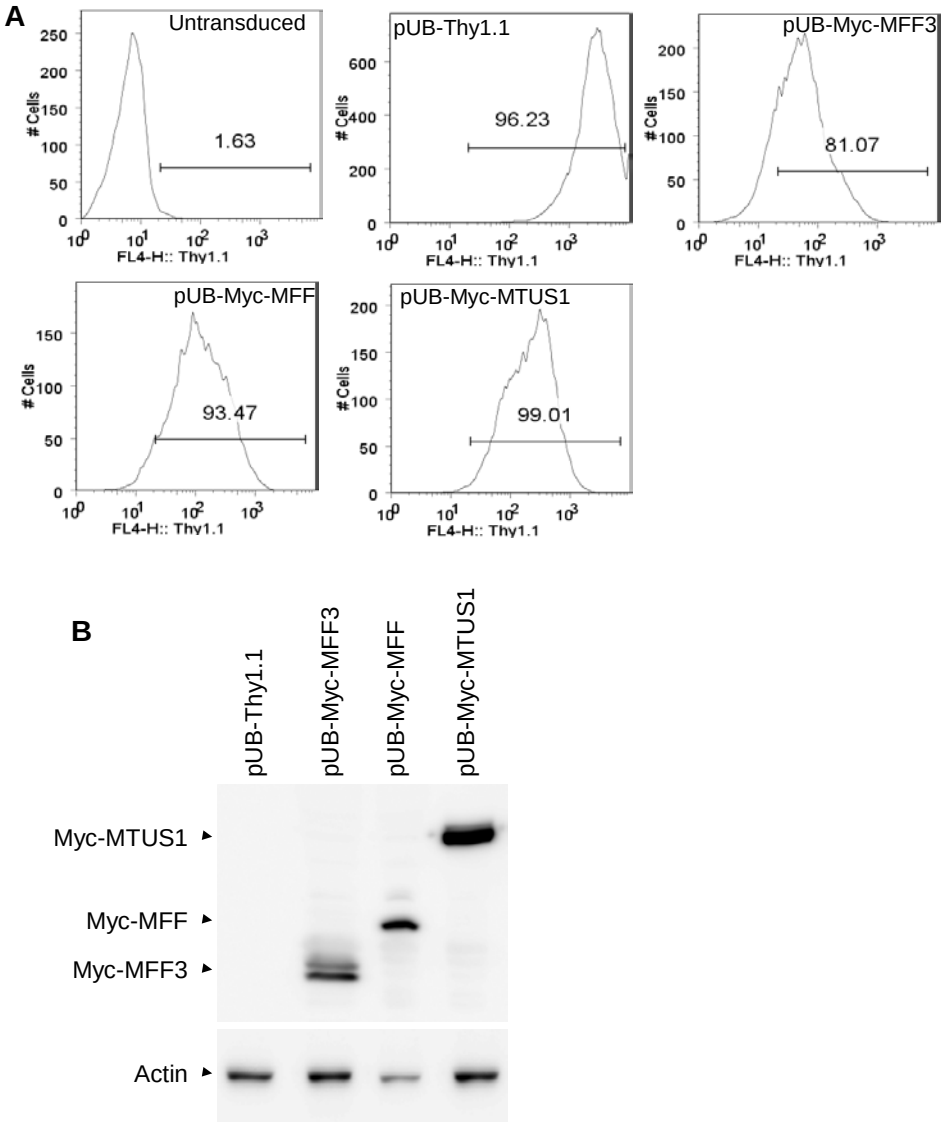

Fig. 11. **Lentiviral vector-mediated ectopic overexpression of MFF and MTUS1 in human MM cells.** Human MM 8226 cells were transduced with individual lentiviral expression vector of MFF3 (pUB-Myc-MFF3), MFF (pUB-Myc-MFF), or MTUS1 (pUB-Myc-MTUS1), or an empty lentiviral expression vector

(pUB-Thy1.1). (**A**) Transduction efficiency analyzed by Thy1.1 staining and flow cytometry. Cells were analyzed at 72 hours post transduction. Gated population (Thy1.1+) indicates cells that were successfully transduced with the lentiviral expression vector. (**B**) Expression of the transduced Myc-tagged proteins analyzed by Western blot analysis. Total cellular proteins were prepared at 4 days post transduction, and then immunoblotted for Myc tag, followed by actin. Bands of Myc-MTUS1, Myc-MFF and Myc-MFF3 are indicated with arrowheads. Actin blot was used as loading control.

In summary, we have completed the research goals and most proposed experiments of this DoD grant. Our findings and data obtained from this DoD project allow us to formulate additional hypotheses to further our study, and to apply for new federal research grants such as NIH R01 grants.

(3) What opportunities for training and professional development has the project provided?

This DoD award has provided critical support for the career development of the PI, Dr. Ping Xie. Supported by this grant, Dr. Xie is able to establish a new collaboration with Drs. David Perlman and Saw Kyin at Princeton University, and has learned from him a number of bioinformatic and proteomic analytical tools. Dr. Xie has given 3 conference talks and 12 seminar presentations at other institutions to promote our research programs. With the support of this award, Dr. Xie has the opportunity to mentor one new postdoctoral fellow, two new graduate students, and five new undergraduate students. This DoD grant also allows Dr. Xie to better integrate her educational and research efforts, including the undergraduate courses of Immunology and Immunology Lab, and the graduate courses of Current Concepts in Immunology and Advanced Topics in Immunology. Taken together, this DoD award has been essential for Dr. Xie to acquire the leadership and expertise to advance her professional development at the forefront of cancer research.

Trainees of this project:
Postdoctoral fellow:
Yingying Liu

Graduate students performing PhD thesis research in the lab:
Shanique Edwards: PhD thesis successfully defended on March 13, 2015.
Almin Lalani

Undergraduate students performing honors thesis research in the lab:
Xin Chen
Debanjan Saha
Vidish Pandya
Ghanwa Khawaja
Vishnu Venkatesh

Awards received by trainees of this project:

2013	Shanique Edwards, Graduate Student, Gallo Award for Scientific Excellence, the 2015 Annual Retreat On Cancer Research In New Jersey, the New Jersey Commission on Cancer Research and the Cancer Institute of New Jersey
2014	Shanique Edwards, Graduate Student, Anne B. and James B. Leathem Fellow, Rutgers University
2014-2015	Xin Chen, Undergraduate Student, Aresty Research Grant, Rutgers University
2015	Debanjan Saha, Undergraduate Student, Summer Undergraduate Research Fellowship, Rutgers University

2015 Almin Lalani, Graduate Student, Gallo Award for Scientific Excellence, the 2015 Annual Retreat On Cancer Research In New Jersey, the New Jersey Commission on Cancer Research and the Cancer Institute of New Jersey
2015 Almin Lalani, Graduate Student, Travel Award, the American Association of Immunologists (AAI)
2015 Almin Lalani, Graduate Student, Arthur McCallum Fellow, Rutgers University
2015-2016 Debanjan Saha, Undergraduate Student, Aresty Research Grant, Rutgers University
2015-2016 Ghanwa Khawaja, Undergraduate Student, Aresty Research Grant, Rutgers University2015
2015-2016 Vishnu Venkatesh, Undergraduate Student, Aresty Research Grant, Rutgers University

Meeting organization:

1. Moderator, Session of Hematologic Malignancies, the 2nd International Conference on Hematology & Blood Disorders, Baltimore, MD. Sep. 29-Oct. 1, 2014.
2. Chair, Block Symposia of Innate Immune Responses in Monocytes/Macrophages, Dendritic Cells, and Myeloid Cells, the Annual Meeting of American Association of Immunologists (AAI), New Orleans, LA. May 12, 2015.

Invited talks at conferences:

1. **Xie P**. TRAF3 in innate immunity and inflammation. Infectious and Inflammatory Disease Retreat, Institute of Infectious Disease of New Jersey, Newark, NJ. July 10, 2014
2. **Xie P.** Molecular mechanisms of B lymphomagenesis induced by TRAF3 inactivation. Invited honorable guest speaker, the 2nd International Conference on Hematology& Blood Disorders, Baltimore, MD. Sep. 29, 2014
3. **Xie P.** Targeting TRAF3 downstream signaling pathways in B cell neoplasms. Honorable guest speaker, the 4th World Congress on Cancer Science & Therapy, Chicago, IL. Oct. 21, 2014

Participation in conferences:

1. Edwards S, Desai A, Liu Y, Moore C, and **Xie P**. Expression and function of a novel isoform of Sox5 in malignant B cells. The 101th Anniversary Meeting of American Association of Immunologists, USA, *J. Immunol.* 192: HUM7P.314, May 5, 2014
2. Edwards S, Desai A, Liu Y, Moore C, and **Xie P**. Expression and function of a novel isoform of Sox5 in malignant B cells. The 2014 Annual Retreat On Cancer Research In New Jersey, the New Jersey Commission on Cancer Research and the Cancer Institute of New Jersey, Piscataway, May 21, 2014.
3. Lalani A, Moore CR, Luo C, Kreider BZ, Liu Y, Morse HC 3rd, and **Xie P**. Myeloid Cell TRAF3 Regulates Immune Responses and Inhibits Inflammation and Tumor Development in Mice. Abstract #: Z4 3027. Keystone Symposia: Mechanisms of Pro-Inflammatory Diseases, Olympic Valley, CA. April 22, 2015.
4. Lalani AI*, Luo C, Morse HC 3rd, and **Xie P**. Specific deletion of TRAF3 in myeloid cells leads to spontaneous inflammation and tumor development in mice. The Annual Meeting of American Association of Immunologists (AAI), New Orleans, LA. May 12, 2015. **Selected for Oral Presentation*.
5. Lalani AI*, Luo C, Morse HC 3rd, and **Xie P**. Specific deletion of TRAF3 in myeloid cells leads to spontaneous inflammation and tumor development in mice. The annual retreat of New Jersey Commission on Cancer Research and the Cancer Institute of New Jersey, Piscataway, NJ. May 20, 2015. **Selected for Oral Presentation*.

(4) How were the results disseminated to communities of interest?

Dr. Xie was invited to give 3 conference talks and 12 seminar presentations at other institutions to share our recent results and findings with communities of interest. In addition, our lab was opened to visitors on Rutgers University Open House day on April 5, 2014 and April 11, 2015. Dr. Xie has introduced our research activities and reported our new results to visitors, including parents and high school students. The objective of such outreach activities is to promote public understanding and increasing interest in scientific research.

(5) What do you plan to do during the next reporting period to accomplish the goals?

Not applicable.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Aberrant B cell survival is one important pathogenic factor that leads to B cell malignancies, which comprise >50% of blood cancers. Despite recent advances, many types of B cell neoplasms remain incurable, highlighting a clear need for new therapeutic approaches. TRAF3 inactivation frequently occurs in a variety of B cell neoplasms, including B-CLL, NHL, MM and WM. Notably, TRAF3 inactivation has particular relevance to military personnel, veterans and their family members: (1) The Epstein-Barr virus (EBV)-encoded protein LMP1 sequesters over 80% of cellular TRAF3 in B cells. EBV reactivation is more common in soldiers and veterans, and is associated with increased risk of B lymphoma. (2) Infections and inflammations trigger TRAF3 degradation through signaling by the TNF-Rs (such as CD40) or Toll-like receptors (such as TLR4). Soldiers and veterans have increased risk of infections and inflammations, due to duty environment, bioterror weapons, physical injury, and stress, etc. (3) Soldiers and veterans exposed to radioactive and chemical weapons/equipment have increased risk of mutations in blood cells, including those of *TRAF3*. In this study, we have identified 150 novel TRAF3-interacting proteins in purified mitochondria of human multiple myeloma cells. Many of these TRAF3 interactors are novel regulators of B cell survival. Our ongoing study aims to further elucidate the mechanisms of how the TRAF3 and its interactors regulate mitochondrial physiology to inhibit B cell survival. Our studies have identified new diagnostic markers and therapeutic targets, and have also opened up new avenues for the prevention and treatment of B cell malignancies, the most common blood cancers.

What was the impact on other disciplines? Nothing to Report.

What was the impact on technology transfer? Nothing to Report.

What was the impact on society beyond science and technology?

Our lab was opened to visitors on Rutgers University Open House day on April 5, 2014 and April 11, 2015. Dr. Xie has introduced our research activities and reported our new results to visitors, including parents and high school students. The objective of such outreach activities is to promote public understanding and increasing interest in scientific research.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change: Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them: Nothing to report.

Changes that had a significant impact on expenditures: Nothing to report.

Significant changes in use or care of human subjects: Nothing to report.

Significant changes in use or care of vertebrate animals: Nothing to report.

Significant changes in use or care of biohazards and/or select agents: Nothing to report.

6. PRODUCTS

(1) Publications, conference papers, and presentations

Journal publications:

1. **Xie P**. TRAF molecules in cell signaling and in human diseases. *J. Mol. Signal.* 8:7, pages 1 – 31, doi:10.1186/1750-2187-8-7, 2013. (Acknowledged this DoD grant; please see the appendix.)
2. Edwards S, Baron J, Moore CR, Liu Y, Perlman DH, Hart RP, and **Xie P**. Mutated in colorectal cancer (MCC) is a novel oncogene in B lymphocytes. *J. Hematol. Oncol.*, 7:56, pages 1-24. doi:10.1186/s13045-014-0056-6, 2014. (Acknowledged this DoD grant; please see the appendix)
3. Moore CR, Edwards S, and **Xie P**. Targeting TRAF3 downstream signaling pathways in B cell neoplasms. *J. Cancer Sci. and Ther.* 7: 67-74, 2015. (Acknowledged this DoD grant; please see the appendix.)
4. Lalani A, Luo C, Han Y, and **Xie P**. TRAF3: a novel tumor suppressor gene in macrophages. *Macrophage*, 2: e1009, pages 1-13, 2015. (Acknowledged this DoD grant; please see the appendix.)

Books or other non-periodical, one-time publications: Nothing to Report.

Invited conference talks and seminar presentations by Dr. Xie:

1. **Xie P.** TRAF3-mediated regulation of innate immunity and inflammation. The Child Health Institute of New Jersey, New Brunswick, NJ. Feb. 17, 2014
2. **Xie P.** TRAF3-mediated regulation of innate immunity and inflammation. Research Forum, Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, NJ, USA. April 7, 2014
3. **Xie P.** Molecular mechanisms of TRAF3 in immune regulation and cancer pathogenesis. Seminar Series, Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, NJ, USA. May 16, 2014
4. **Xie P.** Molecular mechanisms of TRAF3 in immune regulation and cancer pathogenesis. Cardiovascular Seminar Series, Department of Internal Medicine, Saha Cardiovascular Center, the University of Kentucky, Lexington, KY. July 7, 2014
5. **Xie P.** TRAF3 in innate immunity and inflammation. Infectious and Inflammatory Disease Retreat, Institute of Infectious Disease of New Jersey, Newark, NJ. July 10, 2014

6. **Xie P.** Mechanisms of TRAF3 inactivation-initiated B lymphomagenesis. Department of Systems and Computational Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY. July 15, 2014
7. **Xie P.** TRAF3: a tumor suppressor gene in B lymphocytes. Department of Pharmacology, the University of Illinois at Chicago, Chicago, IL. Aug. 11, 2014
8. **Xie P.** TRAF3: a regulator of innate immunity and inflammation. Department of Cell & Molecular Physiology, University of Loyola, Chicago, IL. Aug. 27, 2014
9. **Xie P.** Molecular mechanisms of B lymphomagenesis induced by TRAF3 inactivation. Invited honorable guest speaker, the 2nd International Conference on Hematology& Blood Disorders, Baltimore, MD. Sep. 29, 2014
10. **Xie P.** Regulatory mechanisms of innate immunity and inflammation. Department of Biological Sciences, Chicago State University, Chicago, IL. Oct. 20, 2014
11. **Xie P.** Targeting TRAF3 downstream signaling pathways in B cell neoplasms. Honorable guest speaker, the 4th World Congress on Cancer Science & Therapy, Chicago, IL. Oct. 21, 2014
12. **Xie P.** TRAF3 in B lymphomagenesis. Basic Science Seminar Series, Robert Wood Johnson School of Medicine, Piscataway, NJ, USA. Dec. 11, 2014
13. **Xie P.** TRAF3 and MCC in B lymphomagenesis. Bioinformatics Seminar Series, Tsinghua University, Beijing, China. May 21, 2015
14. **Xie P.** TRAF3: a novel tumor suppressor gene in myeloid cells. Ernest Mario School of Pharmacy, Rutgers University, Piscataway, NJ, USA. Sep. 15, 2015
15. **Xie P.** Molecular mechanisms of TRAF3 and MCC in B cell malignant transformation. LIFS Seminar Series, Division of Life Science, The Hong Kong University of Science & Technology, Hong Kong, China. Sep. 24, 2015

Conference presentations by Dr. Xie's trainees:

1. Edwards S, Desai A, Liu Y, Moore C, and **Xie P**. Expression and function of a novel isoform of Sox5 in malignant B cells. The 101th Anniversary Meeting of American Association of Immunologists, USA, *J. Immunol.* 192: HUM7P.314, May 5, 2014
2. Edwards S, Desai A, Liu Y, Moore C, and **Xie P**. Expression and function of a novel isoform of Sox5 in malignant B cells. The 2014 Annual Retreat On Cancer Research In New Jersey, the New Jersey Commission on Cancer Research and the Cancer Institute of New Jersey, Piscataway, May 21, 2014.
3. Lalani A, Moore CR, Luo C, Kreider BZ, Liu Y, Morse HC 3rd, and **Xie P**. Myeloid Cell TRAF3 Regulates Immune Responses and Inhibits Inflammation and Tumor Development in Mice. Abstract #: Z4 3027. Keystone Symposia: Mechanisms of Pro-Inflammatory Diseases, Olympic Valley, CA. April 22, 2015.
4. Lalani AI*, Luo C, Morse HC 3rd, and **Xie P**. Specific deletion of TRAF3 in myeloid cells leads to spontaneous inflammation and tumor development in mice. The Annual Meeting of American Association of Immunologists (AAI), New Orleans, LA. May 12, 2015. **Selected for Oral Presentation.*
5. Lalani AI*, Luo C, Morse HC 3rd, and **Xie P**. Specific deletion of TRAF3 in myeloid cells leads to spontaneous inflammation and tumor development in mice. The annual retreat of New Jersey Commission on Cancer Research and the Cancer Institute of New Jersey, Piscataway, NJ. May 20, 2015. **Selected for Oral Presentation.*

(2) Website(s) or other Internet site(s): Nothing to Report.

(3) Technologies or techniques: Nothing to Report.

(4) Inventions, patent applications, and/or licenses: Nothing to Report.

(5) Other Products:

Research materials: We have generated a number of lentiviral expression vectors of TRAF3, including wild type and mutant TRAF3 with or without tags, which allow affinity purification and structure-function study of TRAF3. We will make these DNA vectors available to other scientists upon request.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

(1) What individuals have worked on the project?

Ping Xie, PhD, PI: No change.
David Perlman, PhD, collaborator: No change

Name: Yingying Liu, PhD
Project Role: Postdoctoral associate
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 24
Contribution to Project: Dr. Liu has performed most experiments described in this progress report, including mitochondrial function analyses, generation of lentiviral expression vectors of TRAF3 for TAP and TRAF3 mutants, affinity purification of mitochondrial TRAF3-interacting proteins from transduced human multiple myeloma cells, verification of TRAF3-interacting proteins by co-immunocprecipitation and GST pull-down experiments, and functional studies of the novel TRAF3-interacting proteins.
Funding Support: this DoD grant.

Name: Yan Liu
Project Role: Senior technician
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 12
Contribution to Project: Yan Liu has maintained the B-TRAF3-/- mouse breeding colony and carried , and has carried out PCR genotyping for each individual mouse of this project. Yan also performed Western blot analysis to verify TRAF3-interacting proteins of co-immunoprecipitation and GST pull-down experiments.
Funding Support: 50% support from this DoD grant, and 50% support from Dr. Xie's start-up funds.

Name: Shanique Edwards, PhD
Project Role: Graduate student
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 6
Contribution to Project: Shanique has helped to test and troubleshoot the tandem affinity purification protocol to identify TRAF3-interacting proteins from purified B cell mitochondria, and has also applied this strategy to discover mitochondrial proteins interacting with a novel oncogene, MCC, that we recently identified in $TRAF3^{-/-}$ mouse B lymphomas.
Funding Support: Shanique has been supported by a Teaching Assistantship.

(2) Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

(3) What other organizations were involved as partners?

Partner Organization Name: Princeton University
Location of Organization: Princeton Collaborative Proteomics & Mass Spectrometry Center, Lewis-Sigler Institute of Integrative Genomics, Princeton University, Princeton, NJ 08544

Partner's contribution to the project:
Facilities: performed protein sequencing by LC-MS/MS at Princeton University.
Collaboration:
Dr. David Perlman analyzed LC-MS/MS data.
Dr. Saw Kyin performed LC-MS/MS sequencing and analyzed data.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable.

QUAD CHARTS: Not applicable.

9. APPENDICES: All published papers of this grant are attached.

JOURNAL OF MOLECULAR SIGNALING

REVIEW Open Access

TRAF molecules in cell signaling and in human diseases

Ping Xie

Abstract

The tumor necrosis factor receptor (TNF-R)-associated factor (TRAF) family of intracellular proteins were originally identified as signaling adaptors that bind directly to the cytoplasmic regions of receptors of the TNF-R superfamily. The past decade has witnessed rapid expansion of receptor families identified to employ TRAFs for signaling. These include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), T cell receptor, IL-1 receptor family, IL-17 receptors, IFN receptors and TGFβ receptors. In addition to their role as adaptor proteins, most TRAFs also act as E3 ubiquitin ligases to activate downstream signaling events. TRAF-dependent signaling pathways typically lead to the activation of nuclear factor-κBs (NF-κBs), mitogen-activated protein kinases (MAPKs), or interferon-regulatory factors (IRFs). Compelling evidence obtained from germ-line and cell-specific TRAF-deficient mice demonstrates that each TRAF plays indispensable and non-redundant physiological roles, regulating innate and adaptive immunity, embryonic development, tissue homeostasis, stress response, and bone metabolism. Notably, mounting evidence implicates TRAFs in the pathogenesis of human diseases such as cancers and autoimmune diseases, which has sparked new appreciation and interest in TRAF research. This review presents an overview of the current knowledge of TRAFs, with an emphasis on recent findings concerning TRAF molecules in signaling and in human diseases.

Keywords: TRAFs, TNF-Rs, TLRs, NLRs, RLRs, E3 Ubiquitin ligases, DUBs

Background

The tumor necrosis factor receptor (TNF-R)-associated factor (TRAF) family of intracellular proteins were originally identified as signaling adaptors that bind directly to the cytoplasmic regions of receptors of the TNF-R superfamily [1-3]. There are six known members of the TRAF family (TRAF1 to 6) in mammals. Although a novel protein was named TRAF7 [4], this claim is controversial as the protein does not have the TRAF homology domain that defines the TRAF family (Figure 1). The distinctive feature of all TRAF proteins is a C-terminal TRAF domain, which is composed of an N-terminal coiled-coil region (TRAF-N) and a C-terminal β-sandwich (TRAF-C) [2,3,5]. The TRAF domain mediates protein-protein interactions, including TRAF oligomerization as well as interactions with upstream regulators and downstream effectors [2,3,5]. For example, the eight-stranded β-sandwich structure of the TRAF-C domain mediates the interaction with receptors, and the minor structural differences in this domain among TRAFs (as revealed by X-ray crystallography) define the specificity of each TRAF binding to various receptors [6,7]. Therefore, one important role of TRAFs is to serve as adaptor proteins in the assembly of receptor-associated signaling complexes, linking upstream receptors to downstream effector enzymes. Most TRAFs, with the exception of TRAF1, contain an N-terminal RING finger domain, followed by a variable number of zinc fingers [2,3,8]. The RING finger is found in many E3 ubiquitin ligases and comprises the core of the ubiquitin ligase catalytic domain. Indeed, increasing evidence indicates that in addition to their role as adaptor proteins, TRAFs (including TRAF2, 3, 5 and 6) also act as E3 ubiquitin ligases [3,8,9]. Thus, TRAFs function as both adaptor proteins and E3 ubiquitin ligases to regulate signaling.

The past decade has witnessed rapid expansion of receptor families identified to employ TRAF proteins for signaling. In addition to the TNF-R superfamily, TRAFs are now recognized as signal transducers of a wide variety of other

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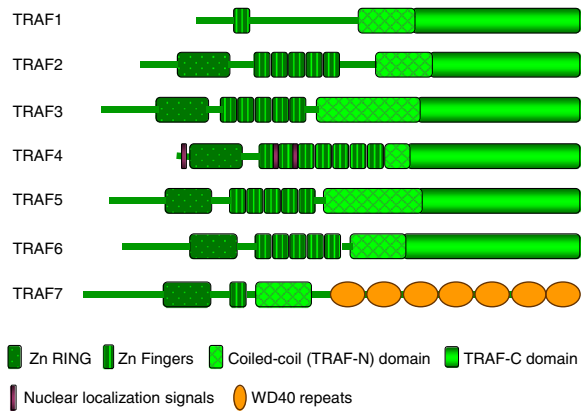

Figure 1 Domain structure of the seven TRAF proteins. Symbols for different domains are shown, including zinc RING (Zn RING), zinc fingers (Zn Fingers), coiled-coil (TRAF-N) domain, TRAF-C domain, nuclear localization signals, and WD40 repeats.

receptor families, including innate immune receptors, adaptive immune receptors, cytokine receptors, and C-type lectin receptors [9-14]. For example, three major families of pattern recognition receptors (PRRs) of the innate immune system recruit TRAFs via additional adaptor proteins: Toll-like receptors (TLRs) via MyD88 or TRIF, nucleotide binding-oligomerization domain (NOD)-like receptors (NLRs) via RIP2, and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) via MAVS [10,15,16]. TRAF-dependent signaling pathways typically lead to the activation of nuclear factor-κBs (NF-κB1 and NF-κB2), mitogen-activated protein kinases (MAPKs), or interferon-regulatory factors (IRFs). Acting alone or in combination, TRAFs are highly versatile regulators that control diverse cellular processes, including survival, proliferation, differentiation, activation, cytokine production, and autophagy [3,9,16,17].

Despite the similarities in the signaling pathways activated by different TRAF proteins, each TRAF appears to play obligatory and non-redundant physiological roles. Germ-line and conditional knockout mice have been instrumental in revealing the overlapping yet distinct roles of different TRAFs in whole animals. Compelling evidence from these studies demonstrates that TRAFs critically regulate a plethora of physiological processes, including innate and adaptive immunity, embryonic development, tissue homeostasis, stress response, and bone metabolism [3,18,19]. The pivotal roles of TRAFs in host immunity are further highlighted by the discoveries that pathogens adopt deliberate strategies to subvert TRAF functions [20,21]. An emerging paradigm of TRAF functions is that alterations in TRAFs may contribute to the pathogenesis of important human diseases, including cancers, autoimmune diseases and immunodeficiencies [18,22,23]. This has sparked new appreciation and interest in TRAF research during the past few years. Here I attempt to provide an overview of the current knowledge of TRAFs, with an emphasis on recent advances in understanding TRAFs in receptor signaling and in human diseases as well as recent insights into the regulatory mechanisms of TRAF ubiquitination.

TRAFs in signaling by the TNF-R superfamily

Receptors of the TNF-R superfamily have wide tissue distribution and regulate diverse biological functions, including immune responses, inflammation, lymphoid organ and brain development, osteoclastogenesis, and tissue homeostasis [3,24-26]. Structurally, these receptors are characterized by the presence of conserved cysteine-rich domains (CRDs) in their extracellular region that are responsible for the binding of their ligands of the TNF superfamily. Based on the intracellular structures, the TNF-R superfamily is categorized into two main groups. The first group of receptors, termed death receptors, contain a death domain in the intracellular region. The second group, also the majority of the TNF-R superfamily, do not have a death domain but contain TRAF-interacting motifs (TIMs) in their intracellular region [3,24,26]. TRAF2, 3 and 5 usually have overlapping binding motifs, whereas TRAF6 has a distinct interacting motif on these receptors [3,27].

Receptors of this family do not have kinase activity and depend on the binding of adaptor proteins to assemble signaling complexes to activate downstream pathways [3,24,26]. Signaling by death receptors mainly relies on adaptor proteins containing a death domain, such as TRADD or FADD, thereby culminating in caspase activation and cell apoptosis. In contrast, signaling by the TIM-containing receptors is mediated primarily, albeit not exclusively, via TRAFs [3,24,26]. These include TRAFs that can interact with the receptors either directly through TIMs or indirectly through other TRAFs or adaptor proteins (Table 1). Binding of TRAFs to TNF-Rs typically induces signaling cascades leading to the activation of NF-κB and MAPKs, including ERK, p38 and JNK, and ultimately regulates cell survival or functionality depending on the cell type and the context [3,24,26]. Notably, TRAF2 and TRAF5 can also modulate signaling by death receptors through association with TRADD, FADD or RIP1 (Table 1). Most TRAF-dependent receptors of this family trigger the canonical NF-κB pathway (RelA/p50, NF-κB1). In contrast, the alternative NF-κB pathway (RelB/p52, NF-κB2) is activated by a subset of TNF-Rs, including CD40, BAFF-R, the lymphotoxin-β receptor (LTβR), 4-1BB, and Fn14 [28-32]. Interestingly, however, unlike CD40 or BAFF-R, TWEAK-induced Fn14 signaling promotes NF-κB2 activation through a distinct mechanism that induces lysosomal degradation of cIAP1-TRAF2 in a cIAP1-dependent manner

Table 1 TRAFs directly and indirectly employed by the TNF-R superfamily

Receptors	TRAFs	References
Receptors containing TRAF-interacting motifs		
TNF-R2	TRAF2	[26,34]
	TRAF1, 3 via TRAF2	[26]
CD40	TRAF2, 3, 5, 6	[27,28,35]
	TRAF1 via TRAF2	[28]
BAFF-R	TRAF3, 6	[36-38]
	TRAF2 via TRAF3	[28]
BCMA	TRAF1, 2, 3, 5, 6	[39,40]
TACI	TRAF2, 3, 5, 6	[2,28,41]
LTβR	TRAF2, 3, 5	[29,42-47]
CD27	TRAF2, 3, 5	[1,48,49]
CD30	TRAF1, 2, 3, 5	[1,2]
4-1BB	TRAF1, 2, 3	[1,2]
OX40	TRAF1, 2, 3, 5, 6	[1,2,50-52]
GITR	TRAF1, 2, 3, 4, 5	[1,2,53,54]
RANK	TRAF1, 2, 3, 5, 6	[1,2,55,56]
HVEM	TRAF1, 2, 3, 5	[1,2,57]
Troy	TRAF2, 5, 6	[58]
XEDAR	TRAF3, 6	[59]
Fn14	TRAF2, 6	[33,60]
Death receptors		
TNF-R1	TRAF2 via TRADD	[61-63]
	TRAF5 via RIP1	[64]
p75NTR	TRAF1, 2, 3, 4, 5, 6	[2,65,66]
EDAR	TRAF1, 3, 6	[63]
FAS	TRAF2 via FADD	[63]
DR3	TRAF2 via TRADD	[63]
DR6	TRAF2 via TRADD	[63]
TRAIL-R1	TRAF2 via RIP1	[67]

[33]. The distinct TWEAK/Fn14 paradigm is covered in detail in a recent review by Silke and Brink [32].

Using CD40 and BAFF-R as examples, here I briefly summarize recent advances in understanding how TRAFs regulate the two NF-κB pathways and activation of MAPKs (Figure 2). In the absence of stimulation, TRAF3 constitutively binds to NIK (the upstream kinase of the NF-κB2 pathway) and TRAF2 (which associates with cIAP1/2). In this complex, cIAP1/2 induces K48-linked polyubiquitination of NIK, and thus targets NIK for proteasomal degradation and inhibits NF-κB2 activation [37,68-71]. Following BAFF or CD154 stimulation, trimerized BAFF-R or CD40 recruits TRAF3, TRAF2, cIAP1/2 and MALT1 to membrane signaling rafts, releasing NIK from the TRAF3/TRAF2/cIAP1/2 complex [37,72-74]. NIK protein is accumulated in the cytoplasm, induces the activation of IKKα and NF-κB2, and eventually up-regulates the expression of anti-apoptotic proteins of the Bcl-2 family (such as Bcl-2, Bcl-xL, and Mcl-1) to induce cell survival [28]. In the receptor signaling complex, TRAF2 induces K63-linked polyubiquitination of cIAP1/2, which is subsequently activated to catalyze K48-linked polyubiquitination and degradation of TRAF3 and TRAF2 [37,72,74,75]. Following CD40 activation, many other signaling proteins (including TRAF5, TRAF6, TRAF1, Ubc13, MEKK1, TAK1 and NEMO) are also recruited to the cytoplasmic domain of the receptor, and the K63-specific ubiquitin ligase activity of TRAF2 and TRAF6 is rapidly stimulated [27,72,75]. These proteins form several separate multiprotein signaling complexes, which result in the phosphorylation and activation of MEKK1 and TAK1. Activated MEKK1 and TAK1 and their associated protein complexes are subsequently released from the receptor into the cytoplasm to activate MAPKs and NF-κB1, which eventually mediate the effector functions of CD40 [35,72]. Interestingly, the releasing step of MEKK1 and TAK1 is inhibited by TRAF3 via a yet unknown mechanism, but promoted by cIAP1/2-catalyzed K48-linked polyubiquitination and proteasomal degradation of TRAF3 [72,74,75]. In response to BAFF stimulation, a signaling pathway of c-Raf-MEK-ERK-dependent phosphorylation and down-regulation of the pro-apoptotic protein Bim also contributes to B cell survival [76]. In light of the evidence that TRAF1 mediates 4-1BB-induced ERK-dependent phosphorylation and down-regulation of Bim to promote T cell survival [77-79], it would be interesting to investigate the role of TRAF1 in BAFF-induced Bim down-regulation in B cells. Collectively, the above evidence indicates that TRAFs are critical regulators of signaling by the TNF-R superfamily.

TRAFs in TLR signaling

Toll-like receptors (TLRs), the best-studied family of PRRs, recognize conserved structures termed pathogen-associated molecular patterns (PAMPs) of diverse invading microbes, including Gram-positive and -negative bacteria, DNA and RNA viruses, fungi, protozoa, and parasites. They also detect endogenous molecules released from damaged or inflamed self-tissues, referred to as damage-associated molecular patterns (DAMPs) [80-82]. Upon sensing these molecules, TLR signaling induces the production of pro-inflammatory cytokines (such as TNFα, IL-1, IL-6, and IL-12), type I interferons (IFNα and IFNβ), chemokines, antimicrobial enzymes, and other inflammatory mediators. These provoke acute inflammatory responses as well as phagocytosis and autophagy, which represent the first line of innate immunity against pathogens [17,83,84]. TLR signaling also serves to prime the subsequent adaptive immune responses by up-regulating adhesion molecules and co-

stimulatory molecules (such as CD40, CD80, and CD86) on antigen presenting cells [85,86].

TLRs (TLR1, 2, 4–6, 10) that sense lipids or proteins are located on the cell membrane, while those (TLR3, 7, 8, 9) that recognize nucleic acids are resided in intracellular endosomes [8,87]. Each TLR consists of an ectodomain containing leucine-rich repeats (LRR) that mediate sensing of PAMPs or DAMPs, and a cytoplasmic Toll/IL-1 receptor (TIR) domain that mediates downstream signal transduction. Ligand-induced TLR dimerization or oligomerization recruits TIR domain-containing adaptor proteins through TIR-TIR interactions, including MyD88, TRIF, Mal and TRAM [83,88,89]. MyD88 is employed by all TLRs except TLR3. TRIF is only used by TLR3 and endocytosed TLR4. Mal (also known as TIRAP) facilitates the recruitment of MyD88 to TLR4, while TRAM acts as a bridging adaptor between TRIF and endocytosed TLR4. Collectively, two general pathways are used by TLRs: MyD88-dependent (all TLRs except TLR3) and TRIF-dependent (TLR3 and TLR4) pathways. Both pathways initiate complex signaling cascades of phosphorylation and ubiquitination events, which culminate in the activation of transcription factors, including NF-κB, IRFs, and AP-1 family members, leading to innate immune responses [83,88,89].

TRAF6 mediates both MyD88-dependent and TRIF-dependent activation of NF-κB and AP-1 (Figure 3). In MyD88-dependent TLR signaling, TRAF6 is recruited to MyD88-activated IRAK1/2, and oligomerization of TRAF6 stimulates its E3 ubiquitin ligase activity. In coordination with the E2 complex Uev1A:Ubc13, TRAF6 catalyzes the attachment of K63-linked polyubiquitin chains onto its substrates, including itself and NEMO [8,89,90], and synthesis of free, unanchored K63-polyubiquitin chains [91]. Ubiquitinated TRAF6 serves as a signaling scaffold to recruit TAK1 via TAB2/3. TRAF6-generated free K63-polyubiquitin chains also bind to TAB2/3 to activate TAK1, and bind to NEMO to activate IKKα/β in the receptor complex. This ultimately results in MyD88-dependent activation of NF-κB [8,82,90,91]. The TAK1 signaling complex, including TRAF6-TAB2/3-TAB1-TAK1, is subsequently dissociated from the receptor and released into the cytosol, where TAK1 activates MAPK cascades, leading to activation of AP-1. Similar to CD40 signaling, the release of the TAK1 signaling complex from TLR4 is inhibited by TRAF3, which is recruited to TLR4 by MyD88 and IRAK1. However, TRAF6 catalyzes K63-linked polyubiquitination of cIAP1/2, which is also recruited by MyD88 and IRAK1. Activated cIAP1/2 promotes K48-linked polyubiquitination and degradation of TRAF3, allowing activation of MAPKs [92]. In TRIF-dependent TLR signaling, TRIF directly recruits TRAF6 and RIP1, which work cooperatively to activate TAK1, eventually leading to activation of NF-κB and AP-1 [8,82,90]. Interestingly, in response to engagement of TLR1, 2 or 4, TRAF6 is also translocated to mitochondria, where it ubiquitinates evolutionarily conserved signaling intermediate in Toll pathways (ECSIT), resulting in increased reactive oxygen species (ROS) generation and bacteria killing [93]. Notably, TRAF6 is also necessary for IRF7 activation and type I IFN production induced by TLR7 and TLR9 in plasmacytoid dendritic cells (pDCs) [94].

TRAF3 is required for both MyD88-dependent and TRIF-dependent activation of IRF3 and IRF7, and thus production of type I IFNs [95,96], a class of cytokines with potent antiviral and antibacterial activities. In MyD88-dependent signaling downstream of TLR7 and TLR9, TRAF3 is recruited to MyD88 and IRAK1. Activated TRAF3 catalyzes its K63-linked auto-ubiquitination, and assembles a signaling complex with MyD88, IRAK4, IRAK1, IKKα and IRF7. Within this complex, IRF7 is phosphorylated and activated by IRAK1 and IKKα to induce the production of type I IFNs [8,82,86]. In TRIF-dependent signaling downstream of TLR3 and TLR4, TRAF3 interacts with oligomerized TRIF, and activated TRAF3 recruits TBK1 and IKKε through NAP1 and TANK. In this signaling complex, TRAF3, in cooperation with Ubc13 and/or Ubc5, catalyzes K63-polyubiquitination of TRAF3 itself, TBK1 and IKKε, which facilitates the phosphorylation of IRF3 and IRF7. The phosphorylated IRF3 and IRF7, in turn, form homodimers or heterodimers, translocate into the nucleus and induce the expression of type I IFNs as well as IFN-inducible gene [8,82,86] (Figure 3).

Interestingly, TRAF1 was also identified as a TRIF-interacting protein in yeast two-hybrid screens. Overexpression of TRAF1 inhibits TRIF- and TLR3-mediated activation of NF-κB and expression of IFN-β, suggesting that TRAF1 inhibits TRIF-dependent signaling [83,97,98]. Similarly, TRAF4 physically interacts with and functionally counteracts TRAF6 and TRIF in TLR signaling [99]. Taken together, recent advances indicate that TRAF6, TRAF3, TRAF1 and TRAF4 play critical and largely distinct roles in signaling by TLRs.

TRAFs in NLR signaling

NOD-like receptors (NLRs) are a family of cytosolic sensors of PAMPs and DAMPs, and are functionally analogous to TLRs [100-102]. Each NLR appears to be activated by multiple agonists. However, in many cases, evidence of direct interaction between NLRs and PAMPs/DAMPs is lacking [103,104]. Effector functions of NLRs include secretion of pro-inflammatory cytokines, chemokines, antimicrobial peptides and type I IFNs, generation of ROS, autophagy, antigen processing, and expression of MHC class II on antigen presenting cells. These responses induce

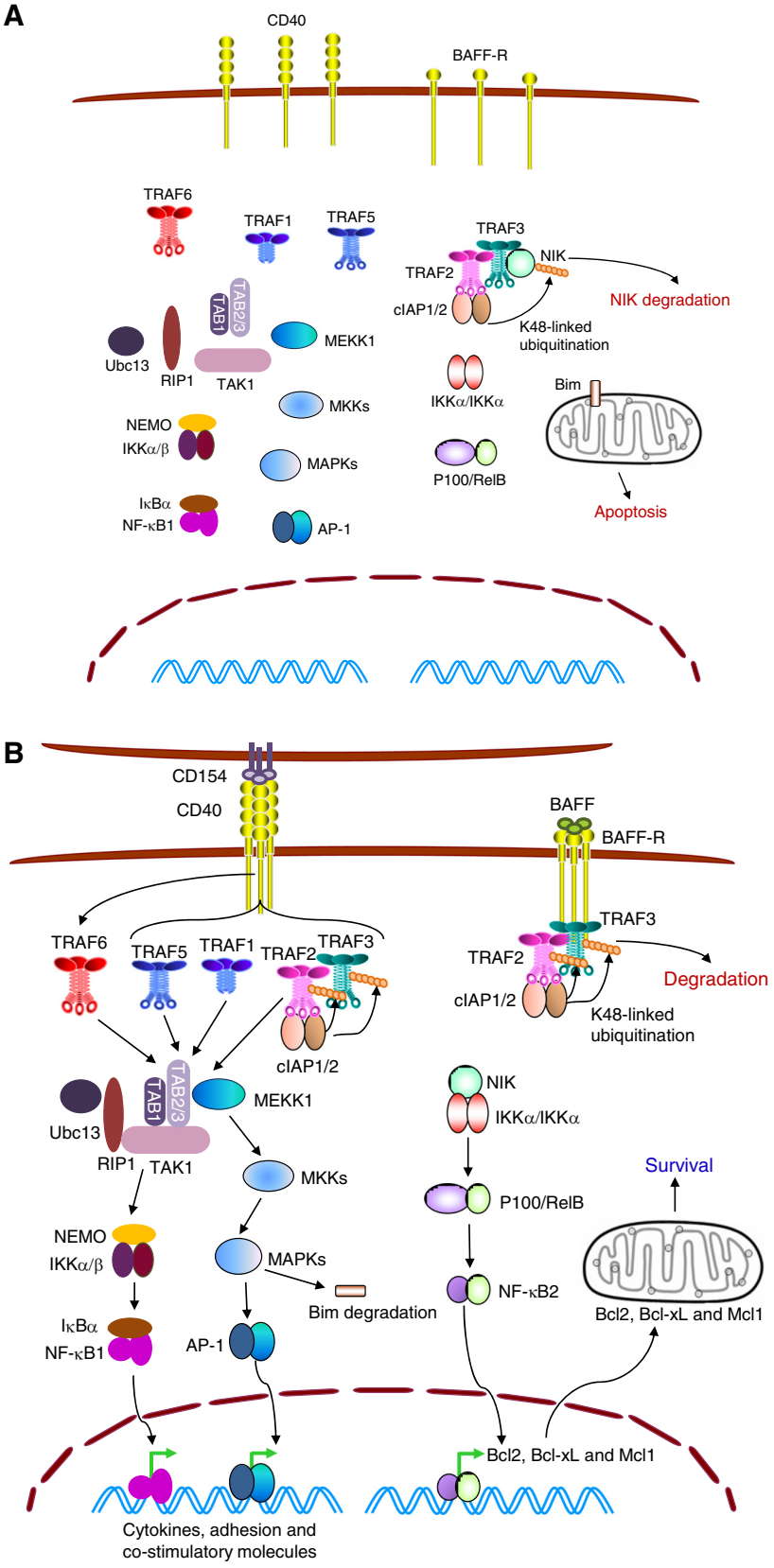

Figure 2 (See legend on next page.)

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Figure 2 TRAFs in BAFF-R and CD40 signaling pathways in B lymphocytes. (**A**) In the absence of stimulation, TRAF3 and TRAF2 promote B cell apoptosis. TRAF3 and TRAF2 constitutively form a complex with cIAP1/2 and NIK, target NIK for K48-linked polyubiquitination and degradation, thereby inhibiting NF-κB2 activation in B cells. (**B**) BAFF-R and CD40 signaling pathways. Upon ligand engagement, BAFF-R or CD40 recruits TRAF3-TRAF2-cIAP1/2 to membrane rafts, thus allowing NIK accumulation and NF-κB2 activation, leading to B cell survival. In addition, TRAF1, 2, 5 and 6 mediate CD40-induced activation of NF-κB1 and MAPKs.

innate immune clearance of the pathogen, and also tailor the adaptive immune system to fight the infection [100-102]. NLRs are characterized by a central NOD domain that mediates nucleotide-binding and oligomerization, and the C-terminal LRRs that possibly mediate ligand detection. In addition, they contain N-terminal effector domains, such as caspase recruitment domains (CARD), pyrin domains (PYD), baculovirus inhibitor of apoptosis repeat (BIR) domains, or an acidic transactivation domain, which recruit downstream signal transduction molecules after ligand sensing [100,101,104]. One well-studied pathway of several NLRs, including NLRP3, NLRP1, NLRP6, and NLRC4, is the assembly of multi-protein complexes called 'inflammasomes', which contain caspase-1 and apoptosis-associated speck-like protein containing a CARD (ASC). Inflammasomes induce proteolytic processing of pro-IL-1β and pro-IL-18 into secretable IL-1β and IL-18, as well as caspase 1-dependent apoptosis termed 'pyroptosis' [15,100,101]. The role of TRAF2 in inflammasome signaling has recently been explored, but the published data are contradictory. Labbe *et al.* reported that depletion of TRAF2 by siRNA inhibits inflammasome signaling in HEK293T cells [105]. However, Vince *et al.* found that inflammasome activation is normal in TRAF2$^{-/-}$ bone marrow-derived macrophages (BMDMs) [71]. Potential involvement of other TRAFs in inflammasome signaling remains to be elucidated.

TRAF2, TRAF5, and TRAF6 are required for NF-κB and MAPK activation induced by NOD1 and NOD2 (Figure 4), the founding members of the NLR family [15,102,106]. Upon detection of *meso*-diaminopimelic acid (DAP) by NOD1 or muramyl dipeptide (MDP) by NOD2 at the vicinity of plasma membranes, oligomerization of NOD1 or NOD2 recruits the dual specificity kinase RIP2 (also called RICK) via a homotypic CARD-CARD interactions [101-103]. Activated RIP2 induces the formation of the signaling complex containing TRAF2, TRAF5, TRAF6, TRAF4, CARD9, cIAP1/2, and Ubc13/Uev1A. In this complex, cIAP1/2, in coordination with Ubc13/Uev1A, catalyze K63-linked polyubiquitination of RIP2, which further recruits TAB2/3-TAB1-TAK1 and NEMO-IKKα/β, leading to NF-κB activation [15,100,107-109]. Interestingly, a recent study by Damgaard *et al.* demonstrated that XIAP is also recruited to the NOD2 signaling complex, in which XIAP primarily conjugates ubiquitin chains on RIP2 that are linked through lysine residues other than K63 and K48 [110]. Thus, XIAP, together with cIAP1/2, constitutes the major ubiquitin ligase activity that ubiquitinates RIP2 in NOD2 signaling, and cIAP1/2 appear to be rate limiting only when XIAP is not present [110]. It has been shown that TRAF2 and TRAF5 are required for NOD-induced NF-κB activation, while TRAF6, CARD9, and ITCH are important for p38 and JNK activation in NOD signaling [15,111,112]. However, the exact mechanism of how these occur is still unknown. Interestingly, TRAF4 is identified as a key negative regulator of NOD2 signaling. TRAF4 binds directly to NOD2 in an agonist-dependent manner, and inhibits NOD2-induced NF-κB activation and bacterial killing [109]. This inhibitory effect of TRAF4 requires its phosphorylation at Ser426 by IKKα, which is also recruited to the NOD2 signaling complex [113].

TRAF3 mediates type I IFN production induced by NOD1 [114], and presumably also that induced by NOD2 (Figure 4). NOD1 and NOD2 induce type I IFN production through distinct mechanisms. Upon sensing DAP, oligomerization of NOD1 recruits TRAF3 via RIP2. TRAF3 in turn activates TBK1 and IKKε, leading to subsequent activation of IRF7 and type I IFN production in epithelial cells [100,102,114]. In contrast, NOD2 induces type I IFN production only in response to viral ssRNA, but not in response to MDP, via a RIP2-independent pathway [102,115]. Following the detection of viral ssRNA, NOD2 engages a signaling complex containing MAVS on mitochondria, which induces IRF3 activation and type I IFN production [115]. TRAF3 has been shown to directly interact with MAVS to mediate RLR-induced type I IFN production [116]. It is thus speculated that TRAF3 may similarly activate TBK1 and IKKε in NOD2-MAVS signaling, but this awaits experimental investigation.

Interestingly, TRAF3 and TRAF6 are involved in the cross-talk between several NLRs and TLRs or RLRs. TRAF3 regulates NLRP12-mediated suppression of TLR-driven NF-κB activation, as NLRP12 interacts with both NIK and TRAF3 [117]. TRAF6 interacts with NLRX1, which negatively regulates NF-κB activation induced by RIG-I or TLR4 [118,119]. Similarly, NLRC3 also inhibits TLR-induced NF-κB activation by interacting with TRAF6 and reducing K63-linked polyubiquitination of TRAF6 [120].

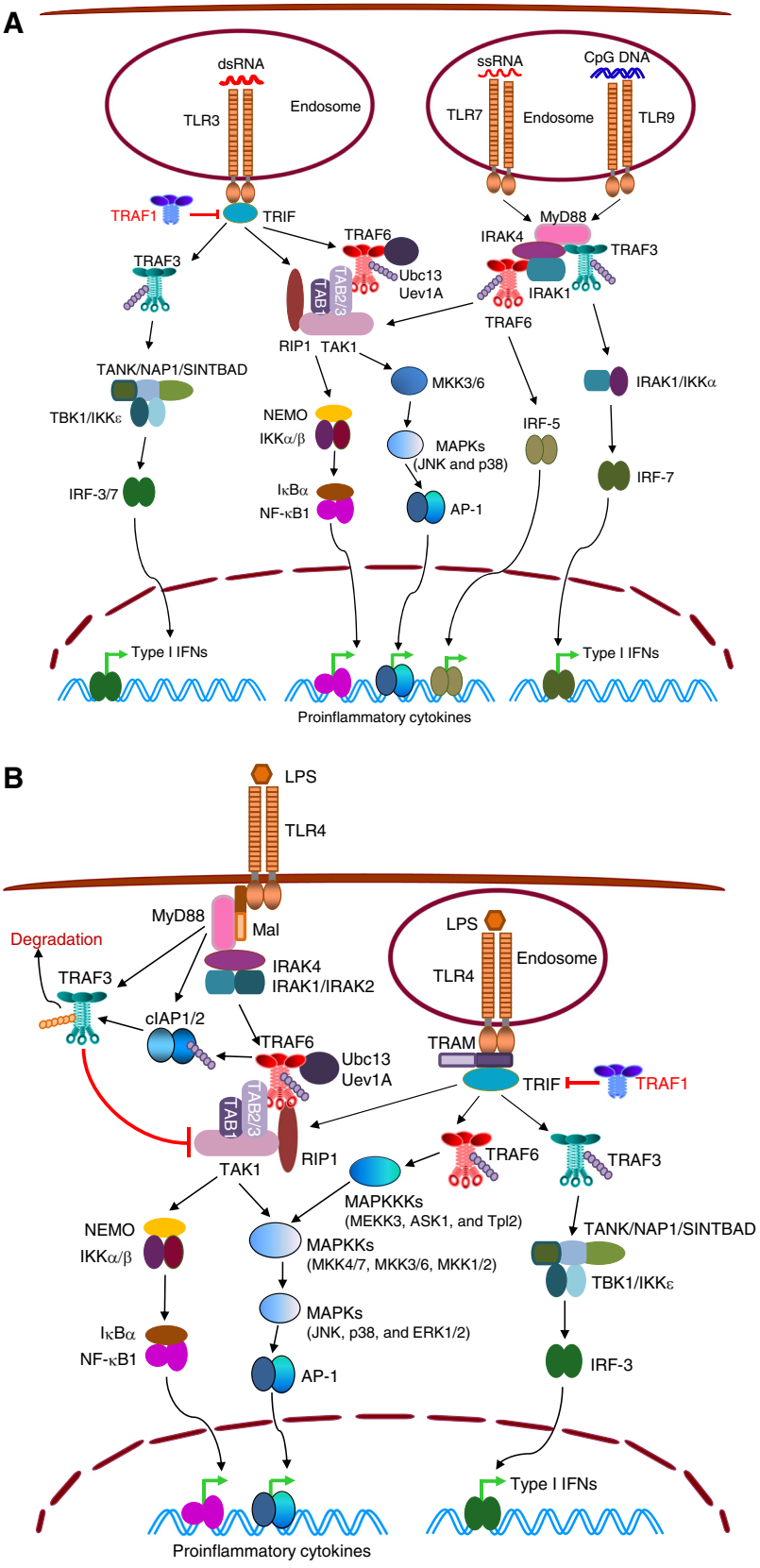

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Figure 3 TRAFs in signaling by TLRs. (**A**) TLR3, 7 and 9 signaling pathways. Upon ligand binding in endosomes, TLR3 recruits TRAF3 and TRAF6 via TRIF, while TLR7 and TLR9 recruit TRAF3 and TRAF6 via MyD88-IRAK1. (**B**) TLR4 signaling pathways. Upon LPS engagement on the plasma membrane, TLR4 recruits TRAF6 and TRAF3 via MyD88-IRAK1. Internalized TLR4 recruits TRAF3 and TRAF6 to endosomes via TRIF. TRAF6 mediates MyD88- and TRIF-induced activation of NF-κB1 and MAPKs, while TRAF3 mediates MyD88- or TRIF-induced activation of IRF-3/7 in signaling by TLRs. In contrast, TRAF1 inhibits TRIF signaling.

TRAFs in RLR signaling

RIG-I like receptors (RLRs), including RIG-I, MDA5, and LGP2, are a family of cytosolic RNA helicases that detect viral RNA PAMPs accumulated during viral infection or replication. RLRs are indispensable for antiviral responses in most cell types except pDCs [116,121,122]. RIG-I/MDA5 signaling rapidly elicits the production of type I and type III IFNs and proinflammatory cytokines. RIG-I and MDA5 exhibit different ligand specificity and respond to different viruses, whereas LGP2 facilitates or antagonizes recognition of viral RNA by MDA5 and RIG-I [116,121,122]. RLRs are structurally characterized by a central DExD/H box RNA helicase domain involved in RNA binding and ATPase function, and a carboxyl-terminal domain (CTD) that contains a positively charged RNA binding pocket. RIG-I and MDA5, but not LGP2, also possess two N-terminal CARDs that are required to trigger downstream signaling [116,123,124]. Upon detection of RNA PAMPs, RIG-I/MDA-5 undergoes conformational change that leads to dimerization and association with the mitochondrial antiviral signaling adaptor (MAVS, also called IPS-1, VISA, or Cardif) through homotypic CARD-CARD interactions [116,121,122]. MAVS consists of an N-terminal CARD domain, a central proline-rich region (PRR), several TRAF-interacting motifs, and a C-terminal transmembrane domain, which anchors the protein on the outer membranes of mitochondria. Dimerization of MAVS directly recruits TRAF2 [125], TRAF3 [126], TRAF5, TRAF6 [127], CARD9 and TRADD, which serve as a platform to assemble signaling complexes at mitochondrial outer membranes [123,124,128,129]. These signaling

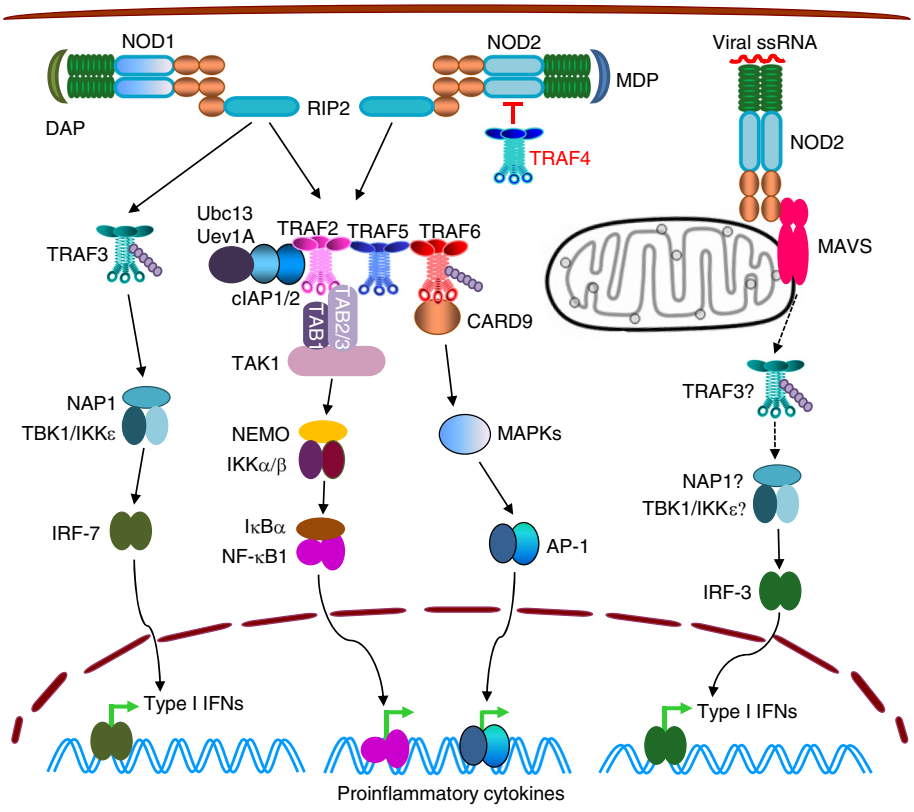

Figure 4 TRAFs in signaling by NOD1 and NOD2. Upon DAP engagement, NOD1 recruits TRAF2, TRAF5, TRAF6 and TRAF3 via RIP2. TRAF2, 5 and 6 mediate NOD1-induced activation of NF-κB1 and MAPKs, while TRAF3 mediates NOD1-induced activation of IRF7. In response to MDP binding, NOD2 also recruits TRAF2, 5 and 6 via RIP2, and thus induces activation of NF-κB1 and MAPKs. When engaged by viral ssRNA, NOD2 binds to MAVS on mitochondria and induces IRF3 activation and Type I IFN production, which is likely mediated by TRAF3.

complexes contain players that are further recruited by TRAFs or TRADD, including cIAP1/2, TANK-NAP1-SINTBAD, TBK1-IKKε, NEMO, IKKα/IKKβ, TAB2/3-TAB1-TAK1, MEKK1, Bcl10, and RIP1-FADD-Casp8-Casp10. RIG-I/MDA5 signaling cascades culminate in the phosphorylation and activation of IRF3, IRF7, NF-κB and AP-1, which work cooperatively to induce the expression of IFNs and proinflammatory cytokines (Figure 5) [123,124,128,129].

TRAF3 is essential for RLR-induced IRF3 but not NF-κB activation, and TRAF3 deficiency results in impaired type I IFN induction in response to RNA virus infection [126]. MAVS has a TRAF3-interacting motif in the C-terminus that is verified by crystallography [130,131], and Tyr9 phosphorylation on MAVS also facilitates the recruitment of TRAF3 [132]. Additionally, TRAF3-MAVS interaction requires the assistance of another TRAF3-interacting protein, UXT-V1 [133]. Following its recruitment to MAVS and in conjunction with Ubc5, TRAF3 undergoes K63-linked auto-ubiquitination, which enhances its ability to bind to NEMO and TANK-NAP1-SINTBAD, thus allowing the recruitment and activation of TBK1 and IKKε [116,128,134-137]. Interestingly, a recent study shows that linear ubiquitination of NEMO switches it from a positive to a negative regulator of RIG-I signaling, as linear ubiquitinated NEMO associates with TRAF3 but disrupts the MAVS-TRAF3 complex [138]. The NEMO-like adaptor proteins TANK, NAP1, and SINTBAD are constitutively bound to both TBK1 and IKKε [135]. Autoubiquitinated TRAF3 activates TBK1 and IKKε to induce the phosphorylation, dimerization and nuclear translocation of IRF3, which triggers the production of type I IFNs [16,128,129].

Depletion of either TRAF2 or TRAF5 leads to reduced IRF3 and NF-κB activation upon RIG-I stimulation, and TRAF2 and TAK1 are important for p38 activation [125,128,139,140]. Biochemical studies revealed TRAF2 and TRAF5 interaction motifs in the C-terminal region

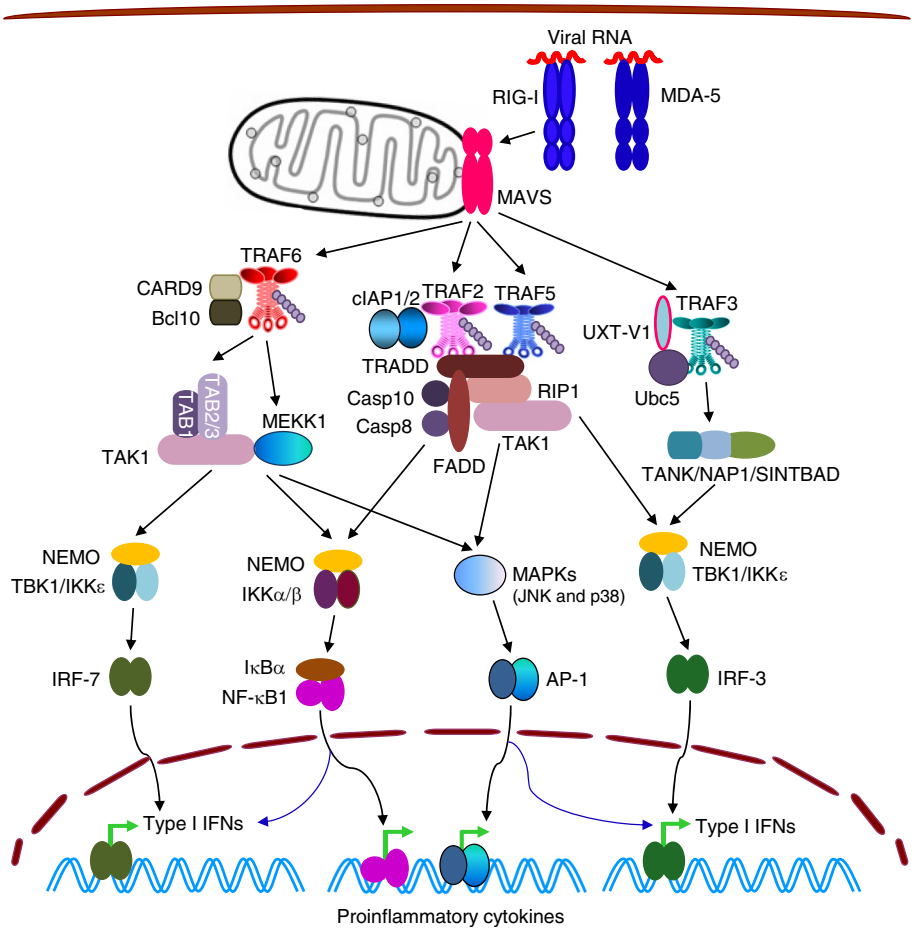

Figure 5 TRAFs in signaling by RIG-I. Upon ligand binding, RIG-I recruits TRAF3, TRAF6, TRAF2 and TRAF5 to mitochondria via MAVS. TRAF3 mediates RIG-I-induced IRF3 but not NF-κB1 activation. TRAF6 mediates RIG-I-induced IRF7 activation and also contributes to activation of NF-κB1, JNK, and p38. TRAF2 is important for p38 activation, and both TRAF2 and TRAF5 also contribute to activation of IRF3 and NF-κB1 in RIG-I signaling.

of MAVS. Upon RIG-I signaling, interaction of TRAF5 with MAVS induces K63-linked TRAF5 auto-ubiquitination and subsequent NEMO-dependent activation of IRF3 and NF-κB [139]. Similarly, activation of p38 by RIG-I proceeds via a TRAF2-TAK1-dependent pathway. The p38 activation in turn stimulates the production of IFNs and IL-12 [125]. Nonetheless, details of TRAF2- or TRAF5- signaling pathways downstream of MAVS remain to be elucidated.

TRAF6 is required for RLR-induced IRF7 activation and also contributes to activation of NF-κB, JNK, and p38 by directly interacting with MAVS, which has two TRAF6-interacting motifs [127,141]. Activation of IRF7 after viral infection resembles IRF3 activation, and involves the direct phosphorylation of IRF7 by TBK1 and IKKε. However, activation of IRF7 but not IRF3 is impaired in TRAF6$^{-/-}$ fibroblasts, and TRAF6 mediates IRF7 ubiquitination [141,142]. Thus, MAVS-induced IRF7 activation is transduced through a unique TRAF6-dependent pathway. Uncoupling IRF3 from the IRF7 activation pathway might be a way of avoiding their simultaneous inhibition by virus-encoded inhibitory proteins [128]. TRAF6 and MEKK1 are also important for RLR-induced activation of NF-κB and MAPKs [127,141]. Interestingly, RIG-I-MAVS-TRAF6 signaling leads to IKKβ-mediated phosphorylation of p65 at ser536, which is under the control of the NADPH oxidase NOX2 [143].

Notably, cIAP1 and cIAP2 are also recruited to MAVS, and mediate K48- and K63- linked polyubiquitination of TRAF3 and TRAF6 in response to viral infections [144]. However, the kinetics of these two types of ubiquitination on TRAF3 and TRAF6 is still unclear. Interestingly, viruses also induce IRF3-dependent apoptosis in infected cells, which require the presence of RIG-I, MAVS, TRAF3, TRAF2, TRAF6 and TBK1, as demonstrated by studies using genetically defective mouse and human cell lines [140]. Apoptosis is triggered by direct interaction of IRF3, through a newly identified BH3 domain, with the pro-apoptotic protein Bax. Co-translocation of IRF3 and Bax to mitochondria results in the induction of mitochondria-dependent apoptosis, and transcriptionally inactive IRF3 mutants could efficiently mediate apoptosis [140]. Although why TRAF3, TRAF2 and TRAF6 are all required for IRF3-induced apoptosis awaits further clarification, it appears that these TRAF molecules cooperate in this process.

TRAFs in cytokine receptor signaling

It was initially recognized that TRAF6 is utilized for signaling by the IL-1R family (IL-1R, IL-18R and IL-33R), which also contain TIR domains found in TLRs [23,145,146]. However, recent evidence indicates that TRAFs also directly regulate signaling by a variety of other cytokine receptors, including receptors for the proinflammatory IL-17 family, anti-viral IFNs, anti-inflammatory TGFβ, and the T cell cytokine IL-2.

IL-17 receptors

The IL-17 family are important in host defense against bacterial, fungal and helminthic parasite infections [147-149]. The founding member of this family, IL-17, is the defining cytokine of a new T helper cell population termed "Th17", which contributes significantly to the pathogenesis of multiple autoimmune and inflammatory diseases [150,151]. Signature target genes of IL-17 include chemokines, proinflammatory cytokines, inflammatory mediators, anti-microbial peptide, and matrix metalloproteases (MMPs) [147-149]. IL-17 (A/F) signals through a heteromeric receptor complex formed by IL-17RA and IL-17RC. IL-17Rs have two extracellular fibronectin III-like domains and a cytoplasmic SEF/IL-17R (SEFIR) domain [149,150]. Ligand-induced association of IL-17RA and IL-17RC recruits a novel adaptor protein Act1 through SEFIR domain-mediated homotypic interaction. Act1 is a U-box E3 ubiquitin ligase that contains both a SEFIR domain and TIMs, and further recruits TRAF6, TRAF2 and TRAF5 [12,152,153]. In cooperation with Ubc13/Uev1A, Act1 catalyzes K63-linked polyubiquitination of TRAF6, which in turn mediates the ubiquitination of IL-17RA and induces the activation of NF-κB through TAK1 and IKKs. Activated NF-κB further induces the expression of IkBζ, C/EBPδ and C/EBPβ, transcription factors that work in concert with NF-κB to induce the expression of signature target genes of IL-17 [12,147,154-158]. On the other hand, TRAF6 also induces GSK3β activation likely through PI-3K, and ERK1/2 activation likely through Raf1 [12,151,159]. Activated GSK3β and ERK induce dual phosphorylation of C/EBPβ and thereby inhibit its activity [12,151,160]. Thus, TRAF6 is essential for IL-17 signaling (Figure 6).

Interestingly, TRAF2 and TRAF5 transduce the IL-17 signals to stabilize mRNA transcripts of chemokines (such as CXCL1) and cytokines (such as IL-6) by recruiting the splicing factor SF2 (also known as alternative splicing factor, ASF) into the IL-17R-Act1 signaling complex [151,153,161]. The IL-17R-Act1-TRAF2-TRAF5 complex also induces the activation of MAPKs, which further enhance mRNA stability. Notably, formation of this complex requires IKKε, an inducible IKK that mediates Act1 phosphorylation at Ser311, adjacent to a putative TRAF-binding motif. Substitution of Ser311 of Act1 with alanine impairs the IL-17-induced Act1-TRAF2-TRAF5 interaction and inflammatory gene expression [161,162]. In contrast, TRAF3 and TRAF4 are negative regulators of IL-17R signaling [12,163,164]. Upon IL-17 stimulation, IL-17RA and IL-17RC directly recruit TRAF3 via a distal C-terminal TRAF3-binding site. The binding of TRAF3 to

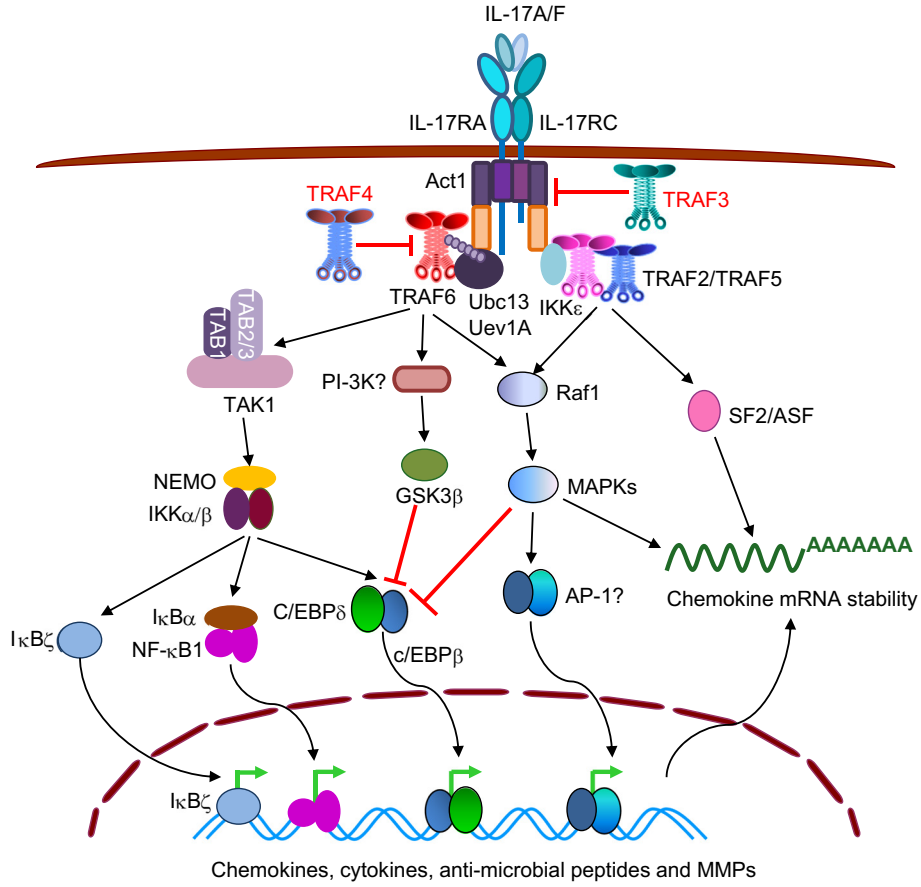

Figure 6 TRAFs in signaling by IL-17R. Upon ligand binding, heteromeric IL-17RA and IL-17RC recruit TRAF6, TRAF2 and TRAF5 via Act1. TRAF6 mediates IL-17-induced activation of NF-κB1, IκBζ, C/EBPδ and C/EBPβ. TRAF2 and TRAF5 transduce the IL-17 signals to stabilize mRNA transcripts of chemokines and cytokines by recruiting SF2 and by inducing activation of MAPKs. In contrast, TRAF3 and TRAF4 inhibit IL-17 signaling. TRAF3 interacts with IL-17RA and IL-17RC and thus interferes with the recruitment of Act1 by IL-17Rs, while TRAF4 binds to Act1 and interrupts the recruitment of TRAF6 by Act1.

IL-17Rs interferes with the formation of the activation signaling complex of IL-17R-Act1-TRAF6, resulting in suppression of downstream signaling, including NF-κB and MAPK activation, and production of inflammatory cytokines and chemokines [12,163]. TRAF4 exerts its negative regulation on IL-17 signaling by competing with TRAF6 for the interaction with Act1, as TRAF4 and TRAF6 use the same TIMs on Act1. Indeed, primary epithelial cells derived from TRAF4$^{-/-}$ mice display markedly enhanced IL-17 signaling [164]. Thus, both TRAF3 and TRAF4 restrict IL-17 signaling at receptor proximal steps (Figure 6).

IFN receptors

Interferons induce the synthesis of a variety of antiviral proteins that mediate swift innate immune responses to control virus replication and spread, and also shape the adaptive immune response by acting directly on T and B cells [116]. TRAF2 and TRAF6 are recognized as direct signal transducers of IFN receptors. Upon IFN engagement, TRAF2 directly binds to the membrane proximal half of the signal-transducing subunit of the IFN receptor, IFNAR1, and is required for IFN-induced NF-κB2 activation and anti-viral responses [13,165]. Similarly, direct interaction of TRAF6 with the intracellular domain of IFNλR1 regulates NF-κB activation and IFNλR1 stability in response to type III IFNs (IFNλ1, IFNλ2, and IFNλ3) [166]. Whether other TRAFs contribute to the regulation of IFN signaling remains to be determined.

TGFβ receptors

The anti-inflammatory cytokine TGFβ binds to type II and type I serine/threonine kinase receptors (TβRII and TβRI). TRAF6 interacts with a consensus TIM present in TβRI [14,167,168]. The TβRI-TRAF6 interaction induces auto-ubiquitination of TRAF6. TβRI kinase activity is required for activation of the canonical Smad pathway, whereas TRAF6 regulates the activation of TAK1 in a receptor kinase-independent manner. Activated TRAF6 mediates K63-linked polyubiquitylation of TAK1 at Lys34 and Lys158, and results in subsequent

activation of p38 and JNK, leading to cell apoptosis [14,167,168]. Thus, TRAF6 is specifically required for the Smad-independent activation of JNK and p38 in response to TGFβ. However, in cancer cells, TRAF6-mediated K63-linked polyubiquitination of TβRI also promotes cleavage of TβRI by TNFα converting enzyme (TACE) in a PKCζ-dependent manner. The liberated intracellular domain of TβRI associates with the transcriptional regulator p300 to activate genes involved in tumor invasiveness, such as Snail and MMP2 [169]. In this case, TRAF6 is critical for TGFβ-induced invasion of cancer cells. Additionally, TRAF6 mediates the suppressive effect of IL-1β or LPS on TGFβ-induced signaling through interaction with the type III TGF-β receptor (TβRIII), an accessory receptor that presents the TGFβ ligand to TβRII. Co-treatment with TGFβ and IL-1β or LPS promotes the interaction between phosphorylated TβRIII and ubiquitinated TRAF6, and thereby sequesters TβRIII from the TβRII/TβRI complex, resulting in inhibition of Smad2/3 activation [170]. Taken together, TRAF6 plays multiple roles in signaling by TGFβ receptors. Interestingly, TGFβ also induces the posttranslational loss of TRAF1, whereas IL-7 restores TRAF1 levels in T cells [171]. No evidence is available about the participation of other TRAFs in TGFβ signaling.

IL-2 receptor

The binding of TRAF6 to the TIM of the IL-2R β-chain negatively regulates IL-2-induced Jak1 activation in CD4 T cells, which is likely involved in the proper regulation of T cell activation and development [172].

TRAFs in other signaling pathways

T cell receptor

TRAF1, TRAF3, and TRAF6 are able to regulate signaling by the T cell receptor (TCR). TRAF1 inhibits CD3-induced NF-κB2 activation and proliferation in T cells [31,173]. TRAF3 is recruited to the signaling rafts, and mediates the synergistic activation of ERK, LAT, PLCγ1 and ZAP70 as well as cytokine production and proliferation in T cells following co-stimulation with TCR and CD28 [11]. TRAF6 is also recruited to the TCR signaling rafts containing CARMA1-MALT1-Bcl10-PKCθ-IKK-Caspase 8 via interaction with the paracaspase MALT1, and contributes to the induction of NF-κB activation and IL-2 production in T cells [174,175]. Interestingly, a recent study by Xie *et al* has shown a distinct mechanism of TRAF6 in TCR signaling, in which TRAF6 is recruited to the TCR/CD28 signaling complex by LAT and promotes the ubiquitination and phosphorylation of LAT as well as the activation of NF-AT in T cells [176].

C-type lectin receptors

Using macrophages derived from TRAF6$^{-/-}$ mice, it has been shown that TRAF6 is required for NF-κB and JNK activation, and expression of proinflammatory cytokines in response to engagement of C-type lectin receptors during fungal infection [177]. This will elicit further studies of other TRAFs in signaling by C-type lectin receptors.

DNA damage response

TRAF6 is essential for DNA damage-induced NF-κB activation. In this process, TRAF6 is activated by the kinase ataxia telangiectasia mutated (ATM), which is a DNA strand break sensor. Following DNA damage, ATM translocates in a calcium-dependent manner to cytosol and membrane fractions, and interacts with TRAF6 via a TIM, resulting in K63-linked polyubiquitination of TRAF6 and recruitment of cIAP1 [178]. The ATM-TRAF6-cIAP1 module stimulates TAB2-dependent TAK1 phosphorylation, and cIAP1 catalyzes monoubiquitination of NEMO at Lys285. NEMO monoubiquitination is a prerequisite for genotoxic NF-κB activation and DNA damage response [178]. Potential involvement of other TRAFs in this response awaits further investigation.

Substrates, E3 ligases and deubiquitinases of TRAFs

Ubiquitination has emerged as a key regulatory mechanism of TRAFs in signaling. As mentioned above in receptor signaling sections, E3 ligase activity has been demonstrated for TRAF2, TRAF3, TRAF5 and TRAF6, which catalyze non-degradative K63-linked polyubiquitination of their substrates. This is mediated in cooperation with the E2 ubiquitin-conjugating enzymes Ubc13-Uev1A or UbcH5c. It is believed that K63-linked polyubiquitin chains serve as docking sites for formation of signaling complexes, facilitate the recruitment and activation of effector kinases, and thus enable the propagation of signals [9,89,179]. The substrates of TRAFs include TRAF themselves, receptors, kinases, adaptor proteins, transcription factors, E3 ubiquitin ligases, and other functional proteins involved in autophagy or ROS production (Table 2). However, in many cases, substrates of TRAFs (especially those of TRAF2, TRAF3 and TRAF5) have not been unequivocally demonstrated by *in vitro* ubiquitination assays using purified proteins. Interestingly, a recent study has shown that TRAF2 becomes a highly active K63-specific ubiquitin ligase when bound to sphingosine-1-phosphate (S1P), which appears to be a co-factor for TRAF2 E3 ligase activity [180]. This suggests that addition of S1P may improve the efficiency of *in vitro* ubiquitination assays for TRAF2. Future studies need to determine whether similar cofactors exist for TRAF3 and TRAF5. Interestingly, however, the crystal structure of the RING domain of TRAF2 [181] and the phenotype of the ΔRING TRAF2 mutant [61,182] suggest that TRAF2 may

Table 2 Substrates of the E3 ligase activity of TRAFs

Substrates (Lys residues of ubiquitination)	E3 ligases	Receptor signaling	References
TRAFs			
TRAF2	TRAF2	TNF-R1/2	[184]
TRAF3	TRAF3	TLR3, TLR4	[92,185]
TRAF5	TRAF5	RIG-I	[139]
TRAF6	TRAF6	TLRs, IL-1R	[17,23,82,89,179,186]
Receptors			
IL-17R	TRAF6	IL-17	[156]
p75 (Lys274, 280 and 283)	TRAF6	NGF	[65]
TβRI	TRAF6	TGFβ	[169]
Kinases			
TAK1 (Lys158)	TRAF6, TRAF2	TNF-R1/2 and IL-1R	[187]
RIP1 (Lys377)	TRAF2	TNF-R1 and IL-1R	[179,180]
TBK1	TRAF3	TLR3, TLR4	[17,23,81]
IKKε	TRAF3	TLR3, TLR4	[17,23,81]
IRAK1 (Lys134 and 180)	TRAF6	TLR7, TLR9, IL-1R	[179,188,189]
Akt (Lys8 and 14)	TRAF6	IL-1R, IGF-1R	[190]
Fyn (K63)	TRAF6	TLR4	[191]
Adaptor proteins			
NEMO (Lys285, 321, 325, 326 and 399)	TRAF6	TLRs, IL-1R, NOD2	[17,23,82,111,192]
TRIF	TRAF2, TRAF6	TLR3, TLR4	[98]
NESCA	TRAF6	TrkA and p75	[193]
LAT (Lys88)	TRAF6	TCR	[176]
Other E3 ligases			
cIAP1/2	TRAF2	CD40	[37]
	TRAF6	TLR4-, IL-1R-induced autophagy	[92]
Smurf2	TRAF2	TNF-R2	[194]
Transcription factors			
IRF7 (Lys444, 446, and 452)	TRAF6	TLR7, TLR8, TLR9, LMP1, RIG-I	[94,142,195]
IRF5 (Lys410 and 411)	TRAF6	NOD2, TLR7, TLR9	[196,197]
Regulators of mRNA stability			
Tristetraprolin	TRAF2	TNF-R1	[198]
Autophagy proteins			
Beclin 1 (Lys117)	TRAF6	TLR4-, IL-1R-induced autophagy	[199]
NDP52	TRAF6	TLR3-induced autophagy	[200]
Regulators of ROS production			
ECSIT	TRAF6	TLR1, 2, 4-induced ROS production	[93]

not function as an E3 ligase at all. The controversy about whether TRAF2 is actually a RING E3 ligase is described in detail in an excellent review by Silke [183].

While serving as E3 ligases themselves, TRAFs are also substrates of other E3 ligases that catalyze K63-linked or K48-linked polyubiquitination (Table 3). K63-linked polyubiquitination of TRAFs usually leads to protein-protein interactions and promotes signal transduction. For example, Act1-mediated K63-linked ubiquitination of TRAF6 recruits TAB2/3-TAK1 and NEMO to activate NF-κB in IL-17R signaling [201], while cIAP1/2-catalyzed K63-linked ubiquitination of TRAF3 recruits TBK1 and IKKε to induce type I IFN production in RIG-I signaling [144]. In an exceptional case, Pellino3-induced ubiquitination of TRAF6 at Lys124 suppresses the ability of TRAF6 to interact with and ubiquitinate IRF7, and thus inhibits type I IFN production in TLR3 signaling [202]. In contrast, K48-linked polyubiquitination of

Table 3 E3 ligases that catalyze the ubiquitination of TRAFs

E3 ligases	Target TRAFs (Lys of ubiquitination)	Receptor signaling	References
K-63 linked polyubiquitination			
Act1	TRAF6 (Lys124)	IL-17R	[201]
	TRAF5	IL-17R	[204]
cIAP1/2	TRAF3 and TRAF6	RIG-I	[144]
Pellino3	TRAF6 (Lys124)	TLR3	[202]
K-48 linked polyubiquitination			
cIAP1/2	TRAF2	TNF-R2	[205,206]
	TRAF2	M-CSFR	[207]
	TRAF2 and TRAF3 (Lys107 and Lys156)	CD40 and TLR4	[72,92]
	TRAF3 and TRAF6	RIG-I	[144]
Triad3A	TRAF3	RIG-I	[203]
AWP1	TRAF2	TNF-R1/2	[208]
SOCS2	TRAF6	AhR	[209]
Siva-1	TRAF2	TCR	[210]
Numbl	TRAF6 and TRAF5	IL-1R	[211,212]
CHIP	TRAF2	Cancer cell invasion	[213]

TRAFs results in degradation of TRAF proteins by the 26S proteasome. K48-linked E3 ligases of TRAFs include cIAP1/2, Triad3A, AWP1, SOCS2, Siva-1, Numbl and CHIP. For example, upon viral infection, Triad3A is up-regulated, and induces K48-linked ubiquitination and degradation of TRAF3, thereby forming a negative feedback loop to halt RIG-I signaling and type I IFN production [203]. Thus, K48-linked ubiquitination and subsequent degradation of TRAFs serve as a negative regulatory mechanism of TRAF-dependent signaling.

A second negative regulatory mechanism of TRAFs is provided by deubiquitinases that cleave K63-linked polyubiquitin chains from TRAFs, which is just beginning to be understood. The known deubiquitinating enzymes (DUBs) of TRAFs include: (1) ubiquitin-specific proteases, such as CYLD, USP2a, USP4, USP20 and USP25; (2) ovarian tumor (OTU) domain-containing DUBs, such as DUBA (also known as OTUD5), OTUB1, OTUB2, and A20; (3) a novel DUB named monocyte chemotactic protein-induced protein 1 (MCPIP1) (Table 4). CYLD, a tumor suppressor and a target gene of NF-κB, negatively regulates NF-κB and JNK activation by removing K63-linked polyubiquitin chains from TRAF2 and TRAF6 as well as several other signaling proteins [214,215]. Expression of DUBA is up-regulated in TLR and IL-1R stimulated cells. DUBA specifically targets and de-conjugates the K63-linked polyubiquitin chains from TRAF3, resulting in TBK1-IKKε dissociation from TRAF3 and inhibition of type I IFN production induced by TLRs and RLRs [128,185,216]. However, DUBA does not affect NF-κB2 activation, which is entirely dependent on K48-linked degradative ubiquitination of TRAF3 [128,185,216]. Interestingly, A20, an unusual enzyme that contains both ubiquitinating and deubiquitinating activities, negatively regulates inflammation by inhibiting NF-κB activation in TNF-R and TLR signaling. A20 is a target gene of NF-κB, and able to remove K63-linked polyubiquitin chains from TRAF6 to turn off NF-κB activation. A20 also inhibits the E3 ligase activities of TRAF6, TRAF2, and cIAP1 by promoting K48-linked polyubiquitination and degradation of the E2 enzymes Ubc13 and UbcH5c [8,128,217]. Furthermore, A20 is capable of targeting an associated signaling molecule such as TRAF2 to the lysosomes for degradation, a process that does not require A20 ubiquitin modifying activity [218]. Notably, $A20^{-/-}$ and $MCPIP1^{-/-}$ mice spontaneously develop severe inflammatory syndrome [219,220], while $CYLD^{-/-}$ and $Usp25^{-/-}$ mice are more susceptible to inflammation [204,221]. Thus, negative regulation of TRAF signaling is necessary to prevent harmful immune responses and inflammatory diseases.

In addition to ubiquitination, other post-translational modifications, including phosphorylation and glutathionylation, are also reported to regulate TRAFs in signaling. Phosphorylation of TRAF1 (at Ser 139 in mouse and Ser 146 in human by PKN1) inhibits TNF-R2-dependent tonic NF-κB and JNK signaling in HeLa cells [233], and also has a negative impact on the recruitment of TBK1 to the 4-1BB signaling complex and the subsequent NF-κB activation in T cells [234]. Phosphorylation of TRAF2 (at Ser11 and Ser55 by PKCζ or IKKε, and at Thr117 by PKCδ and PKCε), which promotes K63-linked ubiquitination of TRAF2 and NF-κB activation, has been demonstrated in TNFα signaling or in transformed cells [235-238]. Following NOD2 activation, phosphorylation

Table 4 Deubiquitinating enzymes that target TRAFs

DUBs	TRAFs	Receptor signaling	References
Ubiquitin-specific proteases			
CYLD	TRAF2, TRAF6	CD40, XEDAR, EDAR, RANK	[222,223]
	TRAF2, TRAF6	IL-1β, TNFα	[224]
USP2a	TRAF2	TNFR1	[225]
	TRAF6	IL-1β, RIG-I	[226]
USP4	TRAF2, TRAF6	TNFα	[227]
	TRAF6	TLR4, IL-1R	[228]
USP20	TRAF6	IL-1β	[229]
USP25	TRAF5, TRAF6	IL-17R	[204]
Ovarian tumor (OTU) family of DUBs			
DUBA (OTUD5)	TRAF3	IL-1β, TLR9	[216]
	TRAF3	TLR3, TLR4, TLR7, RIG-I, MDA-5	[185]
OTUB1	TRAF3, TRAF6	RIG-I	[230]
OTUB2	TRAF3, TRAF6	RIG-I	[230]
A20	TRAF6	TLR4, TLR2	[231,232]
Novel cellular DUBs			
MCPIP1	TRAF2, TRAF3, TRAF6	IL-1, TLR4	[219]

of TRAF4 (at Ser 426 by IKKα) negatively regulates NOD2 signaling in macrophages, including NF-κB activation, cytokine production and antibacterial activity [113]. Tyrosine phosphorylation of TRAF6 by Fyn and c-Src has been shown following LPS stimulation [191]. Interestingly, a recent study reported that TRAF6 is S-glutathionylated under normal conditions. Upon IL-1 stimulation, TRAF6 undergoes deglutathionylation catalyzed by glutaredoxin-1 (GRX-1), a process that is essential for TRAF6 auto-ubiquitination and subsequent NF-κB activation [239]. These findings suggest that different post-translational modifications of TRAF proteins coordinate to regulate the activity of TRAFs in signaling in a dynamic manner.

Viral proteins that target or hijack TRAFs

TRAFs are critical players in host immunity, as demonstrated by their shared usage by both innate immune receptors (such as TLRs, NLRs, RLRs, and cytokine receptors) and adaptive immune receptors (such as CD40, BAFF-R, OX40, 4-1BB, and TCR). Interestingly, viruses and bacteria have developed a variety of strategies to target or hijack TRAFs to evade host immune responses and to promote their own propagation or persistence (Table 5). (1) Several viral and bacterial proteins can function as DUBs to deubiquitinate TRAFs and thus inhibit type I IFN production in RIG-I or TLR signaling. Examples include Lb(pro) of foot-and-mouth disease virus, X protein (HBx) of hepatitis B virus, and YopJ of the Gram- bacterium Yersinia pestis [21,90,240,241]. (2) Some viral proteins can specifically interact with TRAFs and disrupt the formation of TRAF signaling complexes. For example, Gn protein of NY-1 hantavirus and M protein of severe acute respiratory syndrome (SARS) coronavirus disrupt or prevent the formation of TRAF3-TBK1-IKKε complex to inhibit type I IFN production [242,243]. Similarly, A52R and K7 proteins of vaccinia virus disrupt signaling complexes containing TRAF6 and IRAK2 to block NF-κB activation and antiviral defense [20,244]. (3) Some viral proteins usurp TRAFs for viral signaling to promote their own propagation or persistence. The best example of this group is latent membrane protein 1 (LMP1) of Epstein-Barr virus, which sequesters most cellular TRAF3, and hijacks TRAF1, 2, 3, 5 and 6 to mimic constitutively activated CD40 signaling [245-249]. (4) The v-FLIP member MC159 of the human molluscum contagiosum virus mediates the recruitment of both TRAF2 and TRAF3 into the Fas death inducing signaling complex to modulate Fas signaling, and powerfully inhibits both caspase-dependent and caspase-independent cell death induced by Fas [250]. (5) Some viruses up-regulate the expression of specific miRNAs to target TRAFs. For example, the Tat protein of HIV-1 and VSV infection up-regulate miR-32 and miR-146a, which directly target the protein expression of TRAF3 and TRAF6, respectively [251-253]. Together, the above evidence further highlights the crucial importance of TRAFs in host immunity against pathogens.

In vivo functions of TRAFs in mice

The *in vivo* functions of TRAFs in whole animals have been explored by gene targeting in mice. Mice genetically

Table 5 Pathogenic proteins that target TRAFs

Viral or bacterial proteins	TRAFs	Mechanisms	Ref.
Function as DUBs of TRAFs			
Lb(pro) of foot-and-mouth disease virus	TRAF3, TRAF6	Deubiquitinates TRAF3 and 6 to inhibit RIG-I signaling	[21]
X protein (HBx) of hepatitis B virus	TRAF3	Deubiquitinates TRAF3 to inhibit RIG-I signaling	[240]
YopJ of the Gram- bacterium Yersinia pestis	TRAF3, TRAF6	Deubiquitinates TRAF3 and 6 to inhibit TLR signaling	[241]
Disrupt the formation of TRAF signaling complex			
Gn protein of NY-1 hantavirus	TRAF3	Disrupts the interaction of TRAF3 and TBK1-IKKε	[242]
M protein of severe acute respiratory syndrome (SARS) coronavirus	TRAF3	Prevents the formation of TRAF3-TBK1-IKKε complex	[243]
A52R of vaccinia virus	TRAF6	Disrupts the signaling complex of TRAF6 and IRAK2	[244]
K7 of vaccinia virus	TRAF6	Disrupts the signaling complex of TRAF6 and IRAK2	[20]
Usurp TRAFs for viral signaling			
LMP1 of Epstein-Barr virus	TRAF1, 2, 3, 5, 6	Sequesters cellular TRAF3, and usurps TRAF1, 2, 3, 5 and 6 to mimic constitutively activated CD40 signaling	[245-249]
BRRF1 lytic gene product (Na) of Epstein-Barr virus	TRAF2	Utilizes TRAF2 for JNK activation and lytic gene expression	[254]
v-FLIP of Kaposi's sarcoma herpesvirus (human herpesvirus 8)	TRAF2, TRAF3	Recruits TRAF2 and 3 to activate NF-κB and JNK, and to induce cell survival in primary effusion lymphomas	[255]
U(L)37 tegument protein of the herpes simplex virus (HSV)	TRAF6	Activates TRAF6 and NF-κB to induce IL-8 expression	[256]
Modify TRAF signaling complex			
MC159 of human molluscum contagiosum virus	TRAF2, TRAF3	Recruits TRAF2 and 3 to Fas signaling complex and inhibits Fas-induced apoptosis	[250]
Induce miRNAs to target TRAFs			
Tat protein of HIV-1	TRAF3	Up-regulates miR-32 that directly targets TRAF3	[251]
VSV	TRAF6	Up-regulates miR-146a that targets TRAF6 and IRAK1	[252]

deficient in individual TRAFs have been generated. Among these knockout mice, only TRAF1$^{-/-}$, TRAF5$^{-/-}$, and 67% of TRAF4$^{-/-}$ mice could survive to adulthood. In contrast, mice deficient in TRAF2, 3, or 6 exhibit perinatal death with multiple organ abnormalities, indicating that TRAF2, 3, and 6 are indispensable in early development. Although viable, mice deficient in TRAF1, 4 or 5 exhibit distinct phenotypes (Table 6). For example, skin of TRAF1$^{-/-}$ mice is hypersensitive to TNF-induced necrosis [173], and these mice are resistant to allergic lung inflammation in an experimental model of asthma [257]. TRAF4$^{-/-}$ mice suffer respiratory disorder and wheezing caused by tracheal ring disruption, and exhibit numerous developmental abnormalities, including defects in the development of the axial skeleton and in the closure of the neural tube as well as myelin perturbation [258-260]. For mice with early lethality, the causes of death appear to be different. TRAF2$^{-/-}$ mice succumb to severe colitis that result from apoptosis of colonic epithelial cells and accumulation of IL-10-secreting neutrophils, which can be ameliorated by deletion of TNFR1 or combined treatment with neutralizing antibodies against TNFα and IL-10 [261,262]. The early lethality of TRAF3$^{-/-}$ mice is rescued by compound loss of the NF-κB2 gene, suggesting that constitutive NF-κB2 activation leads to the lethal phenotype of TRAF3$^{-/-}$ mice [263]. In contrast, TRAF6$^{-/-}$ mice die of severe osteopetrosis, splenomegaly, and thymic atrophy [264,265]. Taken together, these findings demonstrate that although TRAFs have overlapping functions, each TRAF molecule also plays unique and distinct roles that could not be compensated or substituted by other TRAFs in whole animals.

During the past few years, different laboratories have employed the conditional gene targeting strategy to circumvent the early lethality of TRAF$^{-/-}$ mice. These new mouse models allow more detailed analyses and direct comparison of specific functions of TRAFs in different cell types of whole animals (Table 6).

B lymphocytes

TRAF2, 3, 5 and 6 are important in the survival, development, and activation of B cells. In the absence of either TRAF2 or TRAF3, B cells exhibit remarkably prolonged survival independent of BAFF, which result from the constitutive NF-κB2 activation [36,270,271].

Table 6 In vivo functions of TRAFs in mice

Genotype	Type of knockout	Phenotype	References
TRAF1			
TRAF1−/−	Germline	Viable and normal lymphocyte development	[173]
		Skin hypersensitive to TNF-induced necrosis	[173]
		Hyperproliferation in response to T cell receptor signaling	[173]
		Enhanced Th2 responses	[266]
		Lack of 4-1BB-mediated survival responses in CD8 and memory T cells	[78,79,171]
		Required for 4-1BB-induced NF-κB1 activation in T cells	[31]
		Constitutive NF-κB2 activation in CD8 T cells	[31]
TRAF2			
TRAF2−/−	Germline	Progressively runted and die within 3 weeks after birth	[267]
		Atrophy of the thymus and spleen; depletion of B cell precursors	[267]
		Elevated serum TNF levels; cells sensitive to TNF-induced apoptosis	[267]
		Severe reduction in TNF-mediated JNK activation	[267]
		Severe colitis; drastic changes in the colonic microbiota	[261]
		Increased number of Th17 cells in the colonic lamina propria	[261]
		Apoptosis of colonic epithelial cells due to TNFR1 signaling	[261]
		IL-10-secreting neutrophils accumulated in peripheral blood and bone marrow	[262]
TRAF2flox/flox, CD19-Cre	B cell-specific	Prolonged B cell survival independent of BAFF	[36]
		Splenomegaly and lymphadenopathy	[36]
		Constitutive NF-κB2 activation in B cells	[36]
		Slower and decreased CD40-induced phosphorylation of JNK, p38 and ERK	[74]
		Reduced germinal center formation following SRBC immunization	[74]
TRAF2flox/flox, Lck-Cre	T cell-specific	Normal T cell survival; constitutive NF-κB2 activation in T cells	[36]
TRAF2flox/flox, Albumin-Cre	Hepatocyte-specific	Severely impaired hyperglycemic response to glucagon	[268]
TRAF3			
TRAF3−/−	Germline	Progressively runted; die by 10 days after birth	[269]
		Impaired T cell responses	[269]
TRAF3flox/flox, CD19-Cre	B cell-specific	Prolonged B cell survival independent of BAFF	[36,270]
		Splenomegaly and lymphadenopathy	[36,270]
		Autoimmune manifestations and hyperimmunoglobulinemia	[270]
		Increased T-independent antibody responses	[270]
		Development of B1 lymphomas and splenic marginal zone lymphomas	[271]
		Enhanced signaling by TLR3, TLR4, TLR7, and TLR9 in B cells	[272]
		Accelerated CD40-induced phosphorylation of JNK, p38 and ERK	[74]
TRAF3flox/flox, Lck-Cre	T cell-specific	Normal T cell survival; constitutive NF-kB2 activation in T cells	[36]
TRAF3flox/flox, CD4-Cre	T cell-specific	Normal T cell survival; constitutive NF-kB2 activation in T cells	[11]
		Normal CD4 and CD8 T cell development; increased number of Treg cells	[11]
		Defective T-dependent antibody responses	[11]
		Impaired T cell-mediated immunity to bacterial infection	[11]
		Defective T cell responses to co-stimulation by T cell receptor and CD28	[11]
TRAF4			
TRAF4−/−	Germline	Embryonic lethal but with great individual variation	[258,259]
		Respiratory disorder and wheezing caused by tracheal ring disruption	[258,259]
		Surviving mutant mice manifest numerous developmental abnormalities	[258,259]

Table 6 In vivo functions of TRAFs in mice *(Continued)*

		Altered locomotion coordination typical of ataxia	[258,259]
		High incidence of spina bifida	[258,259]
		Degeneration of a high number of Purkinje cells	[260]
		Increased rates of pulmonary inflammation	[260]
		Reduced migration of DCs; normal development of T and B lymphocytes	[273]
		Inhibits IL-17 signaling and Th17-mediated autoimmune encephalomyelitis	[164]
TRAF5			
TRAF5−/−	Germline	Viable and normal development	[274]
		Mild defect in T-dependent antibody responses	[274]
		Defective in Th1/Th2 differentiation	[275]
TRAF6			
TRAF6−/−	Germline	Perinatal and postnatal lethality	[264,265]
		Severe osteopetrosis; defective in osteoclast formation	[264,265]
		Defective IL-1, CD40 and LPS signaling in lymphocytes	[264,265]
		Defective in lymph node organogenesis	[265]
		Reduced number of immature B cells in the bone marrow	[265]
		Severe defect in the Treg development in thymus	[276]
		Defective development, maturation and activation of DCs	[277]
		Impaired cytokine production in mast cells following FcεRI aggregation	[278]
		Hypohidrotic ectodermal dysplasia	[279]
TRAF6flox/flox, CD19-Cre	B cell-specific	Reduced number of mature B cells in the bone marrow and spleen	[280]
		Impaired T-dependent and T-independent antibody responses	[280]
		Lack of CD5+ B-1 cells	[280]
TRAF6flox/flox, CD4-Cre	T cell-specific	Multiorgan inflammatory disease; hyperactivation of the PI3K-Akt pathway	[281]
		Resistant to suppression by CD4+CD25+ regulatory T cells	[281]
		Resistant to anergizing signals	[282]
		A profound defect in generating CD8 memory T cells;	[283]
		Defective AMPK activation and mitochondrial fatty acid oxidation	[283]
		Specific increase in Th17 differentiation	[284]
		More sensitive to TGFβ-induced Smad2/3 activation and proliferation arrest	[284]
		A severe defect in the Treg development	[276]
TRAF6flox/flox, MCK-Cre	Skeletal muscle-specific	Improved muscle preservation in response to starvation or cancer cachexia	[60,285,286]
		Improved regeneration of myofibers upon injury	[60,285,286]
		Augmented the M2 macrophage phenotype in injured muscle tissues	[60,285,286]
		Upregulated Notch signaling and reduced inflammatory cytokine production	[287,288]

This is further corroborated by the evidence that deletion of cIAP1 and cIAP2 (constitutive interacting partners of TRAF2) also renders BAFF-independent survival of B cells in mice due to constitutive NF-κB2 activation [74]. Strikingly, the development of mature B cells, including the follicular and marginal zone subpopulations of the spleen, are unimpaired in BAFF-R$^{-/-}$ mice that also lack B cell expression of either TRAF2, TRAF3, or cIAP1/cIAP2 [74]. Thus, the survival and maturation pathways normally activated by physiologic triggering of BAFF-R by BAFF are constitutively activated when the expression of TRAF2, TRAF3, or cIAP1/cIAP2 is absent from B cells [74]. Vastly prolonged survival of B cells eventually leads to autoimmune manifestations and B lymphoma development in B cell-specific TRAF3$^{-/-}$ mice [270,271]. Interestingly, TRAF3$^{-/-}$ B cells also display enhanced activation in response to signaling by TLR3, TLR4, TLR7, or TLR9 [272]. Gardam *et al*. further directly compared CD40 signaling in B cell-specific TRAF3$^{-/-}$, TRAF2$^{-/-}$, and cIAP1$^{-/-}$cIAP2$^{-/-}$ mice [74]. Interestingly, loss of TRAF2, TRAF3, or cIAP1/cIAP2 in B cells has very different impacts on CD40 signaling.

TRAF3$^{-/-}$ B cells exhibit accelerated phosphorylation of JNK, ERK, and p38 in response to CD40 signaling. In contrast, TRAF2$^{-/-}$ B cells display slower and decreased CD40 signaling, while cIAP1$^{-/-}$cIAP2$^{-/-}$ B cells show impaired CD40 signaling [74]. Consistent with this, B cell-specific TRAF2$^{-/-}$ and cIAP1$^{-/-}$cIAP2$^{-/-}$ but not TRAF3$^{-/-}$ mice exhibit dramatically reduced germinal center formation following immunization with sheep red blood cells [74]. Notably, TRAF5$^{-/-}$ B cells show defects in proliferation and up-regulation of surface molecules in response to CD40 stimulation, and reduced production of IgM and IgG1 in response to stimulation with CD40 plus IL-4 [274]. Unexpectedly, TRAF6 ablation results in defects in generation of CD5+ B1 cells, reduced number of mature B cells in the bone marrow and spleen, and impaired T-dependent and T-independent antibody responses [280].

T lymphocytes

TRAFs (except TRAF4) play critical roles in regulating T cell immunity. TRAF1$^{-/-}$ T cells exhibit hyperproliferation and increased production of Th2 cytokines (IL-4, IL-5 and IL-13) in response to TCR signaling, but defective 4-1BB-mediated survival responses in effector and memory CD8 T cells [77-79,171,266]. Hyperproliferation of TRAF1$^{-/-}$ T cells is due to constitutive activation of the NF-κB2 pathway [31]. Paradoxically, TRAF2$^{-/-}$ or TRAF3$^{-/-}$ T cells display neither prolonged survival (as that observed in B cells) nor hyperproliferation (as that observed in TRAF1$^{-/-}$ T cells), despite their constitutive processing and activation of NF-κB2 [11,36]. However, the TRAF2-NIK-NFκB2 pathway does drive the development of fatal autoimmune inflammatory disorder in TRAF2$^{-/-}$TNFα$^{-/-}$ mice [289]. Surprisingly, T cell-specific TRAF3$^{-/-}$ mice have increased frequency of regulatory T (Treg) cells, and exhibit defective T-dependent IgG1 responses and T cell-mediated immunity to infection with *Listeria monocytogenes*, which is due to impaired TCR/CD28 signaling [11]. Similarly, CD27-mediated co-stimulatory signaling was reduced in TRAF5$^{-/-}$ T cells [274]. In contrast, TRAF6$^{-/-}$ mice show a severe defect in Treg development in thymus [276]. T cell-specific deletion of TRAF6 results in the development of multiorgan inflammatory disease [281]. TRAF6$^{-/-}$ T cells exhibit hyperactivation of the PI3K-Akt pathway, resistance to suppression by Treg cells, and also resistance to anergizing signals [281,282]. TRAF6$^{-/-}$ CD4 T cells display increased Th17 differentiation, due to enhanced sensitivity to TGFβ-induced Smad2/3 activation and IL-2 down-regulation [284]. Interestingly, activated TRAF6$^{-/-}$ CD8 T cells exhibit defective AMP-activated kinase activation and mitochondrial fatty acid oxidation (FAO) in response to growth factor withdrawal, resulting in a profound defect in memory CD8 T cell development after infection [283].

DCs and mast cells

TRAF1, 2, 3, 4 and 6 regulate the functions of dendritic cells (DCs). Arron *et al*. demonstrated the cooperation of TRAF1 and TRAF2 in DCs [290]. TRAF1$^{-/-}$ DCs matured in CD154 display impaired NF-κB activation and survival but increased TRAF2 degradation in response to CD154 re-stimulation [290]. TRAF3$^{-/-}$ DCs produce increased amounts of IL-12 but reduced amounts of IL-10 and little type I IFN in response to TLR7 and TLR9 signaling [18,95,96]. TRAF3$^{-/-}$ DCs also display constitutive NF-κB2 activation but not prolonged survival [18]. TRAF4$^{-/-}$ DCs exhibit reduced migration both in transwell experiments and *in vivo* [273]. Interestingly, TRAF6 is required for DC maturation and activation. In response to either microbial components or CD40L, TRAF6$^{-/-}$ DCs fail to up-regulate surface expression of MHC class II and CD86, or produce inflammatory cytokines [277]. Similarly, TRAF6$^{-/-}$ mast cells exhibit impaired production of IL-6, CCL-9, IL-13, and TNF following FcεRI aggregation by IgE [278].

Hepatocytes and skeletal muscles

Hepatocyte-specific TRAF2$^{-/-}$ mice exhibit significantly decreased blood glucose levels under high-fat diet conditions. Although these mice show normal insulin signaling and the hypoglycemic response to insulin, they have severely impaired glucagon signaling and the hyperglycemic response to glucagon. In addition, TRAF2 overexpression significantly increases the ability of glucagon or a cAMP analog to stimulate CREB phosphorylation, gluconeogenic gene expression, and hepatic glucose production in primary hepatocytes. Thus, hepatic cell TRAF2 autonomously promotes hepatic gluconeogenesis, and contributes to hyperglycemia in obesity [268]. Interestingly, skeletal muscle-specific depletion of TRAF6 in mice improves satellite cell activation and skeletal muscle regeneration through up-regulation of Notch signaling and reducing the inflammatory repertoire [287]. TRAF6 deficiency inhibits the induction of atrophy program in response to starvation, denervation, or cancer cachexia by suppressing the expression of key regulators of atrophy, including MAFBx, MuRF1, p62, LC3B, Beclin1, Atg12, and Fn14 [60,285,286]. Ablation of TRAF6 also improves the phosphorylation of Akt and FoxO3a and inhibits the activation of 5′ AMP-activated protein kinase in skeletal muscle in response to starvation. Moreover, K63-linked autoubiquitination of TRAF6 regulates ER stress and unfolding protein response pathways in starvation-induced muscle atrophy [288]. It remains to be elucidated whether other TRAFs regulate hepatocyte and skeletal muscle functions.

Atherosclerosis

Experiments of mouse models of atherosclerosis have provided evidence that TRAF1, 5 and 6 regulate the pathogenesis of this disease. Missiou *et al.* reported that TRAF1 deficiency attenuates atherosclerosis in low-density lipoprotein receptor $(LDLR)^{-/-}$ mice by impairing monocyte recruitment to the vessel wall [291]. Deletion of TRAF1 inhibits adhesion of inflammatory cells to the endothelium, reduces the expression of CD29 in macrophages, and decreases the expression of the adhesion molecules ICAM-1 and VCAM-1 in endothelial cells [291]. In contrast, TRAF5 deficiency accelerates atherogenesis in $LDLR^{-/-}$ mice. Deletion of TRAF5 in endothelial cells or in leukocytes enhances adhesion of inflammatory cells to the endothelium, thus facilitating inflammatory cell recruitment to the atherosclerotic plaques. In addition, TRAF5 deficiency increases the expression of adhesion molecules and chemokines, and potentiates macrophage lipid uptake and foam cell formation [292]. Interestingly, endothelial and myeloid cell TRAF6 proteins have opposite roles in atherosclerosis in $ApoE^{-/-}$ mice. Endothelial TRAF6 deficiency inhibits atherosclerosis by reducing proinflammatory gene expression and monocyte adhesion to endothelial cells. In contrast, myeloid cell-specific TRAF6 deletion exacerbates atherosclerosis. $TRAF6^{-/-}$ macrophages exhibit impaired expression of the atheroprotective cytokine IL-10, elevated ER stress, increased sensitivity to oxidized LDL-induced apoptosis, and reduced capacity to clear apoptotic cells [293]. Similar mouse models of TRAF2, 3 and 4 need to be generated and characterized in future studies.

TRAFs in human diseases

Findings obtained from TRAF-deficient mouse models have laid the basis to understand the roles of TRAFs in the pathogenesis of human diseases. Given their importance in regulating the development, survival and activation of various cell types, it would be expected that aberrant functions of TRAFs may contribute to different diseases. However, the roles of TRAFs in human diseases are just beginning to be revealed. Available evidence implicates TRAFs in the pathogenesis of cancers, autoimmune diseases, immunodeficiencies, and neurodegenerative diseases (Table 7).

B cell malignancies

Growing literature documents the prominent relevance of TRAF3, TRAF2 and TRAF1 in B cell malignancies. As predicted from their critical roles in inhibiting B cell survival, biallelic deletions or inactivating mutations of TRAF3 and TRAF2 frequently occur in primary human samples of B cell neoplasms. Deletions and mutations of TRAF3 have been reported in multiple myeloma [294-296], Waldenström's macroglobulinemia [300], Hodgkin lymphomas (HLs) [301], and a variety of non-Hodgkin lymphomas (NHLs), including splenic marginal zone lymphoma, B cell chronic lymphocytic leukemia (B-CLL), and mantle cell lymphoma [298,299]. Similarly, inactivating mutations of TRAF2 have been identified in multiple myeloma [294-296] and diffuse large B-cell lymphoma (DLBCL) [302]. Single nucleotide polymorphisms (SNPs) of TRAF3 are also associated with altered risk of multiple myeloma [297]. In contrast, TRAF1 expression is ubiquitously elevated in both HLs [314] and NHLs, especially in CLL and mediastinal large B-cell lymphoma [315-317]. In addition, TRAF1 SNPs are associated with NHLs [303]. Thus, TRAF3 and TRAF2 are tumor suppressive, whereas TRAF1 appears to be oncogenic in B cells.

Carcinomas

Overexpression and gene amplification of TRAF4 and TRAF6 have been reported in human carcinomas. TRAF4 is overexpressed in breast and lung carcinomas [304,318,319]. TRAF4 protein overexpression is limited to cancer cells and the subcellular localization is consistently cytoplasmic in a large majority of cases. Increased TRAF4 gene copy number is one major mechanism responsible for TRAF4 protein overexpression in human cancers. Indeed, TRAF4 is located at chromosome 17q11.2 in a region of amplification devoid of other known oncogenes [304,318,319]. Intriguingly, TRAF4 is a target gene of the p53 family of transcription factors, including p63, p73 and p53, in squamous cell carcinoma of the head and neck (SCCHN). TRAF4 locates in the nucleus in normal oral epithelium and highly/moderately differentiated cells, but is localized in the cytoplasm in poorly differentiated SCCHN. Overexpression of TRAF4 in SCCHN induces apoptosis and suppresses colony formation [320-322]. Thus, TRAF4 overexpression has different outcomes in different carcinomas. Notably, TRAF6 gene is located in another frequently amplified region at chromosome 11p13. TRAF6 exhibits overexpression and gene amplification in lung cancer and osteosarcoma cells [305,306,323]. Downregulation of TRAF6 in human lung cancer and osteosarcoma cells suppresses NF-κB activation, cell survival and proliferation, and tumor formation and invasion. These observations suggest that TRAF6 overexpression may promote the tumorigenesis and invasion of lung cancer and osteosarcoma cells [305,306,323].

Autoimmune diseases

Single nucleotide polymorphisms (SNPs) in TRAFs have been linked to autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). SNPs of TRAF6 are associated with both SLE and RA [22]. Similarly, SNPs at the TRAF1/C5 locus are associated with both SLE and RA [307,308,310,311]. A single SNP

Table 7 Genetic variations of TRAFs in human diseases

Diseases	Genetic variations of TRAFs	References
B cell malignancies		
Multiple myeloma	Deletions or inactivating mutations of TRAF3, TRAF2	[294-296]
	SNPs of TRAF3	[297]
Splenic marginal zone lymphoma	Deletions or inactivating mutations of TRAF3	[298,299]
B cell chronic lymphocytic leukemia	Deletions or inactivating mutations of TRAF3	[298]
Mantle cell lymphoma	Deletions or inactivating mutations of TRAF3	[298]
Waldenström's macroglobulinemia	Deletions or inactivating mutations of TRAF3	[300]
Hodgkin lymphoma	Deletion of TRAF3	[301]
Diffuse large B-cell lymphoma	Inactivating mutations of TRAF2, TRAF5	[302]
Non-Hodgkin lymphoma	SNPs of TRAF1	[303]
Carcinomas		
Breast cancers	Amplification of TRAF4	[304]
Lung cancers	Amplification of TRAF4, TRAF6	[304,305]
Osteosarcoma	Amplification of TRAF6	[306]
Autoimmune diseases		
Systemic lupus erythematosus	SNPs of TRAF6, TRAF1/C5	[22,307,308]
Rheumatoid arthritis	SNPs of TRAF5, TRAF6, TRAF1/C5	[22,309-311]
Immunodeficiencies		
HSV-1 encephalitis	Inactivating mutation of TRAF3	[312]
Other		
Hypohidrotic ectodermal dysplasia	Inactivating mutation of TRAF6	[313]

(rs7514863), mapping upstream of the TRAF5 gene and affecting a putative transcription factor binding site, demonstrates a significant association with RA [309]. In addition, decreased expression of TRAF2 has been detected in peripheral blood mononuclear cells of SLE patients [324]. However, further association and functional studies are required to determine whether these TRAFs play causal roles in increasing susceptibility to SLE or RA.

Immunodeficiencies

An autosomal dominant mutation of TRAF3 has been reported in a young adult with a history of herpes simplex virus-1 (HSV-1) encephalitis in childhood [312]. The TRAF3 mutant allele is loss-of-expression, loss-of-function, dominant-negative, and associated with impaired responses upon stimulation of both TNF-Rs and TLRs. The recurrent HSV-1 infection and encephalitis result from the impairment of TLR3-induced type I IFN production [312].

Hypohidrotic ectodermal dysplasia

A heterozygous mutation of TRAF6 has recently been identified in a patient with hypohidrotic ectodermal dysplasia (HED)[325]. The mutant TRAF6 protein is capable of forming a complex with TAK1 and TAB2, but cannot bind to the receptor XEDAR. Furthermore, the mutant TRAF6 protein potently inhibits the interaction between wild type TRAF6 and XEDAR, and suppresses the XEDAR-mediated NF-κB activation. Thus, this mutant TRAF6 protein acts in a dominant-negative manner to affect the XEDAR-mediated NF-κB activation during the development of ectoderm-derived organs, leading to HED phenotype [313].

Neurodegenerative diseases

Interestingly, recent evidence implicates the E3 ligase activity of TRAF6 in the pathogenic aggregation of mutant proteins in neurodegenerative diseases such as Parkinson's disease and Huntington disease. It was found that TRAF6 binds to misfolded mutant DJ-1, aSYN and N-HTT, proteins involved in the pathogenesis of the Parkinson's disease and Huntington disease. Mutant DJ-1, aSYN and N-HTT proteins are all substrates of TRAF6. Instead of conventional K63-linked polyubiquitination, TRAF6 promotes atypical ubiquitination of DJ-1, aSYN and N-HTT with K6, K27, and K29 linkage formation, thereby stimulating aggregate formation of mutant DJ-1, aSYN and N-HTT in neurodegenerative diseases [326,327].

Chronic inflammation and infection

In light of their crucial importance in inflammatory and immune responses, it would be predicted that TRAF molecules may also contribute to chronic inflammation and infection. Although no genetic association of TRAFs and chronic inflammation or infection has been identified, recent evidence of alterations of TRAF protein levels supports this possibility. Notably, TRAF2 and TRAF3 are often degraded in response to signaling by the TNF-R superfamily [3,27,32,328]. In contrast, TRAF1 expression is up-regulated by NF-κB activation in response to signaling by a variety of receptors, including TNF-R superfamily and cytokine receptors, etc. [329-331]. The dynamic change of the stoichiometry of different TRAF molecules inside the cell impacts subsequent cellular responses to inflammatory or infectious stimuli. For example, the presence of TRAF1 stabilizes TRAF2, which plays a role in promoting proinflammtory responses in HeLa cells [332,333]. More direct evidence was provided by a recent study demonstrating that TRAF1 is specifically lost from virus-specific CD8 T cells during the chronic phase of infection with HIV in humans [171]. This area warrants further investigation.

Conclusions

Since the first TRAFs were cloned in the mid 1990s, we have witnessed a remarkable progress in understanding the functions of TRAFs in signaling. TRAFs are now recognized as signal transducers of a wide variety of receptors, including the TNF-R superfamily, TLRs, NLRs, RLRs, IL-1R family, IL-17Rs, IFN receptors, TGFβ receptors, IL-2R, TCR, and C-type lectin receptors. Although initially defined as adaptor proteins, most TRAFs also function as E3 ubiquitin ligases through their RING finger domain. Furthermore, activation of TRAFs is exquisitely regulated by post-translational modifications, especially ubiquitination, which has become the subject of intense investigations during the past few years. Termination of TRAF activation could be achieved through either K48-linked polyubiquitination followed by proteosomal degradation or removal of K63-linked polyubiquitin chains catalyzed by deubiquitinases. Acting alone or in combination, TRAF-dependent signaling pathways regulate the activation of NF-κBs, MAPKs, or IRFs to control diverse cellular processes. Accumulating evidence obtained from TRAF-deficient mice demonstrates that each TRAF plays obligatory and distinct roles critical for innate immunity, adaptive immunity, embryonic development, and tissue homeostasis. The pivotal roles of TRAFs in host immunity are further highlighted by the finding that targeting TRAFs appears to be a common mechanism employed by pathogenic proteins of viruses and bacteria. Furthermore, the interest in TRAFs is also driven by recent discoveries that link TRAF genetic variations to human diseases such as cancers, autoimmune diseases, and immunodeficiencies. In conclusion, TRAFs are versatile and indispensable regulators of signal transduction and immune responses, and aberrant functions of TRAFs contribute to the pathogenesis of human diseases.

Perspectives

Despite the wealth of current knowledge about TRAFs, many key questions remain, which will drive the next stage of research in this important area. (1) What is the stoichiometric composition of TRAFs and other signaling proteins in each signaling complex? What are the dynamic kinetics of activation and spatial regulation of each TRAF molecule in response to each specific stimulus? Cutting-edge biochemical, proteomic, and imaging technologies will be needed to uncover these details. (2) How is the E3 ligase activity of each TRAF regulated precisely? What are the substrates of the E3 ligase activities of TRAF2, 3 and 5? Are there additional E3 ligases, deubiquitinases, kinases, and phosphatases that target different TRAFs? Are the enzymes targeting TRAFs regulated by TRAF-dependent signaling pathways? *In vitro* reconstitution experiments and ligase activity assays, high throughput screens for substrates and enzymes, and systems biology approaches will be needed to address these issues. (3) What are the molecular structures of each TRAF in complex with its specific signaling partner, substrate, or enzyme? This requires access to co-crystals containing TRAFs and their interacting partners, and the crystal structure of full-length TRAFs/substrates remains a challenge. (4) Are there additional pathogenic factors of invading microorganisms that target TRAFs during infections? If so, by what precise mechanisms? Yeast 2-hybrid screen, bioinformatic studies and proteomic approaches may be applied toward this end. (5) During pathogen infections, multiple TRAF-dependent signaling pathways are triggered either sequentially or simultaneously, including innate immune receptors (such as TLRs, NLRs and RLRs), adaptive immune receptors (such as TCR, CD40, OX-40 and 4-1BB), and cytokine receptors (such as IL-1R, IL-17R, IFN-Rs, and TβRs). How does each TRAF act in such complex and concerted signaling pathways in different cellular context during infection? Whether and how does each TRAF regulate the crosstalk between different immune signaling pathways? Responses of $TRAF^{-/-}$ mice, and especially cell type-specific $TRAF^{-/-}$ mice, to infections will be instrumental in addressing these questions. Sequential or simultaneous co-engagement of different immune receptors also needs to be investigated thoroughly in cultured cells. (6) What are the cell type-specific factors that dictate cell type-specific TRAF functions? For example, TRAF2 or TRAF3 deficiency leads to prolonged survival in B cells, but not in T cells or DCs. Genetic

and systems biology approaches will be required for such studies. (7) Are there additional TRAF genetic alterations and SNPs associated with human diseases? Do epigenetic modifications of TRAFs contribute to disease conditions? How? Systematic and comprehensive analyses employing genetic, bioinformatic, and deep sequencing approaches will facilitate such investigation. Generation and examination of $TRAF^{-/-}$ or TRAF-transgenic mouse models of human diseases are also required to decipher the underlying mechanisms. Together, these future studies will undoubtedly yield valuable information to advance our understanding of TRAFs.

Given the importance of TRAFs in host immunity and in human diseases, the above future studies will also provide a platform for the development of therapeutic intervention of TRAF-mediated human diseases. For example, insights gained into the structures of each TRAF in complex with its specific signaling partner, substrate, or enzyme will guide the development of structure-based therapeutics. Small agonists and antagonists of TRAFs may be devised to enhance beneficial signaling pathways and to interfere with harmful ones, respectively. In this regard, cell-permeable TRAF6 decoy peptides potently inhibit TRAF6 signaling in cultured cells, and their therapeutic potential in disease settings are currently under investigation [191,334]. A chemical compound 5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine (P(3)-25), which possesses anti-bacterial and anti-fungal activities, specifically inhibits TRAF2-mediated NF-κB activation while enhancing TRAF2-mediated AP-1 activation [335]. However, the diverse and cell type-specific functions of TRAFs may prevent systemic administration of therapeutic agents that directly target TRAFs, and local or cell-specific drug delivery needs to be exercised. Alternatively, therapeutic strategies may be designed to specifically manipulate TRAF-interacting partners or downstream signaling pathways. For example, pharmacological inhibitors for cIAP1/2 are currently at various stages of clinical trials for cancers [107], and may be applied to other TRAF-mediated diseases too. Further in-depth understanding of TRAF signaling pathways will serve as experimental framework to be translated into such therapeutic development.

Competing interests

The author declares that she has no competing financial interests.

Acknowledgements

I would like to thank anonymous reviewers for their critical reading of this article and for their significant contributions. Research of the author's laboratory is supported by the National Institutes of Health grant CA158402, the Department of Defense grant CA120120, the Cancer Institute of New Jersey through Grant Number P30CA072720 from the National Cancer Institute, and the Arthur Herrmann Endowed Cancer Research Fund.

Received: 22 March 2013 Accepted: 7 June 2013
Published: 13 June 2013

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doi:10.1186/1750-2187-8-7
Cite this article as: Xie: TRAF molecules in cell signaling and in human diseases. *Journal of Molecular Signaling* 2013 **8**:7.

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Mutated in colorectal cancer (MCC) is a novel oncogene in B lymphocytes

Shanique KE Edwards[1,2], Jacqueline Baron[1], Carissa R Moore[1], Yan Liu[1], David H Perlman[4], Ronald P Hart[1,3,5] and Ping Xie[1,5,6*]

Abstract

Background: Identification of novel genetic risk factors is imperative for a better understanding of B lymphomagenesis and for the development of novel therapeutic strategies. TRAF3, a critical regulator of B cell survival, was recently recognized as a tumor suppressor gene in B lymphocytes. The present study aimed to identify novel oncogenes involved in malignant transformation of TRAF3-deficient B cells.

Methods: We used microarray analysis to identify genes differentially expressed in TRAF3$^{-/-}$ mouse splenic B lymphomas. We employed lentiviral vector-mediated knockdown or overexpression to manipulate gene expression in human multiple myeloma (MM) cell lines. We analyzed cell apoptosis and proliferation using flow cytometry, and performed biochemical studies to investigate signaling mechanisms. To delineate protein-protein interactions, we applied affinity purification followed by mass spectrometry-based sequencing.

Results: We identified *mutated in colorectal cancer* (*MCC*) as a gene strikingly up-regulated in TRAF3-deficient mouse B lymphomas and human MM cell lines. Aberrant up-regulation of *MCC* also occurs in a variety of primary human B cell malignancies, including non-Hodgkin lymphoma (NHL) and MM. In contrast, *MCC* expression was not detected in normal or premalignant TRAF3$^{-/-}$ B cells even after treatment with B cell stimuli, suggesting that aberrant up-regulation of *MCC* is specifically associated with malignant transformation of B cells. In elucidating the functional roles of MCC in malignant B cells, we found that lentiviral shRNA vector-mediated knockdown of MCC induced apoptosis and inhibited proliferation in human MM cells. Experiments of knockdown and overexpression of MCC allowed us to identify several downstream targets of MCC in human MM cells, including phospho-ERK, c-Myc, p27, cyclin B1, Mcl-1, caspases 8 and 3. Furthermore, we identified 365 proteins (including 326 novel MCC-interactors) in the MCC interactome, among which PARP1 and PHB2 were two hubs of MCC signaling pathways in human MM cells.

Conclusions: Our results indicate that in sharp contrast to its tumor suppressive role in colorectal cancer, MCC functions as an oncogene in B cells. Our findings suggest that MCC may serve as a diagnostic marker and therapeutic target in B cell malignancies, including NHL and MM.

Keywords: MCC, TRAF3, B lymphoma, Multiple myeloma, PARP1, PHB2

Background

B cell neoplasms account for over 90% of lymphoid tumors worldwide, and comprise >50% of blood cancers. Despite recent advances in treatment, many types of human B cell lymphomas remain incurable, highlighting a clear need for new preventative and therapeutic strategies [1,2]. Increasing evidence indicates the importance of genetic determinants in B lymphomagenesis [3-5]. Identification and validation of new genetic risk factors are imperative for a better understanding of B cell malignant transformation and for the development of new therapeutic strategies. Recent studies from our laboratory and others have identified TRAF3, a critical determinant of B cell survival, as a tumor suppressor gene in B lymphocytes. TRAF3 is a member of the tumor necrosis factor receptor (TNF-R)-associated factor (TRAF) family (TRAF1-6) of cytoplasmic adaptor proteins [6]. All TRAF proteins have the distinctive feature of a C-

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terminal TRAF domain, which mediates the interaction with TRAF-binding motifs of receptors of the TNF-R superfamily [6,7]. Homozygous deletions and inactivating mutations of the TRAF3 gene have been identified in non-Hodgkin lymphoma (NHL), including splenic marginal zone lymphoma (MZL), B cell chronic lymphocytic leukemia (B-CLL) and mantle cell lymphoma (MCL), as well as multiple myeloma (MM) and Waldenström's macroglobulinemia (WM) [8-11].

By generating and characterizing a mouse model that has the *Traf3* gene specifically deleted in B lymphocytes (B-TRAF3$^{-/-}$ mice), we recently reported that TRAF3 deletion leads to spontaneous development of MZL and B1 lymphoma in mice [12,13]. Interestingly however, B lymphoma development in B-TRAF3$^{-/-}$ mice exhibits a long latency (approximately 9 months), indicating that TRAF3 inactivation and its aberrant signaling pathways are not sufficient to induce B lymphomagenesis and that additional oncogenic pathways are necessary for B lymphoma development. Although TRAF3 deletions or mutations exist in human patients with NHL and MM, it is not known whether TRAF3 inactivation is the primary or secondary oncogenic mutation in human samples. Thus, B-TRAF3$^{-/-}$ mice offer the unique advantage to identify secondary oncogenic pathways that drive B lymphomagenesis in the context of TRAF3 inactivation. To identify such secondary oncogenic alterations that mediate the malignant transformation of TRAF3$^{-/-}$ B cells, we performed a transcriptome microarray analysis using TRAF3$^{-/-}$ mouse splenic B lymphomas. Surprisingly, our microarray analysis identified *mutated in colorectal cancer* (*MCC*), a tumor suppressor gene of colorectal cancer, as a strikingly up-regulated gene in B lymphomas spontaneously developed in different individual B-TRAF3$^{-/-}$ mice.

The *MCC* gene was discovered in 1991 through its linkage to the region showing loss of heterozygosity (LOH) in familial adenomatous polyposis (FAP) [14-17]. Subsequent studies revealed that the *adenomatous polyposis coli* (*APC*) gene and not *MCC* is responsible for FAP. The APC gene is mutated somatically in 60–80% of sporadic colorectal cancers (CRCs), whereas somatic mutation of MCC is relatively rare, 3–7%, in sporadic CRCs [14-18]. However, it was subsequently reported that the MCC gene is silenced through promoter methylation in approximately 50% of primary sporadic CRCs and 80% of serrated polyps, suggesting that the silencing of MCC is important in early colon carcinogenesis via the serrated neoplasia pathway [19-22]. Furthermore, loss-of-function mutations, LOH, or decreased expression of the *MCC* gene are also detected in a number of other human cancers, including lung cancer [17,23], gastric carcinoma [24], esophageal cancer [25], and hepatocellular carcinoma [26,27]. In addition, an SNP of the MCC gene (rs11283943) is significantly associated with increased risk of breast cancer [28]. Although an inactivating *MCC* mutation in mice alone failed to induce any evident CRCs, the homozygous mice displayed a slightly higher proliferation rate of the epithelial crypt cells [29,30]. Interestingly, an unbiased genetic screening of a mouse model of CRC implicated *MCC* mutation as a key event in colorectal carcinogenesis [18]. Consistent with the genetic evidence, functional studies revealed that MCC blocks cell cycle progression in NIH3T3 fibroblasts and CRCs [31,32], inhibits cell proliferation and migration in CRCs [20,32-34], and is required for DNA damage response in CRCs [35]. MCC appears to specifically target and negatively regulate the oncogenic NF-κB and β-catenin pathways in CRCs and hepatocellular carcinoma [20,27,32,36]. Mutation studies have revealed that the N-terminal domain (130–278 aa) of MCC is required for repressing the Wnt/β-catenin signaling pathway [20] and that the PDZ-binding motif at the extreme C-terminus of MCC mediates its interaction with Scrib-Myosin IIB to regulate cytoskeletal reorganization and cell migration in CRCs [34]. Collectively, the above genetic and functional evidence indicates that *MCC* functions as a tumor suppressor gene in CRCs by inhibiting cell cycle progression and migration, and by promoting DNA damage-induced cell cycle arrest in colorectal epithelial cells.

It has been shown that during development, MCC is expressed in diverse tissues derived from all three embryonic germ layers, including the developing gut and central nervous system [29]. In adults, MCC is expressed in the surface epithelium of the colon and villi of the small intestines as well as other tissues, including the cerebellar cortex, kidney, pancreas, and liver [31,37]. However, expression of MCC was not reported in lymphocytes, and the function of MCC in lymphocytes or lymphomas has not been explored. In the present study, we aimed to address this gap in knowledge.

Our unexpected finding that *MCC* was strikingly up-regulated in TRAF3$^{-/-}$ mouse B lymphomas prompted us to further examine *MCC* expression in human B cell neoplasms. Our results demonstrated high levels of aberrant *MCC* expression in 6 human patient-derived MM cell lines with TRAF3 deletions or relevant mutations. We also surveyed the public gene expression database of microarray data of human cancers (http://www.oncomine.org), and learned that *MCC* was also aberrantly and significantly elevated in a variety of primary human B cell malignancies. These include primary effusion lymphoma (PEL), centroblastic lymphoma (CBL), diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma (BL), and MM [38-41]. However, expression of the transcript and protein of MCC was not detected in wild type B cells or premalignant TRAF3$^{-/-}$ B cells, even after treatment with a variety of B cell stimuli, including CD40, BCR, LPS, and CpG. These results suggest that aberrant MCC expression is

associated specifically with B cell tumorigenesis. We next investigated the functions of MCC in malignant B cell survival and proliferation, and found that lentiviral shRNA vector-mediated knockdown of MCC induced apoptosis and inhibited proliferation in human patient-derived MM cell lines. Furthermore, we identified MCC signaling pathways and interacting partners in malignant B cells. Our results thus provide novel mechanistic insights into how MCC promotes malignant B cell survival and proliferation. Taken together, our findings indicate that in sharp contrast to its tumor suppressive function in CRCs and other human carcinomas, MCC is oncogenic in B lymphocytes.

Results

Transcriptome microarray analysis of TRAF3$^{-/-}$ mouse B lymphomas

TRAF3$^{-/-}$ B cells purified from young B-TRAF3$^{-/-}$ mice exhibit prolonged survival but do not proliferate autonomously [12], and therefore are premalignant B cells. Consistent with this, no B lymphoma development was observed in B-TRAF3$^{-/-}$ mice younger than 9 months old [13]. The long latency of B lymphoma development observed in B-TRAF3$^{-/-}$ mice suggests that TRAF3 inactivation and its downstream signaling pathways are not sufficient and that additional oncogenic alterations are required to induce B lymphomagenesis. To identify such secondary oncogenic alterations, to provide new insights into the molecular mechanisms of B cell malignant transformation, and to discover new therapeutic targets for the treatment of B cell malignancies, we performed global gene expression profiling of TRAF3$^{-/-}$ mouse B lymphomas by transcriptome microarray analysis. We used RNA samples of 3 representative TRAF3$^{-/-}$ splenic B lymphomas (mouse ID: 6983–2, 7041–10, and 7060–8), in which B lymphomas are >70% of B cells, as assessed by FACS analysis of B cell populations and Southern blot analysis of IgH gene rearrangements [13]. Results of the microarray analysis have identified 160 up-regulated genes and 244 down-regulated genes in TRAF3$^{-/-}$ B lymphomas as compared to LMC spleens (cut-off fold of changes: 2-fold up or down, $p < 0.05$; Additional file 1: Table S1) (NCBI GEO accession number: GSE48818). Selective examples of these identified genes are shown in the heatmap (Figure 1A). Functional clustering and pathway analyses by Ingenuity (http://www.ingenuity.com) revealed that genes differentially expressed in TRAF3$^{-/-}$ B lymphomas include transcription factors, cell surface receptors, enzymes, cell cycle regulators, protein translation regulators, lipid metabolism regulators, and novel genes with unknown functions.

From the genes identified by the microarray analysis, we selected 12 genes up-regulated in TRAF3$^{-/-}$ B lymphomas for further verification by quantitative real time PCR using TaqMan gene expression assay kits. Our data verified the mRNA up-regulation of the 12 genes examined, including *Diras2*, *MCC*, *Tbc1d9*, *Ccbp2*, *Btbd14a*, *Sema7a*, *Twsg1*, *Ppap2b*, *TCF4*, *Tnfrsf19*, *Zcwpw1,* and *Abca3* (Figure 1B). Striking up-regulation of these transcripts was verified in the three splenic B lymphoma samples used for microarray analyses (mouse ID: 6983–2, 7041–10, and 7060–8), and also confirmed in three additional splenic B lymphomas (mouse ID: 7140–7, 7140–3 and 5–5) as well as ascites from two cases (mouse ID: 7060–8 and 7140–3; Figure 1B). Thus, up-regulation of these 12 genes is recurrent in B lymphomas spontaneously developed in different individual B-TRAF3$^{-/-}$ mice.

We next surveyed public gene expression databases of human cancers (http://www.oncomine.org) [42] and searched the literature to investigate whether the genes identified in our microarray analyses exhibit alterations in mRNA expression, DNA copy number variation, or mutations in primary human B lymphomas and other cancers. Interestingly, we found that among the up-regulated genes identified in our microarray analysis, *MCC* is most consistently up-regulated in a variety of primary human B cell malignancies, including PEL, CBL, DLBCL, BL, and MM [38-41]. It has been previously shown that *MCC* functions as a tumor suppressor gene in CRCs and hepatocellular carcinoma [20,27,31-35]. However, the functional roles of MCC in lymphocytes or lymphomas remain unknown. In this context, we selected to further explore the expression and function of MCC in B lymphocytes and B cell malignancies in the present study.

Striking up-regulation of MCC in TRAF3$^{-/-}$ mouse B lymphomas but not in premalignant TRAF3$^{-/-}$ B lymphocytes

We first verified the up-regulation of *MCC* in splenic B lymphomas and ascites spontaneously developed in 8 different individual B-TRAF3$^{-/-}$ mice at the protein level using Western blot analysis (Figure 2A). In contrast, MCC protein expression was not detected in LMC splenic B cells (Figure 2A). We also compared the transcript expression of *MCC* in different mature B cell subsets or B cells of different developmental stages by surveying the public gene expression database of Mouse Immune Genome (http://www.immgen.org). Mature B cell subsets examined include follicular, marginal zone, germinal center, and B1 B cells, and developing B cells examined include common lymphoid progenitor, pre-pro-B, pro-B, pre-B, newly-formed B, and transitional (T1, T2 and T3) B cells. The data in Mouse Immune Genome indicate that the *MCC* transcript is barely detected in any mature B cell subsets or developing B cells of any developmental stages. It has been previously shown that the expression of MCC is gradually up-regulated during differentiation of PC12 cells induced by

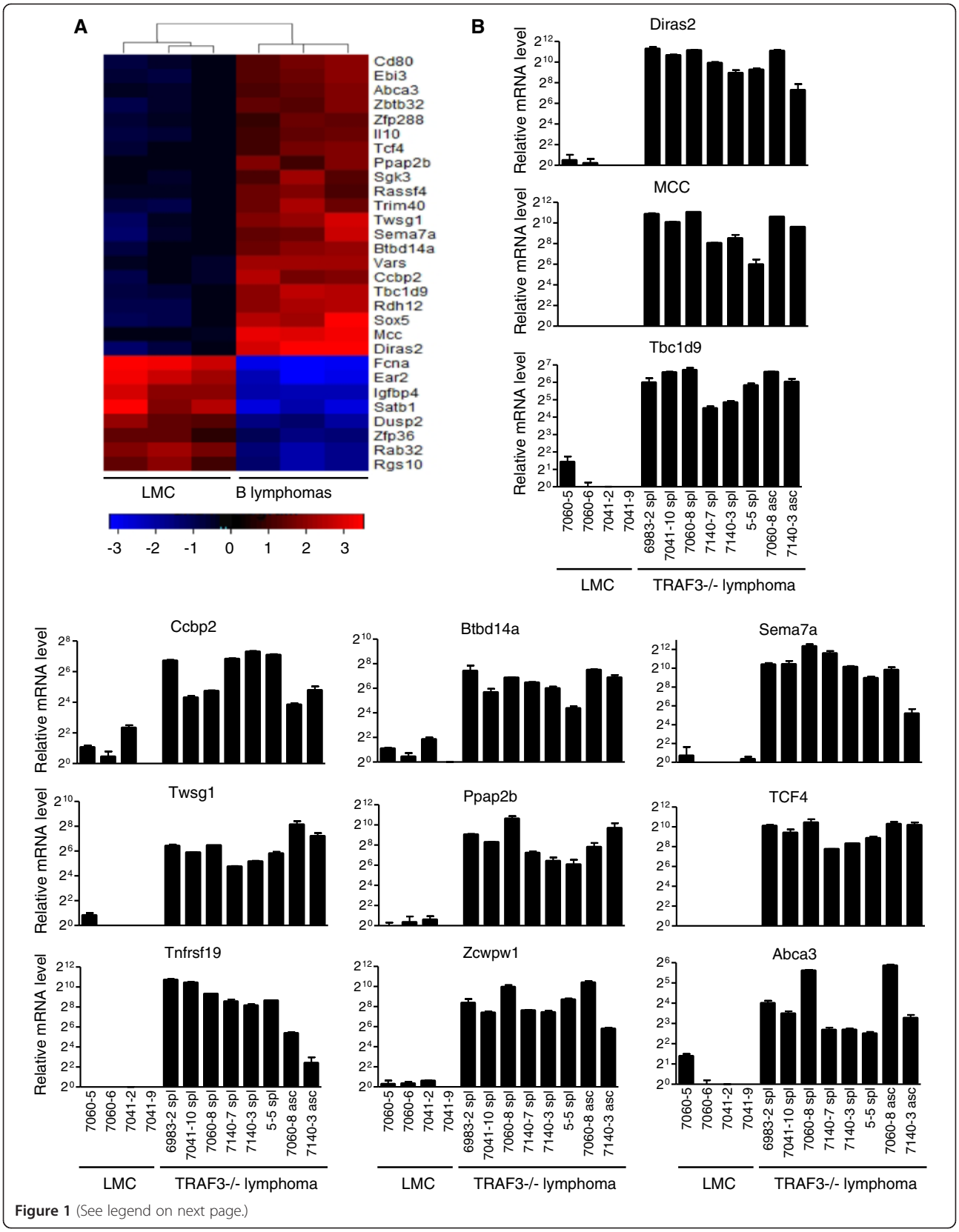

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Figure 1 Representative genes differentially expressed in TRAF3$^{-/-}$ mouse B lymphomas identified by the transcriptome microarray analysis. (A) Heatmap of representative microarray data**.** The mRNA expression profiles of splenocytes from 3 pairs of LMC and tumor-bearing B-TRAF3$^{-/-}$ mice were analyzed by microarray analysis. cRNA was hybridized to the Illumina Sentrix MouseRef-8 24 K Array (Illumina). Genes shown in the heatmap are selected from 160 up-regulated and 244 down-regulated genes: red indicates overexpression, blue indicates underexpression, and black indicates median expression. Values under the color key indicates Log2 (fold of change). Dendrogram on top of the heatmap shows the relatedness between samples as determined by hierarchical clustering. **(B)** Verification of transcript up-regulation of genes identified by the microarray analysis using quantitative real time PCR. Total cellular RNA was prepared from splenocytes of LMC mice, or splenic B lymphomas (spl) and ascites (asc) of diseased B-TRAF3$^{-/-}$ mice, and cDNA was synthesized by reverse transcription. Real time PCR was performed using TaqMan primers and probes (FAM-labeled) specific for mouse *Diras2*, *MCC*, *Tbc1d9*, *Ccbp2*, *Btbd14a*, *Sema7a*, *Twsg1*, *Ppap2b*, *TCF4*, *Tnfrsf19*, *Zcwpw1*, and *Abca3*. Each reaction also included the probe (VIC-labeled) and primers for mouse β-actin mRNA, which served as endogenous control. Relative mRNA expression levels of each gene were analyzed using the Sequencing Detection Software (Applied Biosystems) and the comparative Ct (ΔΔCt) method. Graphs depict the results of two experiments with duplicate reactions in each experiment (mean ± S.D.), and mouse ID of each sample is indicated in the graphs.

NGF [37]. We thus investigated the potential up-regulation of *MCC* during the proliferation, differentiation, and activation of B lymphocytes induced by a variety of B cell stimuli. We purified splenic B cells from LMC or tumor-free young B-TRAF3$^{-/-}$ mice (age: 10–12 weeks; premalignant TRAF3$^{-/-}$ splenic B cells), and then stimulated the cells with various B cell stimuli. These include agonistic anti-CD40 Abs, LPS (TLR4 agonist), B cell receptor (BCR) crosslinking Abs, and CpG2084 (TLR9 agonist), alone or in combination. TLR4 and TLR9 were examined as a representative of plasma membrane- and endosome- localized members of the TLR family, respectively [43]. We found that the transcript of *MCC* was neither up-regulated nor detected in LMC or premalignant TRAF3$^{-/-}$ splenic B cells after treatment with any of the stimuli examined (Figure 2B). In contrast, the expression of *Zcwpw1*, another gene identified by our microarray analysis, was robustly up-regulated in LMC or premalignant TRAF3$^{-/-}$ splenic B cells after treatment with LPS alone, LPS in combination with CD40, or CpG in combination with CD40. This indicates that expression of *Zcwpw1* is induced during the proliferation and activation of B lymphocytes. However, unlike *Zcwpw1*, the expression of *MCC* is only up-regulated and detected in TRAF3$^{-/-}$ B lymphomas, suggesting that MCC up-regulation is selectively associated with B cell malignant transformation.

We next cloned the full-length coding sequence of the MCC transcripts from primary splenic B lymphomas of 4 different individual B-TRAF3$^{-/-}$ mice (mouse ID: 6983–2, 7060–8, 105–8, and 115–6) by reverse transcription and PCR. Our sequencing results demonstrated that the *MCC* gene expressed in TRAF3$^{-/-}$ B lymphomas is predicted to encode a protein of 828 amino acids and does not contain any mutations. In an effort to understand how *MCC* is up-regulated in TRAF3$^{-/-}$ B lymphomas, we first determined the copy number of the MCC gene in primary splenic B lymphomas of 8 different individual B-TRAF3$^{-/-}$ mice using Taqman Copy Number Assay Kit. Our results revealed that the copy number of the MCC gene was not changed in TRAF3$^{-/-}$ mouse B lymphomas as compared to that observed in LMC splenocytes (Figure 2C). We next investigated the involvement of epigenetic alterations in MCC up-regulation by performing chromatin immunoprecipitation (ChIP) analyses. We used antibodies specific for a repressive histone mark (trimethylated Lys 27 of histone 3, H3K27Me3) and an activating histone mark (acetylated Lys 9/14 of histone 3, H3K9/14Ac), respectively. We chose to examine these two epigenetic modifications based on previous evidence that alterations of H3K27Me3 and H3K9/14Ac frequently occur in human B lymphomas and MM [44-51]. Our results showed that the activating H3K9/14 acetylation of the promoter region of the *MCC* gene was not obviously changed in TRAF3$^{-/-}$ B lymphoma cells as compared to LMC or premalignant TRAF3$^{-/-}$ splenic B cells (Figure 2D). In contrast, the repressive H3K27 trimethylation of the *MCC* promoter region was almost completely abolished in TRAF3$^{-/-}$ B lymphoma cells, and also modestly decreased in premalignant TRAF3$^{-/-}$ splenic B cells (Figure 2D). Such changes in H3K27 trimethylation was not observed in the promoter region of *Diras2* (Figure 2D), another gene strikingly up-regulated in TRAF3$^{-/-}$ B lymphomas identified in our study. The trimethylation of H3K27 that is almost completely abolished in the *MCC* promoter region in TRAF3$^{-/-}$ B lymphomas is intriguing, considering that the expression levels and activity of EZH2 of the polycomb repressive complex 2 (PRC2) catalyzing H3K27Me3 are often elevated in human B lymphomas and MM [45-47]. This unexpected decrease of the repressive H3K27Me3 of the *MCC* promoter region may be caused by increased activity of the H3K27 demethylase UTX or altered recognition of the *MCC* promoter by PRC2 in TRAF3$^{-/-}$ B lymphoma cells. Altered recognition of the *MCC* promoter by PRC2 might be brought about by changes in DNA methylation of this region, accessory proteins of the PRC2 complex, or expression of EZH2-binding non-coding RNA surrounding the MCC gene locus in TRAF3$^{-/-}$ B lymphomas [47,52-54]. Regardless

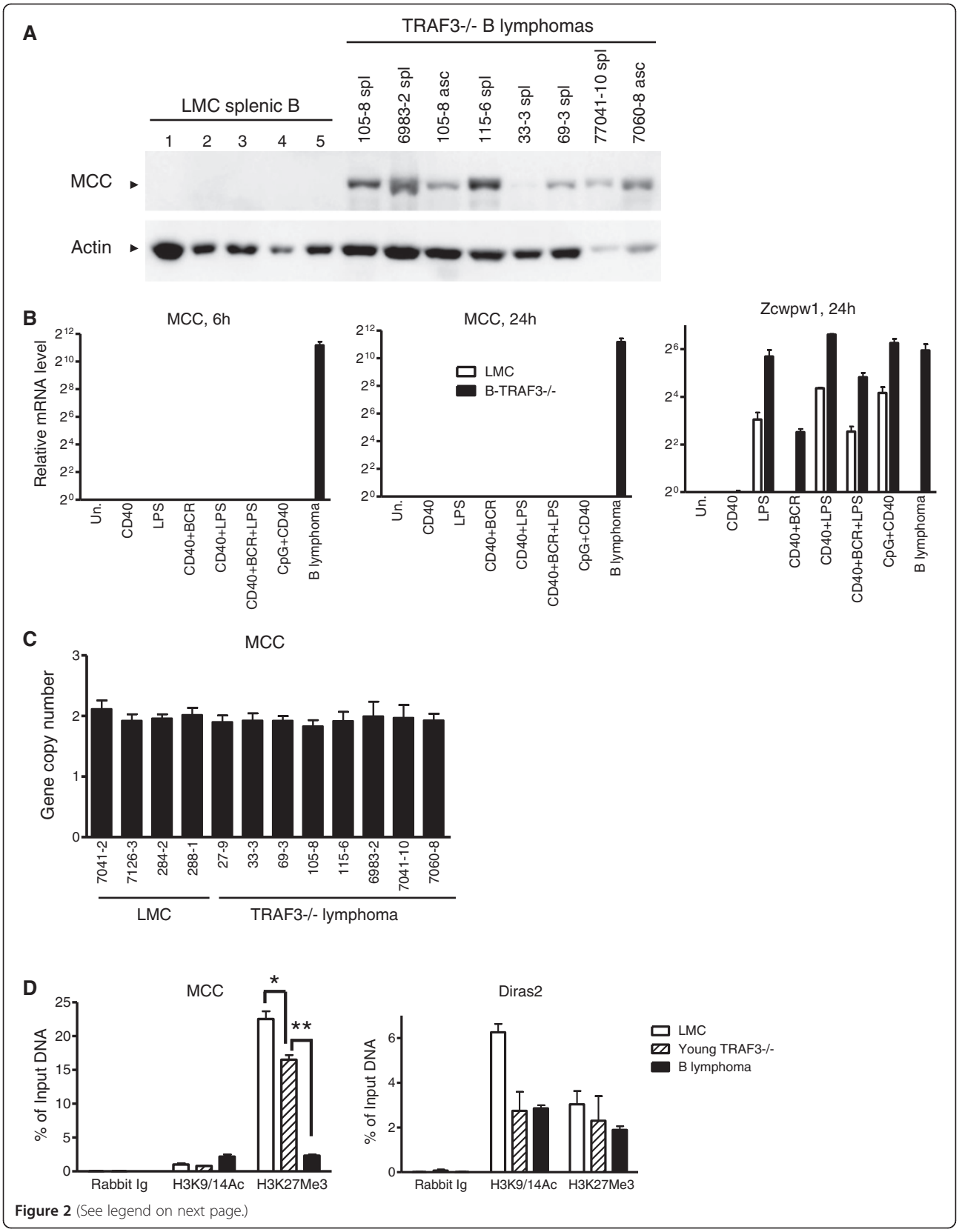

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Figure 2 Striking up-regulation of MCC in TRAF3$^{-/-}$ mouse B lymphomas but not in premalignant TRAF3$^{-/-}$ B lymphocytes. (A) Western blot analysis of the MCC protein. Total cellular proteins were prepared from purified LMC splenic B cells or splenic B lymphomas (spl) or ascites (asc) of different individual B-TRAF3$^{-/-}$ mice. **(B)** Regulation of the expression of *MCC* and *Zcwpw1* in response to B cell stimuli. LMC and premalignant (young) TRAF3$^{-/-}$ splenic B cells were cultured *ex vivo* in the absence or presence of stimuli of B cell proliferation, differentiation and activation for 6 or 24 hours. B cell stimuli examined include: 2 μg/ml anti-CD40, 20 μg/ml LPS, 1 μg/ml anti-BCR, and 100 nM CpG2084, alone or in combination. RNA samples of TRAF3$^{-/-}$ B lymphomas (mouse ID: 7060–8) were used as positive control of the *MCC* and *Zcwpw1* transcripts in Taqman assays. **(C)** Normal copy number of the *MCC* gene in TRAF3$^{-/-}$ mouse B lymphomas. Copy number of the mouse *MCC* gene in genomic DNA samples prepared from LMC splenocytes or TRAF3$^{-/-}$ B lymphomas was determined using the TaqMan Copy Number Assay kit. Mouse ID was indicated in the figure. **(D)** Histone modifications of the promoter region of the *MCC* and *Diras2* genes. Chromatin was prepared from LMC and young TRAF3$^{-/-}$ splenic B cells, or TRAF3$^{-/-}$ B lymphomas. Fragmented chromatin was immunoprecipitated using antibodies specific for histone marks (H3K27me3 or H3K9/14ac) or non-specific rabbit Ig. Immunoprecipitated DNA was quantified by qPCR using primer pairs specific for the promoter region of the *MCC* and *Diras2* genes, respectively. Quantity of immunoprecipitated DNA is presented as percentage of input DNA in graphs. All graphs (B-D) depict the results of three independent experiments with duplicate reactions in each experiment (mean ± S.D.). *$P < 0.05$, and **$P < 0.0001$ by Student's *t* test.

of the exact mechanisms, our results suggest that alterations in epigenetic modifications (such as H3K27Me3) of the *MCC* promoter region contribute at least partially to the up-regulation of MCC observed in TRAF3$^{-/-}$ B lymphomas.

Aberrant up-regulation of MCC in human patient-derived MM cell lines with TRAF3 deletions or relevant mutations

To confirm the clinical relevance of our study, we examined *MCC* expression in 6 human patient-derived MM cell lines with TRAF3 deletions or relevant mutations. These include: two cell lines with TRAF3 bi-allelic deletions (KMS11 and 8226), two cell lines with TRAF3 frameshift mutations (LP1 and U266), and two cell lines with cIAP1/2 bi-allelic deletions (KMS28PE and KMS20). Similar to TRAF3 inactivation, bi-allelic deletions of cIAP1/2 also lead to constitutive activation of the non-canonical NF-κB pathway, NF-κB2 (p52/RelB) [9]. We found that MCC is aberrantly up-regulated in all examined human MM cell lines at both the transcript and protein levels (Figure 3A and B). Robust expression of MCC was also detected in an EBV-transformed B lymphoblastoid cell line C3688 [55], which also exhibits constitutive NF-κB2 activation. In contrast, MCC expression was not detected in normal human blood B lymphocytes. It should be noted that MM is the tumor of terminally differentiated B cells, plasma cells. By directly comparing with purified normal plasma cells, microarray analyses by Zhan *et al.* have previously identified aberrant up-regulation of the *MCC* transcript in MM patient samples [39,40]. Similarly, microarray analyses by Basso *et al*. have demonstrated aberrant up-regulation of the *MCC* transcript in patient samples of B lymphomas (PEL, CBL, BL and DLBCL) as compared with normal B cells of different activation stages, including naive pre-germinal center B cells, centroblasts, centrocytes, and memory B cells [38]. Together, the above evidence indicates that *MCC* is aberrantly up-regulated only in malignant B cells and neoplastic plasma cells, but not in their normal counterparts.

We then cloned the full-length coding sequence of the MCC transcripts from LP1 and KMS11 cells by reverse transcription and PCR. Sequencing results of the PCR products of MCC transcripts demonstrated that the coding cDNAs of the MCC gene cloned from human MM cells are predicted to encode a protein of 829 amino acids and contain no mutations. Our results thus demonstrate that MCC is aberrantly expressed in both mouse and human transformed B cells, but not expressed in normal B lymphocytes.

Subcellular localization of MCC and its regulation in human MM cells during ER stress responses

Subcellular localization of a protein provides important clues about its potential function. It has been previously shown that MCC is localized in the cytosol as well as in the nucleus, and is also associated with the plasma membrane and membrane organelles in epithelial cells [20,31,33,37]. To elucidate where MCC exerts its roles in malignant B cells, we examined the subcellular localization of MCC using a biochemical fractionation method. To investigate whether MCC protein level is regulated in response to apoptosis induction and ER stress, we employed two ER stress inducers, DTT (a chemical that reduces disulfides to thiols and thus affects protein folding or conformation) and thapsigargin (an inhibitor of ER Ca^{2+} transport). We prepared the cytosol, microsomes (rich in ER), mitochondria and nuclei fractions from human MM cells in the absence or presence of treatment with DTT or thapsigargin. Our results revealed that MCC protein was primarily localized in the mitochondria, but also detectable in the ER, cytosol and nucleus in human MM cells (Figure 3C). Interestingly, we noticed that the two ER stress inducers, DTT and thapsigargin, markedly inhibited the protein levels of MCC, but did not change the subcellular localization of MCC in human MM cells (Figure 3C). The ER stress responses induced by DTT and thapsigargin were evident as demonstrated by the cleavage of the

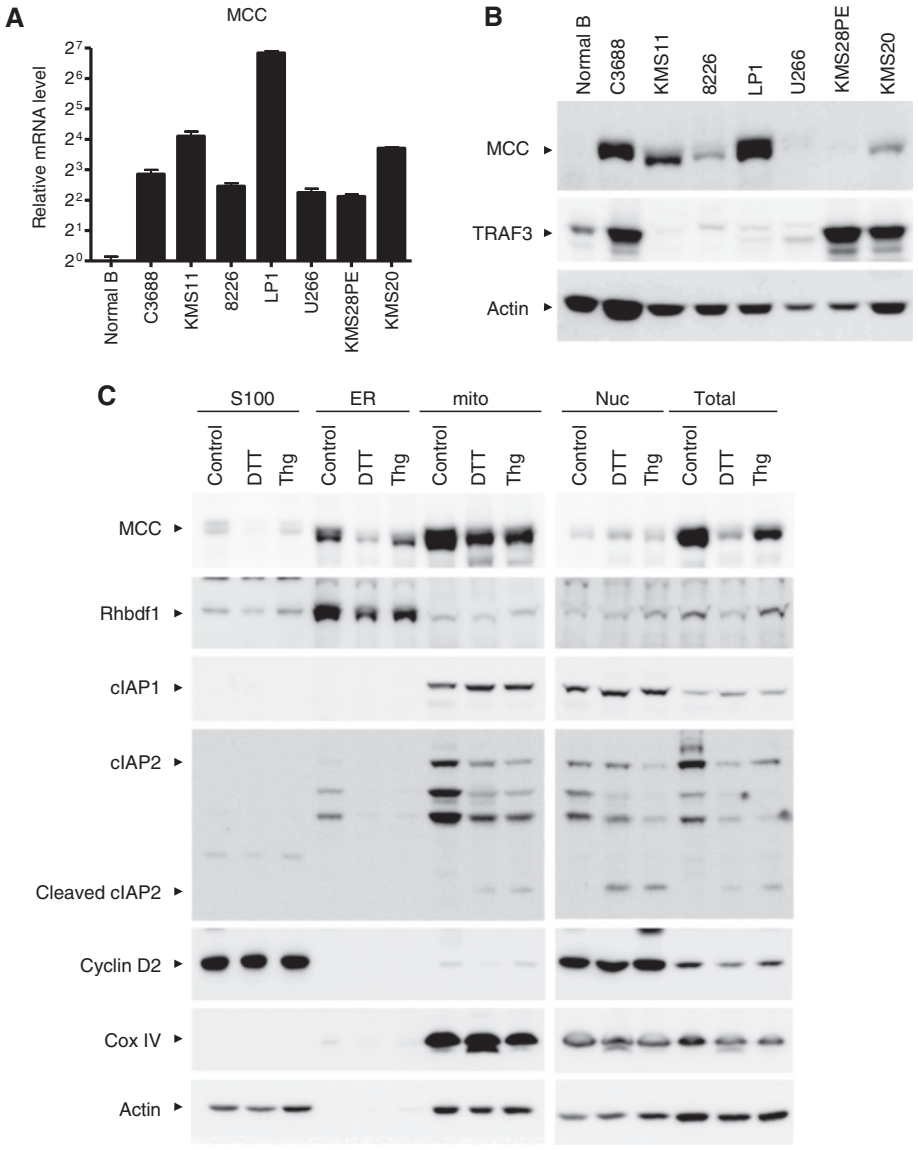

Figure 3 Aberrant expression and subcellular localization of MCC in human MM cells. (A) Taqman assay of the *MCC* transcript. Total cellular RNA was prepared from normal human blood B lymphocytes (Normal B), an EBV-transformed B lymphoblastoid cell line C3688, or human MM cell lines with TRAF3 deletions or relevant mutations. The human MM cell lines include KMS11, 8226, LP1, U266, KMS28PE, and KMS20. Real-time PCR was performed using TaqMan primers and probes (FAM-labeled) specific for human *MCC*. Each reaction also included the probe (VIC-labeled) and primers for human β-actin mRNA, which served as an endogenous control. Relative mRNA expression levels of the *MCC* gene were analyzed using the Sequencing Detection Software (Applied Biosystems) and the comparative Ct (ΔΔCt) method. Graphs depict the results of three independent experiments with duplicate reactions in each experiment (mean ± S.D.). **(B)** Western blot analysis of the MCC protein. Total cellular proteins were prepared from normal B lymphocytes, C3688 cells, or human MM cell lines with TRAF3 deletions or relevant mutations. Proteins were immunoblotted for MCC, followed by TRAF3 and actin. Data shown are representative of 3 experiments. **(C)** Subcellular localization of MCC and its regulation during ER stress responses. LP1 cells were cultured in the absence (control) or presence of ER stress inducers DTT or thapsigargin (Thg). After treatment for 24 hours, cytosol (S100), ER, mitochondria (mito) and nuclei (Nuc) were biochemically fractionated from cells, and an aliquot of cells was used for total protein lysates (Total). Proteins in each fraction or total lysates were immunoblotted for MCC, Rhbdf1 (an ER protein), cIAP1, cIAP2, cyclin D2, COX IV (a mitochondrial protein), and actin. Results shown are representative of 3 independent experiments.

cellular inhibitor of apoptosis, cIAP2, and the induction of apoptosis in human MM cells (Figure 3C and data not shown). Yang *et al*. have previously shown that cIAP2 is cleaved by the mitochondrial serine protease Omi/HtrA2 during apoptosis [56]. Given the predominant mitochondrial localization of MCC, it is likely that the ER stress-induced decrease of MCC protein levels may be mediated through selective mitochondrial autophagy or proteasome-dependent degradation [57-59]. Together, our data demonstrated that MCC exhibits a primary

mitochondrial localization in human MM cells, and that MCC protein levels are down-regulated by ER stress and apoptosis induction in malignant B cells. These results suggest that MCC may play a role in regulating malignant B cell survival and proliferation.

Lentiviral shRNA vector-mediated knockdown of MCC induced apoptosis and inhibited proliferation in human MM cells

Previous studies have shown that MCC blocks cell cycle progression and inhibits cell proliferation in fibroblasts and CRCs [20,31,32,35]. To explore the functional roles of MCC in regulating malignant B cell survival and proliferation, we employed genetic means to manipulate the expression levels of MCC in human MM cells, including lentiviral vector-mediated overexpression and knockdown of MCC, respectively. In contrast to the cell cycle blocking and proliferation inhibitory effects of MCC overexpression reported in fibroblasts and CRCs [20,31,32,35], we found that overexpression of MCC in human MM cell line 8226 cells, which express endogenous MCC at relatively modest levels, did not affect cell cycle progression, cell proliferation, or cell survival at all (data not shown). It has been previously shown that the expression level of ectopically transfected MCC was strongly reduced in CRCs after several passages, suggesting selection against MCC expression in CRC cultures [35]. However, we observed that overexpression of MCC was maintained in human MM 8226 cells after >10 passages. Together, the results of our overexpression studies argue against a negative role for MCC in the survival or proliferation of malignant B cells.

We next screened four lentiviral shRNA vectors specific for human *MCC* using the human MM cell line LP1 cells. Cells transduced with a scrambled shRNA vector were used as control in these experiments. Our results of Western blot analysis showed that the MCC shRNA 1332 knocked down the protein levels of MCC by ~85% reduction, while the MCC shRNA 2689 decreased MCC levels by ~70% reduction in LP1 cells (Figure 4A). We subsequently used these MCC shRNA lentiviruses to transduce human MM cell lines LP1 and KMS11 cells. We found that both MCC shRNAs 1332 and 2689 inhibited growth and induced apoptosis in human MM cells, as demonstrated by growth curve determination, trypan blue or PI staining of dead cells, and annexin V staining of apoptotic cells (Figure 4B and C). Interestingly, MCC shRNA 1332 that resulted in a greater decrease in MCC protein level was also most effective at inducing apoptosis in human MM cells. In contrast, MCC shRNAs 1388 and 2284, which did not markedly knock down MCC protein level, did not drastically induce apoptosis in human MM cells (Figure 4). We further determined whether knockdown of MCC inhibits the proliferation of human MM cells by performing cell cycle analysis and proliferation dye labeling experiments. Results of cell cycle analysis demonstrated that transduction of MCC shRNA 1332 or 2689 resulted in a dramatic decrease in the proliferating cell population (S/G2/M phase: $2n < \text{DNA content} \leq 4n$) and an increase in the apoptotic cell population (DNA content $< 2n$) in human MM cells (Figure 5A). As observed for apoptosis induction, MCC shRNA 1332 was also more potent than shRNA 2689 in decreasing the proliferating cell population of human MM cells (Figure 5A). Our results of the proliferation dye labeling experiments further confirmed that knockdown of MCC by shRNA 1332 remarkably inhibited the proliferation of human MM cells. This was demonstrated both by the reduction of GFP+ shRNA 1332-transduced cells and by the slower dilution of the proliferation dye in shRNA 1332-transduced cells as compared to scrambled shRNA-transduced cells or untransduced cells after culture for 4 days (Figure 5B). Taken together, our results demonstrate that MCC plays a positive role and is required for the survival and proliferation in human MM cells, indicating that MCC acts as an oncogene in B lymphocytes.

MCC signaling pathways in human MM cells

To understand the mechanisms of MCC shRNA-mediated induction of apoptosis and inhibition of proliferation in human MM cells, we investigated the involvement of known MCC targets. In light of the previous evidence that MCC specifically targets and negatively regulates the oncogenic NF-κB and β-catenin pathways in CRCs and hepatocellular carcinoma [20,27,32,36], we determined the effects of MCC knockdown on the levels of these two pathways in human MM cells, including IκBα, IκBβ, RelA, phospho-β-catenin and β-catenin [20,27,32,36]. Our results showed that knockdown or overexpression of MCC did not change any of the proteins of the NF-κB and β-catenin pathways in human MM cells (Figure 6). We further examined another known MCC target involved in DNA damage response in CRCs, phospho-histone H3 (P-HH3) [35], which is a marker of mitosis. Contrary to what was observed in CRCs [35], we found that knockdown of MCC markedly decreased the level of P-HH3 in human MM cells, confirming the proliferation-promoting function of MCC in malignant B cells.

We then measured the levels or activation of a number of regulators of cell apoptosis and proliferation, including caspases, the Bcl-2 family proteins, and cyclins. Our results demonstrated that knockdown of MCC led to activation of both caspases 8 and 3, as evidenced by their cleavage into active caspase fragments (Figure 6). Interestingly, knockdown of MCC decreased the level of Mcl1 (an anti-apoptotic protein of the Bcl2 family), while overexpression of MCC increased the level of Mcl1 in human MM cells (Figure 6). However, MCC knockdown or overexpression did not affect the protein

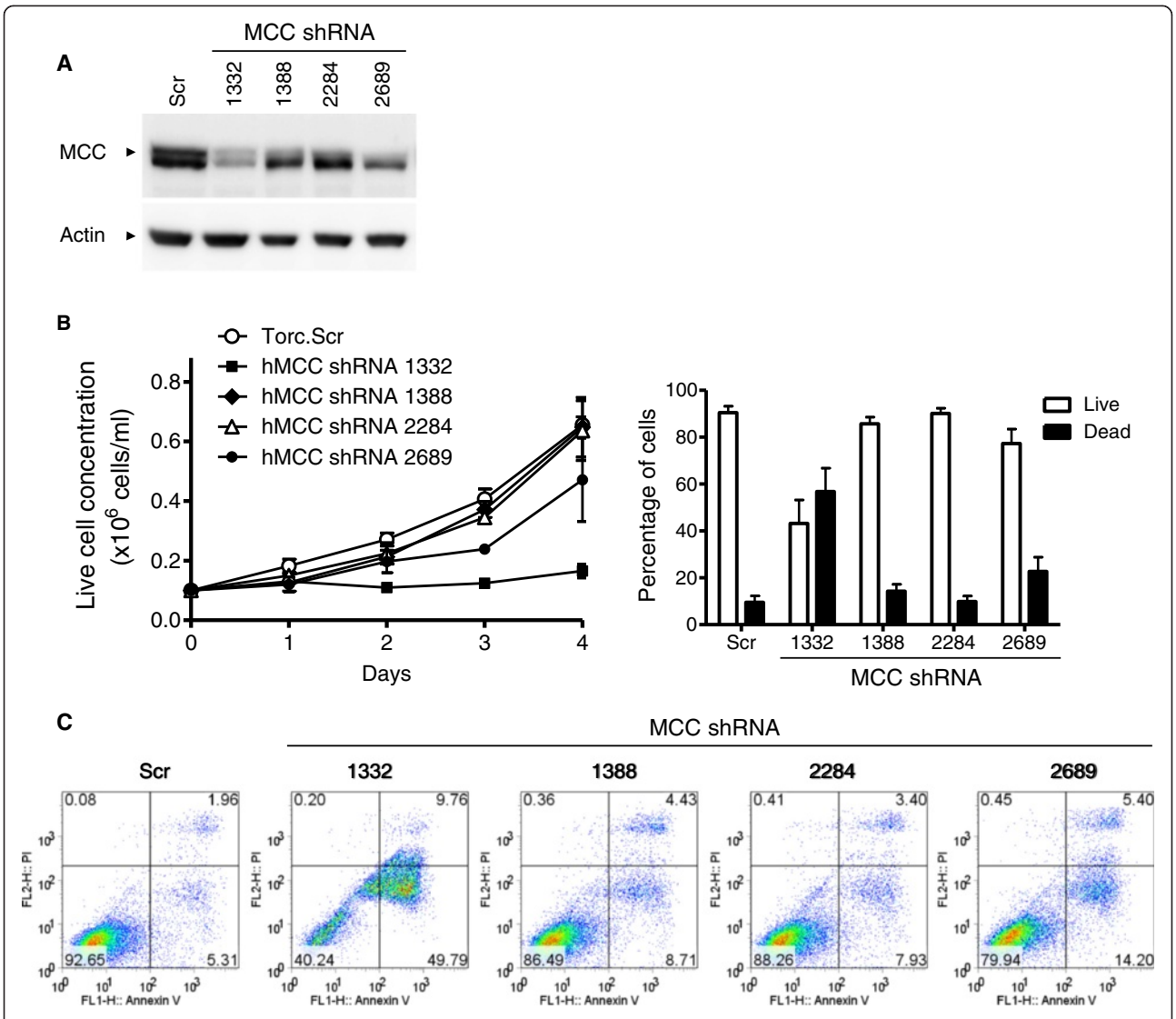

Figure 4 Lentiviral shRNA vector-mediated knockdown of MCC induced apoptosis in human MM cells. The human MM cell line LP1 cells were transduced with lentiviruses expressing MCC shRNAs (including 1332, 1388, 2284 or 2689) or a scrambled shRNA (Scr). **(A)** Knockdown of the MCC protein determined by immunoblot analysis. Total cellular proteins were prepared from LP1 cells successfully transduced with a scrambled shRNA (Scr), or MCC shRNA 1332, 1388, 2284 or 2689 on day 5 post-transduction. Proteins were immunoblotted for MCC, followed by actin. Immunoblot of actin was used as a loading control. Results shown are representative of 3 independent experiments, and similar results were obtained in the human MM cell line KMS11 cells. **(B)** Growth curves of live cells and percentage of live versus dead cells determined by Trypan blue-stained cell counting. On day 4 post-transduction, successfully transduced LP1 cells were cultured in a 6-well plate for growth curve determination. Live and dead cells were counted daily for 4 days using Trypan blue staining and a hemocytometer. The graphs depict the results of 3 independent experiments (mean ± S.D.). **(C)** Representative FACS profiles of transduced cells analyzed by annexin V and PI staining. On day 6 post-transduction, transduced LP1 cells were stained with annexin V and PI, and then analyzed by a flow cytometer. In the FACS profiles, apoptotic cells were identified as annexin V+ PI-, dead cells were annexin V+ PI+, and live cells were annexin V- PI-. Data shown are representative of 3 independent experiments, and similar results were obtained in the human MM cell line KMS11 cells.

levels of other members of the Bcl2 family, including Bcl-xL, Bcl2, Bim, Bad, Bid and Bik (Figure 6). Among the cell cycle regulators examined, knockdown of MCC caused specific down-regulation of the protein levels of cyclin B1 and cyclin A, while overexpression of MCC increased the level of cyclin B1 in human MM cells (Figure 6). Knockdown of MCC also led to specific up-regulation of the protein level of the cell cycle inhibitor p27, which was modestly down-regulated by overexpression of MCC in human MM cells (Figure 6). In contrast, MCC knockdown did not change other cell cycle regulators examined, including cyclin D1, cyclin D2, p21, E2F1, and p53 (Figure 6). Our findings suggest that MCC inhibits apoptosis and induces proliferation

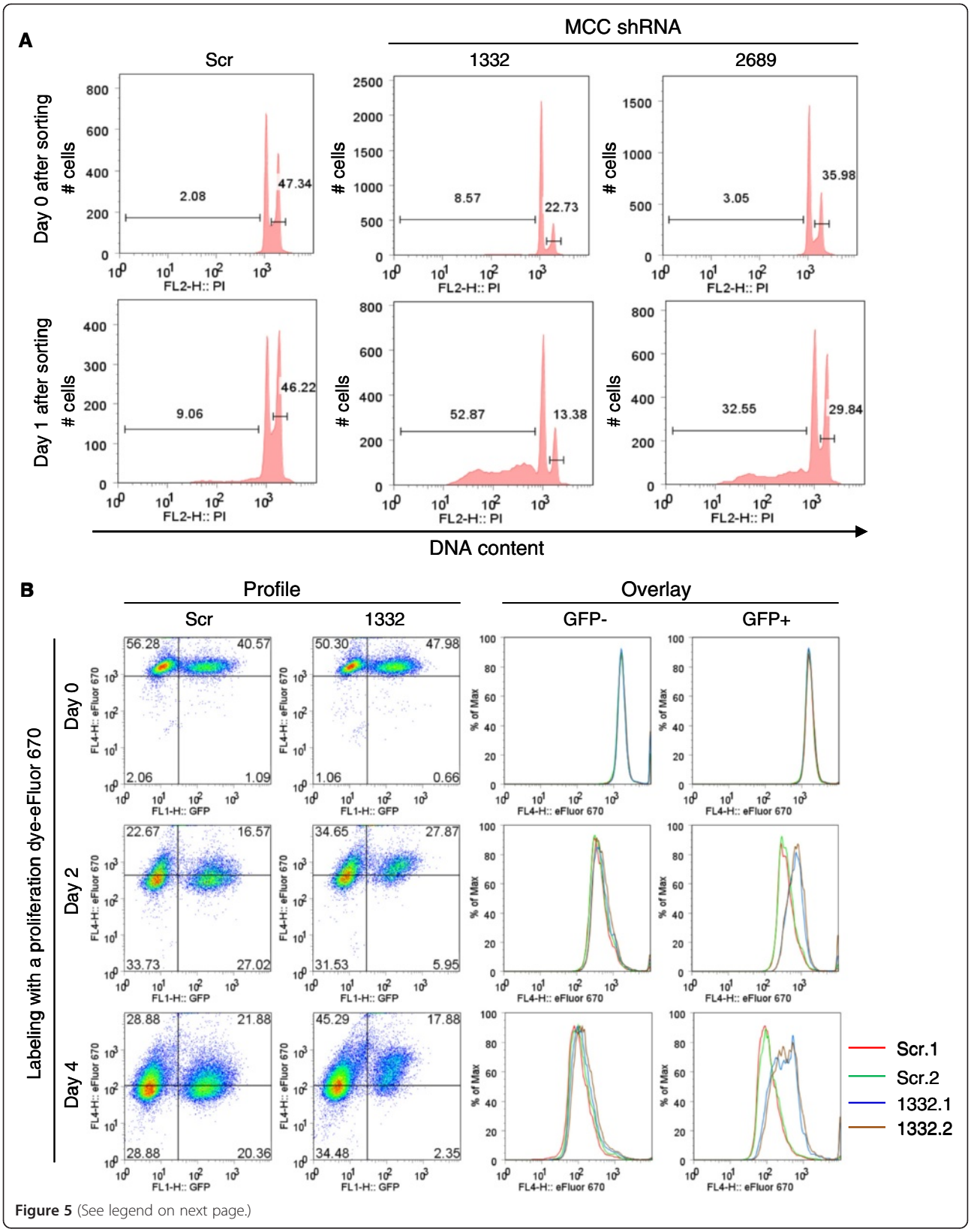

Figure 5 (See legend on next page.)

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Figure 5 Knockdown of MCC induced apoptosis and inhibited proliferation in human MM cells. (A) Cell cycle distribution analyzed by PI staining and flow cytometry. The human MM cell line LP1 cells were transduced with lentiviruses expressing MCC shRNAs (1332 or 2689) or a scrambled shRNA (Scr). Successfully transduced (GFP+) cells were sorted by a FACS sorter on day 4 post-transduction. Top panel shows the FACS histograms of cells right after sorting (Day 0), and bottom panel shows the FACS histograms of sorted cells after cultured for 24 hours (Day 1). Gated populations in the FACS histograms indicate apoptotic cells (DNA content < 2n) or proliferating cells (2n < DNA content ≤ 4n). Results shown are representative of 3 independent experiments, and similar results were also obtained in the human MM cell line KMS11 cells. **(B)** Cell proliferation analyzed by dilution of the proliferation dye (eFluor 670)-labeling and flow cytometry. On day 4 post transduction, LP1 cells transduced with MCC shRNA 1332 (1332) or a scrambled shRNA (Scr) were labeled with a proliferation dye eFluor 670, which binds to any cellular protein containing primary amines. As cells divide, the dye is distributed equally between daughter cells that can be measured as successive halving or dilution of the fluorescence intensity of the dye. Day 0 FACS profiles and overlay histograms show the eFluor 670 signals of freshly labeled cells, while those of Day 2 and Day 4 show diluted eFluor 670 signals of cells after cultured for 2 and 4 days, respectively. Inhibited proliferation of MCC shRNA 1332-transduced cells was demonstrated by the hampered dilution of the proliferation dye as compared to those observed in both the untransduced (GFP-) cells and the scrambled shRNA (Scr)-transduced cells. Results shown are representative of 3 independent experiments with duplicate samples in each experiment.

by inhibiting cleavage of caspases 8 and 3, up-regulating Mcl1 and cyclin B1, and down-regulating p27 in human MM cells.

To further understand the mechanisms of MCC-mediated regulation of cell survival and proliferation, we sought to investigate key signaling pathways that are known to play important roles in regulating B cell survival and proliferation, including the activation of p38, ERK, JNK, Akt and c-Myc [60]. Our results showed that knockdown of MCC led to specific decreases in the phosphorylation level of ERK1/2 and the protein level of c-Myc (a downstream target of ERK), while overexpression of MCC exhibited the opposite effect in human MM cells (Figure 6). In contrast, knockdown or overexpression of MCC did not change the activation of p38, JNK and Akt in human MM cells (Figure 6). These results indicate that ERK-c-Myc is a key signaling pathway underlying the oncogenic roles of MCC in malignant B cells.

MCC interacting proteins in human MM cells

To gain further insights into the molecular mechanisms underlying the oncogenic roles of MCC in B cells, we set out to identify MCC interacting proteins in human MM cells. To facilitate co-immunoprecipitation studies, we constructed two lentiviral expression vectors of tagged human MCC, including pUB-FLAG-hMCC and pUB-hMCC-SBP-6×His, which express an N-terminal FLAG tagged or a C-terminal SBP-6×His tagged MCC, respectively. We used these vectors to transduce human patient-derived MM cell line 8226 cells, which express endogenous MCC at relatively modest levels. Our flow cytometric data demonstrated that the lentiviral transduction efficiency by pUB-FLAG-hMCC and pUB-hMCC-SBP-6×His was > 85% in 8226 cells (data not shown). Transduced FLAG-hMCC and hMCC-SBP-6×His were immunoprecipitated from whole cell lysates using anti-FLAG-agarose or streptavidin-sepharose beads, respectively. We first examined a number of previously known MCC interacting proteins identified in CRCs or 293T cells, including β-catenin, Mst3, VCP, PP2A, DFFA, VHL, VDAC, scribble, and myosin IIb [20,32-34,61]. However, none of these proteins were detected in co-immunoprecipitates with either FLAG-MCC or MCC-SBP-6×His in human MM cells. Thus, our findings suggest that MCC inhibits apoptosis and promotes proliferation likely through interaction with novel, previously unknown partners in malignant B cells.

To delineate the novel MCC-interactome in human MM cells, we turned to employ an unbiased strategy, affinity purification followed by mass spectrometry-based sequencing. Considering the primary mitochondrial localization of MCC in human MM cells, immunoprecipitates of hMCC-SBP-6×His by streptavidin-sepharose beads from both whole cell lysates and purified mitochondria of 8226 cells were analyzed by high resolution LC-MS/MS, respectively. In these experiments, immunoprecipitates of FLAG-hMCC by streptavidin-sepharose beads were used as negative control. Using this strategy, we identified 365 proteins in the MCC-interactome of human MM cells: 333 MCC interacting proteins in whole cell lysates, and 207 MCC interactors in mitochondria (Figure 7A, and Additional file 2: Table S2). Among these, 175 MCC interactors were identified in both whole cell lysates and purified mitochondria of human MM cells. However, only 39 (out of 365, 10.7%) proteins of our study were also previously identified as MCC interacting proteins in CRCs or 293 T cell (Figure 7A, and Additional file 2: Table S2) [20,32-34,36,61]. Thus, most of the proteins identified in our study are novel MCC-interacting partners.

We next performed disease association analyses using Ingenuity (http://www.ingenuity.com), and cancer is identified as the top disease associated with the MCC-interactome identified in our study. Results of Ingenuity analyses showed that 195 of the 333 (58.5%) MCC interactors in whole cell lysates and 91 of the 207 (43.96%) MCC interactors in mitochondria are associated with cancer. Functional annotation and clustering analyses by

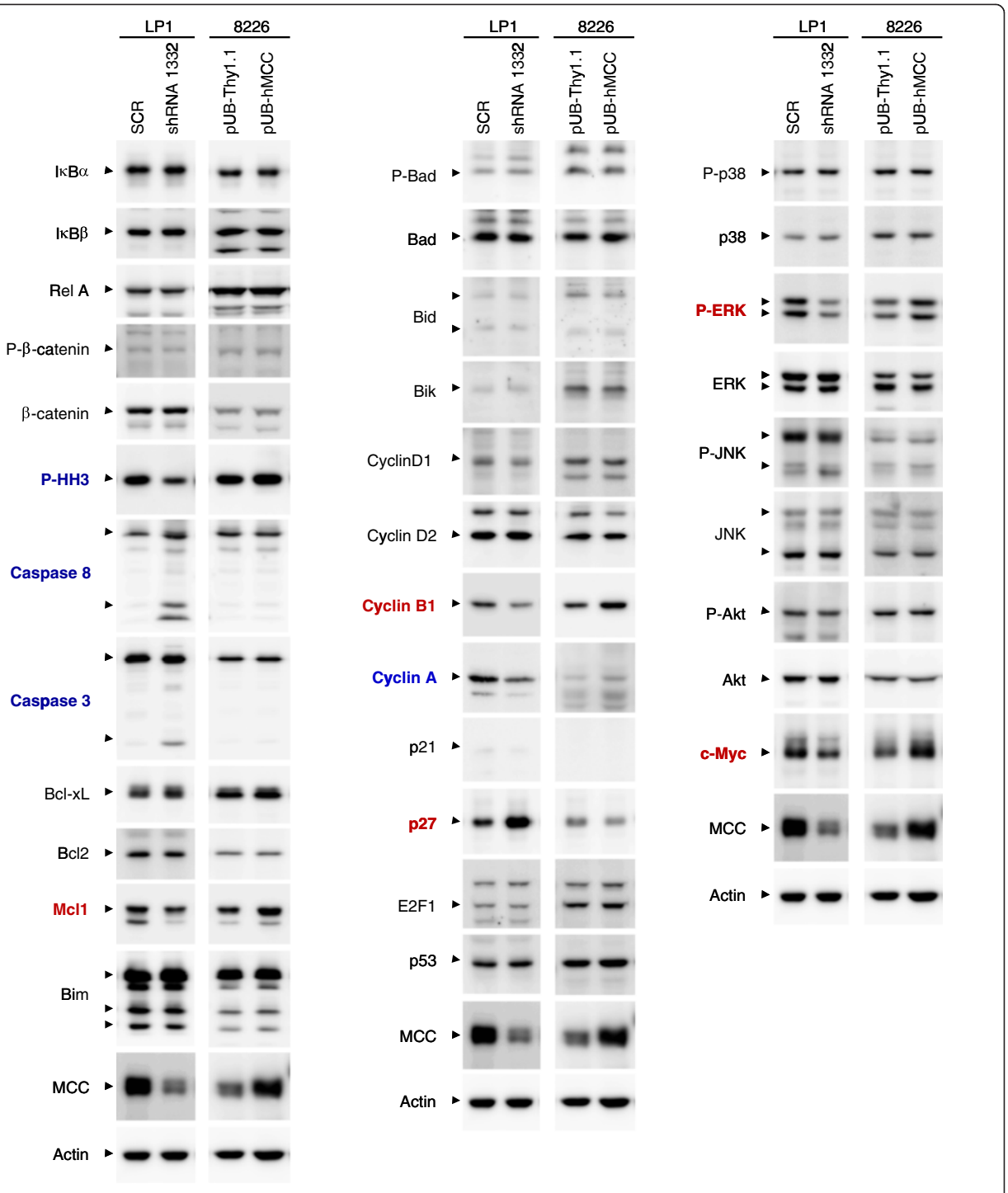

Figure 6 MCC signaling pathways in human MM cells. The human MM cell line LP1 cells, which express high levels of endogenous MCC, were transduced with lentiviruses expressing MCC shRNA 1332 or a scrambled shRNA (SCR). Another human MM cell line 8226 cells, which express modest levels of endogenous MCC, were transduced with lentiviruses expressing hMCC (pUB-hMCC) or Thy1.1 only (pUB-Thy1.1). Total cellular lysates were prepared and immunoblotted for known targets of MCC and regulators of apoptosis or cell cycle. Known target proteins of MCC examined include phosphorylated β-catenin (P-β-catenin), β-catenin, IκBα, IκBβ, RelA, and phosphorylated histone H3 (P-HH3). Regulators of apoptosis examined include caspase 8, caspase 3, Bcl-xL, Bcl2, Mcl1, Bim, P-Bad, Bad, Bid, and Bik. Regulators of cell cycle examined include cyclin D1, cyclin D2, cyclin B1, cyclin A, p21, p27, E2F1, p53, phosphorylated-p38 (P-p38), p38, P-ERK, ERK, P-JNK, JNK, P-Akt, Akt, and c-Myc. The MCC blot was used as a control for MCC shRNA 1332 or pUB-hMCC transduction, and the actin blot was used as a loading control. Proteins that are changed by knockdown of MCC are highlighted in blue, and those that are changed by both knockdown and overexpression of MCC are highlighted in red. Results shown are representative of 3 independent experiments.

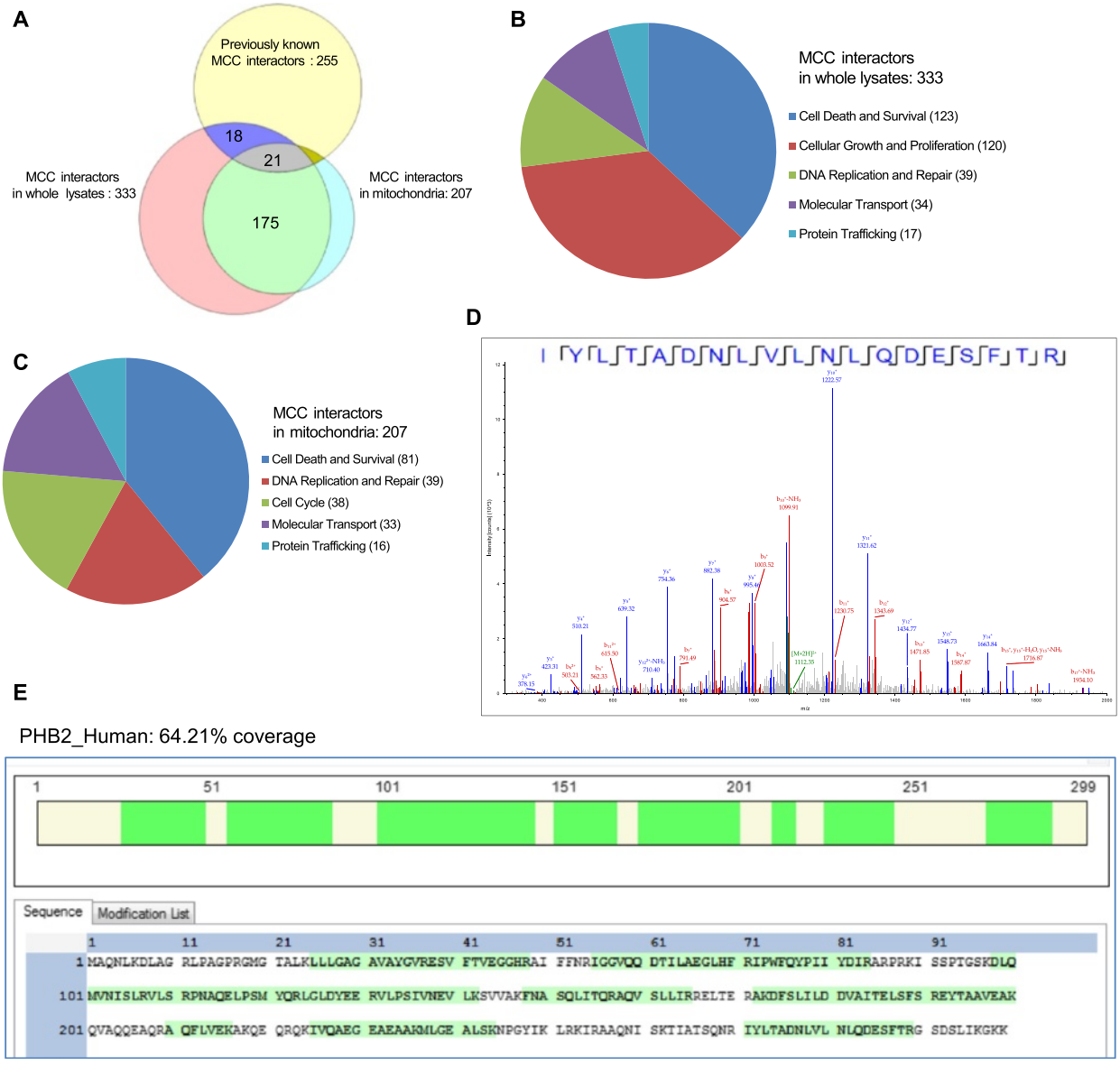

Figure 7 The MCC-interactome in human MM cells identified by affinity purification followed by LC-MS/MS. The human MM cell line 8226 cells were transduced with lentiviruses expressing hMCC-SBP-6xHis or FLAG-hMCC. Immunoprecipitates of hMCC-SBP-6xHis by streptavidin-sepharose beads from whole cell lysates and purified mitochondria of 8226 cells were analyzed by high resolution LC-MS/MS, respectively. Immunoprecipitates of FLAG-hMCC by streptavidin-sepharose beads were used as negative control in these experiments. **(A)** Venn diagram of MCC-interactors identified in the present study and previous studies. The diagram depicts the number of previously known MCC-interactors or MCC-interactors identified in whole cell lysates or mitochondria of human MM cells in our study, as well as the overlap between each category. **(B and C)** Functional clustering and annotation of MCC-interactors identified in whole cell lysates **(B)** or mitochondria **(C)** of human MM cells. The number of proteins of each functional cluster is shown. **(D)** Representative mass spectral profile of a peptide of PHB2 identified by LC-MS/MS. The deduced peptide sequence is shown. **(E)** Schematic diagram of peptide sequences of PHB2 identified by LC-MS/MS. The peptide sequences detected by LC-MS/MS are highlighted in green color.

Ingenuity revealed that mitochondrial MCC interactors are mainly regulators of cell death and survival (81 of 207, 39.1%) (Figure 7B). Similarly, MCC interactors in whole cell lysates are mainly regulators of cell death and survival (123 of 333, 36.9%) or cellular growth and proliferation (120 of 333, 36%) (Figure 7C). Other MCC interactors include regulators of DNA replication and repair, molecular transport, and protein trafficking (Figure 7B and C). Together, the MCC-interactome identified in our study is consistent with the prominent roles of MCC in promoting survival and proliferation in human MM cells (Figures 4 and 5).

There are two isoforms of *MCC* encoding proteins that differ at their extreme N-terminus due to alternative promoter usage, isoform 1 (828 amino acids in mouse and 829 amino acids in human) and isoform 2 (1019 amino acids). The *MCC* that we cloned from LP1 cells and used to generate FLAG-hMCC and hMCC-SBP-6×His is isoform 1 (829 aa). Interestingly, our LC-MS/MS data identified 6 unique peptides of the isoform 2 of MCC (1019 aa) in the immunoprecipitates of hMCC-SBP-6×His in both whole cell lysates and purified mitochondria (Additional file 3: Figure S1 and Additional file 2: Table S2). These results demonstrate that the two isoforms of MCC form hetero-dimers or hetero-oligomers in human MM cells.

From the top 10 MCC-interactors identified in both whole cell lysates and purified mitochondria of human MM cells, we selected to further verify the interaction of MCC with two important regulators of cell survival and proliferation, PARP1 and prohibitin-2 (PHB2, Figure 7D and E) [62-65], by co-immunoprecipitation and Western blot analyses. Our results confirmed the interactions of MCC with PARP1 and PHB2 in both whole cell lysates and purified mitochondria of human MM cells (Figure 8A and B). We further performed an interaction network analysis using the top 100 MCC-interactors of human MM cells (60 proteins identified in both whole cell lysates and mitochondria, 10 proteins identified in mitochondria only, and 30 proteins identified in whole cell lysates only) and the STRING analysis tool (http://www.string-db.org) [66], which builds protein interaction networks based on known and predicted protein-protein interactions. As shown in Figure 8C, 61 proteins of the top 100 MCC-interactors form an interaction network centered upon PARP1 and PHB2. Interestingly, PARP1 and PHB2 have also been previously shown to directly or indirectly interact with and/or regulate MCC targets identified by knockdown and overexpression of MCC studies in human MM cells (Figure 6), including ERK, c-Myc, p27, cyclin B1, Mcl-1, caspase 8, and caspase 3 [62-65]. Taken together, our findings indicate that MCC promotes cellular survival and proliferation by associating with and modulating the interaction network centered at PARP1 and PHB2 in malignant B cells.

Discussion

The late onset and long latency of lymphoma development observed in B-TRAF3$^{-/-}$ mice suggest that secondary oncogenic hits are required to promote B cell transformation. In the present study, we identified *MCC* as a gene strikingly up-regulated in TRAF3$^{-/-}$ mouse B lymphomas and human MM cell lines. Aberrant expression of *MCC* has also been documented in primary human B cell malignancies, including PEL, CBL, DLBCL, BL, and MM [38-41]. In contrast, *MCC* expression was not detected in normal or premalignant TRAF3$^{-/-}$ B cells even after treatment with B cell stimuli, suggesting that aberrant expression of *MCC* is specifically associated with malignant transformation of B cells. In elucidating the function of MCC in malignant B cells, we found that lentiviral shRNA-mediated knockdown of MCC induced apoptosis and inhibited proliferation in human MM cells. Experiments of knockdown and overexpression of MCC allowed us to identify downstream targets of MCC in human MM cells, including ERK, c-Myc, p27, cyclin B1, Mcl-1, caspase 8, and caspase 3. Furthermore, we delineated the profile of proteins assembled in the MCC signaling complex in whole cells or mitochondria by employing affinity purification followed by mass spectrometry-based sequencing. Our results indicate that MCC associates with the interaction network centered upon PARP1 and PHB2 to promote cellular survival and proliferation in malignant B cells. Collectively, our findings indicate that MCC functions as an oncogene in B cells.

Paradoxically, in contrary to the up-regulation of *MCC* in B cell neoplasms, the *MCC* gene is frequently deleted, mutated, or silenced in other human cancers, including colorectal cancer [14-17,19,20,22], lung cancer [17,23], gastric carcinoma [24], esophageal cancer [25], and hepatocellular carcinoma [26,27]. Functional evidence and signaling pathway studies indicate that *MCC* acts as a tumor suppressor gene by inhibiting the oncogenic NF-κB and β-catenin pathways in CRCs and hepatocellular carcinoma [20,27,31-36]. We also previously observed regulation of β-catenin by Sox5, another gene identified in our microarray analysis, in human MM cells [67]. However, NF-κB and β-catenin levels were not altered by knockdown or overexpression of MCC in human MM cells. Thus, MCC plays distinct roles via different signaling mechanisms in B cell malignancies versus other human cancers.

It has been shown that in CRCs, MCC mainly localizes in the cytoplasm, and is induced to shuttle into the nucleus in response to DNA damage [35]. Interestingly, we found that MCC is primarily localized at mitochondria, but also detectable in the ER, cytosol and nucleus in human MM cells. MCC does not contain any mitochondrial targeting motifs or transmembrane domains. Through delineation of the mitochondrial MCC-interactome, we identified a number of mitochondrial proteins that are associated with MCC in human MM cells, including PHB2, prohibitin (PHB), ECHA, VDAC3, ADT1, ADT2, and ADT3. All of these mitochondrial proteins are known as critical regulators of cell survival and apoptosis (http://www.ingenuity.com). Therefore, MCC is primarily localized at mitochondria to promote cellular survival by interacting with multiple mitochondrial proteins in malignant B cells.

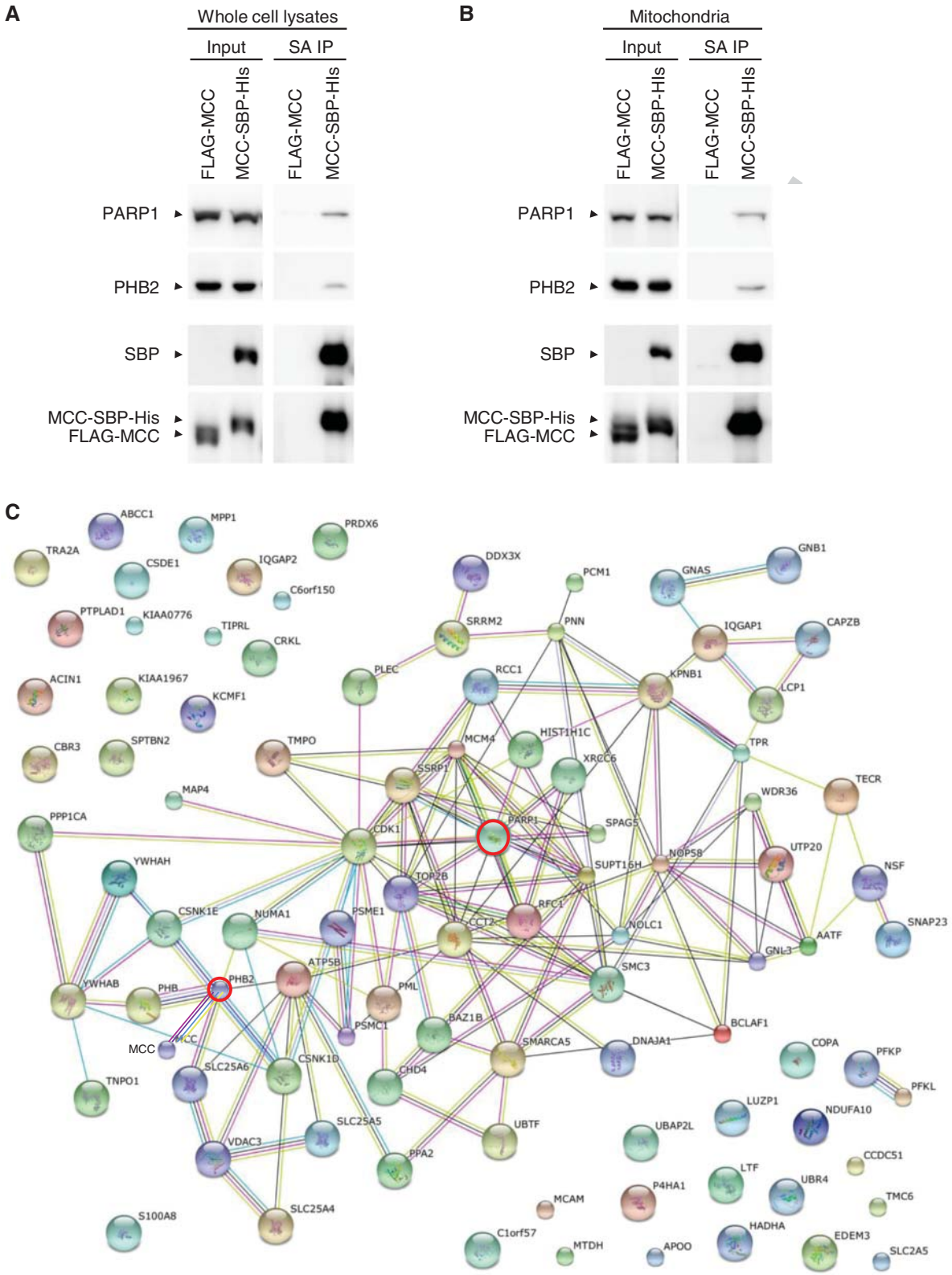

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Figure 8 PARP1 and PHB2 are two hubs of the MCC interaction network in human MM cells. The human MM cell line 8226 cells were transduced with pUB-hMCC-SBP-6xHis or pUB-FLAG-hMCC. Immunoprecipitation was performed as described in Figure 7. **(A and B)** Immunoblot analyses of proteins pulled down by streptavidin-sepharose beads (SA IP) from whole cell lysates **(A)** or purified mitochondria **(B)**. Proteins were immunoblotted for PARP1, PHB2, SBP, and followed by MCC. Blots of an aliquot of whole cell or mitochondrial lysates before immunoprecipitation were used as the input control. **(C)** The MCC interaction network in human MM cells**.** We performed an interaction network analysis using the top 100 MCC-interactors and the STRING analysis tool (http://www.string-db.org). The top 100 MCC-interactors identified in our study and used for the interaction network analysis include 60 proteins identified in both whole cell lysates and mitochondria, 10 proteins identified in mitochondria only, and 30 proteins identified in whole cell lysates only. Sixty-one proteins of the top 100 MCC-interactors of human MM cells form an interaction network centered at PARP1 and PHB2.

Most proteins (326 out of 365, 89.3%) of the MCC-interactome identified in our study are novel, previously unknown MCC-interacting partners. Among these, PARP1 is the top novel MCC-interactor identified in both mitochondrial and whole cell lysates of human MM cells. PARP1, the most abundant member of the polyADP-ribose polymerases (PARP) family, catalyzes post-translational modification of proteins by polyADP-ribosylation. This modification affects protein-protein and protein-DNA interactions. In addition to its pivotal role in DNA repair, PARP1 also critically regulates transcription, cell survival, and proliferation [62,63]. PARP1 can act in both a pro- and anti-tumor manner depending on the context [62]. In this study, we identified PARP1 as a signaling hub of the MCC-interactome in human MM cells (Figure 8C). PARP1 is known to interact with numerous MCC interactors identified in our study, including CDK1, MCM4, XRCC6, RFC1, CCT2, SSRP1, TOP2B, HIST1H1C, and SUPT16H (http://www.string-db.org). Interestingly, PARP1 has been shown to directly or indirectly regulate the activation or expression of multiple MCC targets identified by our knockdown and overexpression experiments, including caspase 8, ERK, c-Myc, p27, cyclin B1, and Mcl1 (Figure 6). Indeed, PARP1 can induce the PARylation of caspase-8, thereby inactivating caspase-8 and inhibiting caspase-mediated apoptotic signaling [62]. PARP1 directly interacts with phosphorylated ERK2 to mediate cell proliferation, whereas PARP1 inhibition causes loss of ERK2 stimulation [62,63]. PARP1 also down-regulates the expression of MKP-1, which dephosphorylates ERK [62]. Through modifications of chromatin or interactions with gene specific promoters/transcription factors, PARP1 regulates in total 3.5% of the transcriptome, including increasing the expression of c-Myc [62,63] and repressing the expression of p27 [68]. Furthermore, PARP1 can indirectly regulate the degradation and anti-apoptotic function of Mcl1 through interaction with CDK1/cyclin B1 [69,70]. Therefore, PARP1 is a novel MCC-interactor that plays a central role in mediating the oncogenic effects of MCC in malignant B cells.

Thirty-nine proteins of the MCC-interactome identified in our study are previously known as MCC-interacting proteins in CRCs or 293T cells. Among these, PHB2 is the top known MCC-interactor identified in both mitochondrial and whole cell lysates of human MM cells. PHB2, a ubiquitously expressed pleiotropic protein, is mainly localized in mitochondria by forming heteromeric complex with PHB, but also present in the cytosol, nucleus and plasma membrane [64,65,71]. PHB2 plays crucial roles in regulating mitochondrial function, cell survival, proliferation, stress response, and development [64,65,71]. PHB2 is expressed at higher levels in proliferating cells, including neoplastic tissues [64,65,71]. Silencing or abrogation of PHB2 induces apoptosis and reduces proliferation in a variety of cancer cells [64,65,71]. Here we identified PHB2 as another hub of the MCC interaction network in human MM cells (Figure 8C). Previous studies have demonstrated the association between PHB2 and multiple MCC-interactors identified in our study, including PHB, CSNK1D (CK1δ), CSNK1E (CK1ε), ATP5B, NUMA1, and SLC25A6 (http://www.string-db.org) [65,72]. PHB2 has also been shown to directly or indirectly regulate the activity or expression of multiple MCC targets identified in our study, including caspase 3, ERK, c-Myc, p27, and cyclin B1 (Figure 6). For example, over-expression of PHB2 inhibits caspase 3 activation and cell apoptosis, whereas down-regulation of PHB2 is associated with increased caspase 3 expression and cell apoptosis [64,65,71]. PHB and PHB2 interact with c-Raf to induce the phosphorylation of ERK1/2 via MEK1 [65]. Through activation of ERK1/2, PHB2 may also indirectly regulate the expression levels of downstream targets of the ERK pathway, including c-Myc, p27 and cyclin B1 [65,71,73,74]. The c-Myc up-regulation mediated by MCC-PHB2 or MCC-PARP1 via ERK signaling pathway in MM cells is functionally analogous to that induced by canonical Wnt-β-catenin signal activation observed in epithelial cells [75,76]. Thus, similar to PARP1, PHB2 and PHB are likely to play essential roles in mediating the oncogenic effects of MCC in malignant B cells.

Interestingly, preclinical evidence indicates that both PARP1 and PHB2/PHB are excellent therapeutic targets in cancer [62,65]. In preclinical studies, PARP inhibitors (such as olaparib) exhibit potent tumoricidal activities on breast cancer, ovarian cancer, pancreatic cancer, prostate cancer, Ewing's sarcoma, small cell lung carcinoma,

and neuroblastoma, among others. The therapeutic effects of olaparib on BRCA-mutated breast cancer have been confirmed in early phase clinical trials with only mild adverse side effects [62]. Notably, PHB ligands (such as flavaglines and capsaicin) display robust cytotoxicity on cancer cells, but have cytoprotective activities on normal cells (e.g. neurons and cardiomyocytes), particularly against oxidative stress [65]. PHB and PHB2 were recently identified as the direct targets of flavaglines (e.g., rocaglamide, rocaglaol and silvestrol), natural products isolated from medicinal plants that show significant anticancer effects but no sign of toxicity in mice [65]. Capsaicin, a component of hot chili peppers, binds to PHB2, and this binding induces apoptosis in human myeloid leukemia cells [65]. Both flavaglines and capsaicin regulate subcellular localization of PHB2 [77-79]. Flavaglines specifically inhibit PHB1/2-c-Raf interaction and prevent PHB1/2-c-Raf membrane localization [79], while capsaicin induces the translocation of PHB2 from mitochondria to the nucleus [77,78]. Such specific targeting mechanisms of PHB ligands suggest that they may exert robust synergistic effects with conventional chemotherapies that target different signaling pathways, including DNA damage response, proteasome, the Bcl-2 family, and NF-κB activation. In this regard, flavaglines have been shown to enhance the efficacy of doxorubicin in mouse lymphoma models [65]. Therefore, identification of PARP1 and PHB2/PHB as hubs of the signaling pathways of MCC in human MM cells implicates potential use of PARP inhibitors and PHB ligands in the treatment of B cell malignancies involving aberrant expression of MCC.

Conclusions

In the present study, we have identified MCC as a novel oncogene in B lymphocytes and provided insights into its signaling mechanisms in human MM cells. In the unique cellular context of malignant B cells, MCC forms an interaction network centered at PARP1 and PHB2 to promote cellular survival and proliferation by up-regulating ERK activation, c-Myc, Mcl1, and cyclin B1, and by down-regulating p27 and suppressing cleavage of caspases 8 and 3. The lack of expression of MCC in normal or premalignant B cells but ubiquitous up-regulation of MCC in primary human B cell malignances suggests that MCC may be a useful diagnostic marker for B cell neoplasms. Our finding that knockdown of MCC induced apoptosis and inhibited proliferation in human MM cells suggests that MCC may also serve as a therapeutic target in B cell malignancies. Furthermore, the central role of PARP1 and PHB2 in the MCC interaction network of human MM cells implies that PARP1 inhibitors and PHB ligands may have therapeutic application in B cell neoplasms, including NHL and MM.

Methods

Mice, cell lines, and reagents

Mice and disease monitoring were as previously described [12,13]. Human patient-derived MM cell lines were generously provided by Dr. Leif Bergsagel (Mayo Clinic, Scottsdale, AZ), including 8226, KMS11, LP1, U266, KMS28PE, and KMS20. The EBV-transformed human B lymphoblastoid cell line C3688 was provided by Dr. Lori Covey (Rutgers University, Piscataway, NJ). All human B cell lines were cultured as described [80]. Most antibodies and reagents used in this study were as previously described [13,67,80]. Mouse monoclonal Abs to MCC were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DTT, thapsigargin, and lentiviral shRNA constructs for human MCC were purchased from Sigma-Aldrich Corp. (St. Louis, MO). The plasmid pENTR1A-NTAP-A containing the SBP tag sequence was purchased from Addgene (Cambridge, MA). PARP1 Abs were from eBioscience (San Diego, CA), and PHB2 Abs were purchased from Bethyl Laboratories Inc (Montgomery, TX). Additional polyclonal rabbit Abs were from Cell Signaling Technology (Beverly, MA).

Transcriptome microarray analysis

Total RNA was extracted from splenocytes of LMC (mouse ID: 6983–6, 7041–9, and 7060–5) and tumor-bearing B-TRAF3$^{-/-}$ mice (mouse ID: 6983–2, 7041–10, and 7060–8) using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA quality was assessed on an RNA Nano Chip using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The mRNA was amplified with a TotalPrep RNA amplification kit with a T7-oligo(dT) primer according to the manufacturer's instructions (Ambion), and microarray analysis was carried out with the Illumina Sentrix MouseRef-8 24 K Array at the Burnham Institute (La Jolla, CA). Results were extracted with Illumina GenomeStudio v2011.1, background corrected and variance stabilized in R/Bioconductor using the lumi package [81,82] and modeled in the limma package [83]. Microarray data are available from NIH GEO Accession GSE48818.

Taqman assays of the transcript expression of identified genes

Complementary DNA (cDNA) was prepared from RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR of specific genes was performed using corresponding TaqMan Gene Assay kit (Applied Biosystems) as previously described [84]. Briefly, real-time PCR was performed using TaqMan primers and probes (FAM-labeled) specific for mouse *MCC, Diras2*, *Tbc1d9*, *Ccbp2*, *Btbd14a*, *Sema7a*, *Twsg1*, *Ppap2b*, *TCF4*, *Tnfrsf19*, *Zcwpw1*, *Abca3*, or human *MCC*. Each reaction also included the probe

(VIC-labeled) and primers for mouse or human β-actin mRNA, which served as an endogenous control. Relative mRNA expression levels of each gene were analyzed using the Sequencing Detection Software (Applied Biosystems) and the comparative Ct method (ΔΔCt) as previously described [84].

Splenic B cell purification and stimulation

Splenic B cells were purified using anti-mouse CD43-coated magnetic beads and a MACS separator (Miltenyi Biotec Inc.) following the manufacturer's protocols as previously described [12,84]. The purity of isolated populations was monitored by FACS analysis, and cell preparations of >95% purity were used for RNA and protein extraction. Purified B cells were cultured *ex vivo* in the absence or presence of B cell stimuli for 6 or 24 hours as described previously [12,84]. B cell stimuli examined include 2 μg/ml anti-CD40, 20 μg/ml LPS, 1 μg/ml anti-BCR, and 100 nM CpG2084, alone or in combination. Total cellular RNA was prepared at 6 or 24 hours after stimulation.

Taqman copy number assay of the mouse *MCC* gene

Genomic DNA was prepared from splenocytes of LMC and tumor-bearing B-TRAF3$^{-/-}$ mice as previously described [13]. Quantitative real-time PCR of the mouse *MCC* gene was performed using the TaqMan Copy Number Assay kit (assay ID: Mm00490037_cn; Applied Biosystems) following the manufacturer's protocols. Briefly, real-time PCR was performed using the TaqMan primers and probe (FAM-labeled) specific for the mouse MCC gene. Each reaction also included the probe (VIC-labeled) and primers specific for the mouse Trfc gene (TaqMan Copy Number Reference Assay, Applied Biosystems), which served as reference control. Relative copy numbers of the MCC gene in genomic DNA samples were analyzed using the Sequencing Detection Software (Applied Biosystems) and the comparative Ct method (ΔΔCt) following the manufacturer's protocols.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed essentially as described previously [85,86]. Briefly, 20 × 10^6 cell equivalents of fragmented chromatins (containing DNA fragments < 400 bp) were immunoprecipitated with Ig isotype control or antibodies specific for histone modifications. We used the following histone antibodies in the ChIP experiments: H3K27me3 (Millipore, Billerica, MA) and H3K9/14ac (Diagenode, Denville, NJ). Immunoprecipitated DNA were purified, and then analyzed by quantitative real time PCR using primers specific for the promoter region of the mouse *MCC* or *Diras2* gene. Primer used are: mMCC-U519F (5'- CAG GGA GGT TGG AGA GGA -3') and mMCC-U446R (5'- AAA CAT GCC CTG CCC TTG -3'); mDiras2-U559F (5'- GCA CAT GTG ACT ACT ATT G -3') and mDiras2-U480R (5'- AAT CTC TCC TCC CAC AAG -3'). The enrichments of each gene promoter immunoprecipitated by histone marks were quantitated relative to the input DNA [86].

Cloning of the full-length cDNA of the *MCC* gene from TRAF3$^{-/-}$ mouse B lymphomas and human MM cell lines

Total cellular RNA was prepared from B lymphomas spontaneously developed in four individual B-TRAF3$^{-/-}$ mice (mouse ID: 6983–2, 7060–8, 105–8, and 115–6), and the corresponding cDNA samples were used as templates to clone the mouse MCC coding sequences using primers mMCC-F (5'- ATG AAT TCT GGA GTT GCG GTG -3') and mMCC-R (5'- TTA GAG TGA CGT TTC GTT GGT G -3'). Similarly, human MCC coding sequences were cloned from human MM cell lines LP1 and KMS11 cells using reverse transcription PCR. Primers used for the cloning of human MCC are hMCC-F (5'- TGC ATC ATG AAT TCC GGA GT -3'), and hMCC-R (5'- TTA AAG CGA AGT TTC ATT GGT GTG -3'). The high fidelity polymerase Pfu UltraII (Santa Clara, CA) was used in these PCR reactions. Sequences of the cloned mouse and human MCC were determined at GenScript (Piscataway, NJ).

Generation of lentiviral MCC expression and shRNA vectors

The coding cDNA sequence of *MCC* cloned from the human MM cell line LP1 cells was subcloned into the lentiviral expression vector pUB-eGFP-Thy1.1 [87] (generously provided by Dr. Zhibin Chen, the University of Miami, Miami, FL) by replacing the eGFP coding sequence with the MCC coding sequence. To facilitate immunoprecipitation experiments, we engineered an N-terminal FLAG tag or a C-terminal SBP-6×His tag [88] in frame with the MCC coding sequence, respectively. We subsequently generated two lentiviral expression vectors of tagged hMCC, including pUB-FLAG-hMCC and pUB-hMCC-SBP-6×His. Lentiviral shRNA vectors specific for human *MCC* (including hMCC shRNA 1332, 1388, 2284 and 2689; all in Torc1 vectors) or a scrambled shRNA vector were purchased from Sigma. To facilitate FACS analysis and cell sorting, we engineered an eGFP-expressing version of all the shRNA vectors by replacing the puromycin resistance gene of Torc1 with the eGFP coding sequence. Each lentiviral expression or shRNA vector was verified by DNA sequencing.

Lentiviral packaging and transduction of human MM cells

Lentiviruses of MCC shRNA vectors and a scrambled shRNA vector were packaged following the manufacturer's protocol (Sigma) as previously described [13,89]. Lentiviruses of MCC expression vectors were packaged

and titered as previously described [67,80,87]. Human MM cells were transduced with the packaged lentiviruses at a MOI of 1:5 (cell:virus) in the presence of 8 μg/ml polybrene [13,87,89]. Transduction efficiency of cells was analyzed by flow cytometry on day 3 post transduction. All shRNA vectors contain an eGFP expression cassette, and were directly analyzed using a flow cytometer. All pUB lentiviral expression vectors have an expression cassette of the marker Thy1.1, and thus allow the transduced cells to be analyzed by Thy1.1 immunofluorescence staining followed by flow cytometry. Transduced cells were subsequently sorted or directly analyzed for apoptosis, cell cycle distribution, proliferation, and protein expression.

Growth curve determination, annexin V staining of apoptotic cells, cell cycle distribution, and cell proliferation analyses

For MCC knockdown studies, on day 4 post-transduction, successfully transduced cells were sorted for GFP+ shRNA expressing populations, and then plated in 6-well plates for growth curve determination, annexin V staining, or cell cycle analysis. For growth curve determination, live and dead cells were differentiated using trypan blue staining, and counted using a hemacytometer. For analysis of apoptosis, cells were stained with annexin V and PI according to the manufacturer's protocol (Invitrogen), and analyzed by flow cytometry as previously described [13]. For cell cycle analysis, cells were fixed with ice-cold 70% ethanol. Cell cycle distribution was subsequently determined by propidium iodide (PI) staining followed by flow cytometry as previously described [12,90]. For cell proliferation analysis, cells were labeled with a cell proliferation dye eFluor® 670 (eBioscience), and dilution of the proliferation dye was analyzed by flow cytometry following the manufacturer's protocol.

Total protein lysates, fractionation of cytosol, mitochondria and microsomes (rich in ER), and immunoblot analysis

For total protein lysates, cell pellets were lysed in 200 μl of 2X SDS sample buffer (0.0625 M Tris, pH6.8, 1% SDS, 15% glycerol, 2% β-mercaptoethanol and 0.005% bromophenol blue), sonicated for 30 pulses, and boiled for 10 minutes.

For biochemical fractionation, human MM cells (30×10^6 cells/condition) were cultured in the absence or presence of 250 μM DTT or 0.5 μM thapsigargin for 24 hours. Cytosol, mitochondria and microsomes (rich in ER) were fractionated from cells as previously described [91-93]. Briefly, cells were washed with ice-cold PBS, swelled in 700 μl of Mitochondria Isolation Buffer (250 mM sucrose, 10 mM HEPES, pH7.5, 10 mM KCl, 1 mM EDTA, and 0.1 mM EGTA with protease and phosphatase inhibitors) on ice for 10 minutes, and then homogenized in a Dounce homogenizer. Cell lysis was checked by trypan blue uptake, and homogenization stopped when 90% of cells were broken. Nuclei were pelleted by centrifugation at 1,000 g for 10 minutes at 4°C. The cleared lysates were then centrifuged at 10,000 g for 25 minutes at 4°C to obtain the pellets of mitochondria. The supernatants were further centrifuged at 100,000 g for 2 hours to separate the pellets of microsomes (rich in ER) from cytosolic proteins (S100 fraction). One-fifth volume of 5X SDS sample buffer was added into each S100 fraction. The pellets of nuclei, mitochondria and microsomes (rich in ER) were lysed and sonicated in 300 μl of 2X SDS sample buffer, respectively. All protein samples were subsequently boiled for 10 minutes.

Total protein lysates, or cytosolic, ER, mitochondrial and nuclear proteins were separated by SDS-PAGE. Immunoblot analyses were performed using specific antibodies as previously described [67,80]. Images of immunoblots were acquired using a low-light imaging system (LAS-4000 mini, FUJIFILM Medical Systems USA, Inc., Stamford, CT).

Co-immunoprecipitation assay in whole cell lysates

Human MM cell line 8226 cells (5×10^7 cells/condition) transduced with pUB-FLAG-hMCC or pUB-hMCC-SBP-6×His were lysed and sonicated in the CHAPS lysis buffer [94] (1% CHAPS, 20 mM Tris, pH 7.4, 150 mM NaCl, 50 mM β-glycerophosphate, and 5% glycerol with freshly added 1 mM DTT and EDTA-free Mini-complete protease inhibitor cocktail). The insoluble pellets were removed by centrifugation at 10,000 g for 20 minutes at 4°C. The CHAPS lysates were subsequently immunoprecipitated with anti-FLAG-agarose beads (Sigma; for FLAG-hMCC), or streptavidin-sepharose beads (Pierce, Rockford, IL; for hMCC-SBP-6×His), separately. Immunoprecipitates were washed 5 times with the Wash Buffer (0.5% CHAPS, 20 mM Tris, pH 7.4, 150 mM NaCl, 50 mM β-glycerophosphate, and 2.5% glycerol with freshly added 1 mM DTT and EDTA-free Mini-complete protease inhibitor cocktail). Immunoprecipitated proteins were resuspended in 2× SDS sample buffer, boiled for 10 minutes, and then separated on SDS-PAGE for mass spectrometry or immunoblot analyses.

Co-immunoprecipitation assay in mitochondrial lysates

Human MM cell line 8226 cells (1.5×10^8 cells/condition) transduced with pUB-FLAG-hMCC or pUB-hMCC-SBP-6×His were used for cytosol, mitochondria and ER fractionation as described above. Mitochondrial pellets were lysed and sonicated in the CHAPS lysis buffer [94], and cleared by centrifugation at 10,000 g for 20 minutes at 4°C. The mitochondrial lysates were subsequently immunoprecipitated with streptavidin-sepharose

beads (Pierce, Rockford, IL; for hMCC-SBP-6×His). Immunoprecipitates were washed 5 times with the Wash Buffer, resuspended in 2× SDS sample buffer, boiled for 10 minutes, and then separated on SDS-PAGE for mass spectrometry or immunoblot analyses.

Mass spectrometry based-sequencing

Whole cell lysates or mitochondrial lysates immunoprecipitated with streptavidin-sepharose beads were used for LC-MS/MS. The entire gel lanes for hMCC-SBP-6×His complex and negative control (FLAG-hMCC) were each sectioned into 15 continuous slices. The gel slice samples were subjected to thiol reduction by TCEP, alkylation with iodoacetamide, and digestion with sequencing-grade modified trypsin [95,96]. Peptides were eluted from the gel slices, desalted, and then subjected to reversed-phase nano-flow ultra high performance capillary liquid chromatography (uPLC) followed by high-resolution/high-mass accuracy MS/MS analysis using an LC-MS platform consisting of an Eksigent Nano Ultra 2D Plus uPLC system hyphenated to a Thermo Orbi Velos mass spectrometer. The MS/MS was set to operate in data dependent acquisition mode using a duty cycle in which the top 15 most abundant peptide ions in the full scan MS were targeted for MS/MS sequencing. Full scan MS1 spectra were acquired at 100,000 resolving power and maintained mass calibration to within 2–3 ppm mass accuracy. LC-MS/MS data were searched against the human IPI and UniProt databases using the Mascot and Proteome Discoverer search engines [95,96]. Protein assignments were considered highly confident using a stringent false discovery rate threshold of <1%, as estimated by reversed database searching, and requiring that ≥2 peptides per protein be unambiguously identified. Rough relative protein amounts were estimated using spectra counting values.

Statistics

Statistical analyses were performed using the Prism software (GraphPad, La Jolla, CA). Statistical significance was assessed by Student t test. P values less than 0.05 are considered significant.

Additional files

Additional file 1: Table S1. List of genes differentially expressed in TRAF3$^{-/-}$ mouse splenic B lymphomas identified by the microarray analysis.

Additional file 2: Table S2. The MCC-interactome in human MM cells identified by affinity purification followed by LC-MS/MS.

Additional file 3: Figure S1. The MCC isoform 2 was identified as an MCC-interacting protein by affinity purification and LC-MS/MS. The two isoforms of MCC proteins differ at their extreme N-terminus due to alternative promoter usage. Both pUB-FLAG-hMCC and pUB-hMCC-SBP-6xHis are cloned from MCC isoform 1 (829 aa). **(A)** Schematic diagram of peptide sequences of MCC isoform 1 identified by LC-MS/MS. **(B)** Schematic diagram of peptide sequences of MCC isoform 2 (1019 aa) identified by LC-MS/MS. The peptide sequences detected by LC-MS/MS are highlighted in green color. The unique region of MCC isofrom 2 is marked with a dashed black box.

Abbreviations

MCC: Mutated in colorectal cancer; TRAF3: Tumor necrosis factor receptor (TNF-R)-associated factor 3; B-TRAF3$^{-/-}$: B cell-specific TRAF3-deficient; NHL: Non-Hodgkin lymphoma; MM: Multiple myeloma; MZL: Splenic marginal zone lymphoma; B-CLL: B cell chronic lymphocytic leukemia; MCL: Mantle cell lymphoma; WM: Waldenström's macroglobulinemia; LOH: Loss of heterozygosity; FAP: Familial adenomatous polyposis; CRCs: Colorectal cancers; PEL: Primary effusion lymphoma; CBL: Centroblastic lymphoma; DLBCL: Diffuse large B-cell lymphoma; BL: Burkitt's lymphoma; NF-κB: Nuclear factor κ light chain enhancer of activated B cells; BCR: B cell receptor; LPS: Lipopolysaccharides; shRNA: Short hairpin RNA; ChIP: Chromatin immunoprecipitation; H3K27Me3: trimethylated lysine 27 of histone 3; H3K9/14Ac: Acetylated lysine 9/14 of histone 3; DTT: Dithiothreitol; ERK: Extracellular signal-regulated kinase; JNK: C-Jun N-terminal kinase; SBP: Streptavidin-binding peptide; SA: Streptavidin; LC-MS/MS: Liquid chromatography-mass spectrometry/mass spectrometry; uPLC: High performance capillary liquid chromatography; PARP1: Poly [ADP-ribose] polymerase 1; PHB2: Prohibitin-2; PHB: Prohibitin; PI: Propidium iodide; FACS: Fluorescence-activated cell sorting; PCR: Polymerase chain reaction.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

SE designed and performed experiments, analyzed data, and wrote the manuscript; JB, CM and YL carried out experiments, analyzed data, and revised the manuscript; DP performed LC-MS/MS sequencing, analyzed data, and revised the manuscript; RH analyzed microarray data and revised the manuscript; PX supervised and designed this study, analyzed data, and wrote the manuscript. All authors read and approved this manuscript.

Acknowledgements

This study was supported by the National Institutes of Health grant CA158402 (P. Xie), the Department of Defense grant W81XWH-13-1-0242 (P. Xie), a Faculty Research Grant (P. Xie), and in part by a grant from NCI (1RC1CA147187, to R. Hart) and an Aresty Research Grant (J. Baron). The FACS analyses described in this paper were supported by the Flow Cytometry Core Facility of the Cancer Institute of New Jersey (P30CA072720).
We would like to express our gratitude to Dr. P. Leif Bergsagel for providing us the human multiple myeloma cell lines used in this study, and to Dr. Lori R. Covey for critical review of this manuscript as well as for providing C3688 cells and normal human blood B cells for our study. We would also like to thank Sukhdeep Grewal, Benjamin Kreider, Punit Arora, Anand Desai, and Ashima Choudhary for technical assistance on this study.

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Received: 7 June 2014 Accepted: 24 July 2014
Published online: 09 September 2014

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doi:10.1186/s13045-014-0056-6
Cite this article as: Edwards *et al.*: Mutated in colorectal cancer (MCC) is a novel oncogene in B lymphocytes. *Journal of Hematology & Oncology* 2014 **7**:56.

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Supplementary Table 1. List of genes differentially expressed in TRAF3$^{-/-}$ mouse splenic B lymphomas identified by the microarray analysis

#	GeneSymbol	Gene Name	LogFC	AveExpr	t	P. Value	Adj. P. Val.
1	Zcwpw1	zinc finger, CW type with PWWP domain 1	3.36	9.50	14.20	4.07E-07	0.000126365
2	Diras2	DIRAS family, GTP-binding RAS-like 2	3.35	10.56	14.57	3.32E-07	0.000126365
3	Serpina3f	serine (or cysteine) peptidase inhibitor, clade A, member 3F	3.03	10.87	9.99	6.54E-06	0.000571
4	Sox5	SRY-box containing gene 5	2.93	9.48	13.97	4.65E-07	0.000130244
5	C130026I21Rik	RIKEN cDNA C130026I21 gene	2.87	9.95	5.58	0.000456113	0.008256021
6	Tnfrsf19	tumor necrosis factor receptor superfamily, member 19	2.87	9.00	13.59	5.78E-07	0.000148597
7	Mcc	mutated in colorectal cancers	2.76	8.68	31.01	6.98E-10	9.15E-06
8	Slamf9	SLAM family member 9	2.75	10.73	4.75	0.00131062	0.016097166
9	Fah	fumarylacetoacetate hydrolase	2.63	11.18	7.83	4.13E-05	0.00178595
10	Rdh12	retinol dehydrogenase 12	2.61	11.29	12.81	9.27E-07	0.000205961
11	Ahnak2	AHNAK nucleoprotein 2	2.54	8.93	12.49	1.13E-06	0.000226433
12	Chst7	carbohydrate (N-acetylglucosamino) sulfotransferase 7	2.42	9.23	7.85	4.05E-05	0.001759595
13	Vars	valyl-tRNA synthetase	2.30	11.46	16.48	1.23E-07	8.74E-05
14	Twsg1	twisted gastrulation homolog 1 (Drosophila)	2.30	10.96	10.51	4.41E-06	0.000441575
15	Tbc1d9	TBC1 domain family, member 9	2.29	9.53	17.05	9.34E-08	7.20E-05
16	Vars	valyl-tRNA synthetase	2.13	9.29	17.17	8.83E-08	7.20E-05
17	Cd59a	CD59a antigen	2.11	10.04	5.72	0.000385181	0.007336917
18	Gnb3	guanine nucleotide binding protein (G protein), beta 3	2.09	8.73	5.99	0.000283427	0.006099042
19	Sspn	sarcospan	2.08	10.14	4.98	0.000958982	0.013242851
20	Lacc1	laccase (multicopper oxidoreductase) domain containing 1	2.08	8.98	19.86	2.70E-08	5.06E-05
21	Gbp1	guanylate binding protein 1	2.06	9.78	4.19	0.00279695	0.02699119
22	Vars	valyl-tRNA synthetase	2.06	12.05	21.24	1.56E-08	3.41E-05
23	Ccbp2	chemokine binding protein 2	1.96	9.96	7.62	5.07E-05	0.002000536
24	Gng13	guanine nucleotide binding protein (G protein), gamma 13	1.94	8.60	7.33	6.70E-05	0.00236437
25	Plscr1	phospholipid scramblase 1	1.94	10.35	6.13	0.000239207	0.005519038
26	Clic4	chloride intracellular channel 4 (mitochondrial)	1.93	12.48	7.57	5.33E-05	0.002054453
27	Sema7a	sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (sema	1.91	9.22	9.70	8.22E-06	0.000643485
28	Chrnb1	cholinergic receptor, nicotinic, beta polypeptide 1 (muscle)	1.89	9.84	8.35	2.55E-05	0.001291342
29	Ppap2b	phosphatidic acid phosphatase type 2B	1.86	10.46	18.00	6.01E-08	7.20E-05
30	Ebi3	Epstein-Barr virus induced gene 3	1.82	9.85	10.82	3.51E-06	0.00039538
31	4930539E08Rik	RIKEN cDNA 4930539E08 gene	1.81	8.92	8.10	3.22E-05	0.001536721
32	Nacc2	nucleus accumbens associated 2, BEN and BTB (POZ) domain containing	1.79	9.23	12.76	9.57E-07	0.000209017
33	Fcrl5	Fc receptor-like 5	1.79	9.62	8.79	1.74E-05	0.001046831
34	Sel1l3	sel-1 suppressor of lin-12-like 3 (C. elegans)	1.76	8.30	7.70	4.70E-05	0.001893452
35	Dnajc7	DnaJ (Hsp40) homolog, subfamily C, member 7	1.76	10.73	6.73	0.000124642	0.003589973
36	Pdlim1	PDZ and LIM domain 1 (elfin)	1.74	11.75	6.79	0.000116482	0.003392213
37	Abca3	ATP-binding cassette, sub-family A (ABC1), member 3	1.71	10.86	11.21	2.66E-06	0.0003633
38	Rassf4	Ras association (RalGDS/AF-6) domain family member 4	1.66	11.54	10.68	3.89E-06	0.000397578
39	Kynu	kynureninase (L-kynurenine hydrolase)	1.65	10.56	14.43	3.57E-07	0.000126365
40	Cd274	CD274 antigen	1.64	11.38	16.17	1.43E-07	8.74E-05

41	Cd80	CD80 antigen	1.64	8.83	12.33	1.25E-06	0.00023571
42	Ccdc28b	coiled coil domain containing 28B	1.64	10.34	7.19	7.74E-05	0.002628686
43	Rassf4	Ras association (RalGDS/AF-6) domain family member 4	1.63	11.53	11.04	3.00E-06	0.000376611
44	Sp140	Sp140 nuclear body protein	1.63	9.80	11.51	2.15E-06	0.000322971
45	AF067061	cDNA sequence AF067061	1.63	8.54	4.77	0.001275097	0.015838995
46	Ero1lb	ERO1-like beta (S. cerevisiae)	1.63	11.22	12.45	1.16E-06	0.000226433
47	Nfatc1	nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1	1.61	9.89	8.27	2.76E-05	0.001378762
48	Hdac4	histone deacetylase 4	1.61	8.78	8.11	3.17E-05	0.001523759
49	Tubb2b	tubulin, beta 2B class IIB	1.60	10.65	5.22	0.000712545	0.010897018
50	Trim40	tripartite motif-containing 40	1.59	8.56	8.79	1.74E-05	0.001046831
51	Plscr1	phospholipid scramblase 1	1.57	9.33	6.70	0.000129091	0.003645988
52	Arhgap24	Rho GTPase activating protein 24	1.52	10.13	11.91	1.65E-06	0.000270244
53	D13Ertd608e	DNA segment, Chr 13, ERATO Doi 608, expressed	1.52	8.97	10.25	5.35E-06	0.0005051
54	Hn1l	hematological and neurological expressed 1-like	1.52	9.27	15.10	2.49E-07	0.000112734
55	Ccdc28b	coiled coil domain containing 28B	1.50	9.62	9.68	8.31E-06	0.000643485
56	Tcf4	transcription factor 4	1.50	12.04	9.19	1.24E-05	0.000847921
57	Zbtb32	zinc finger and BTB domain containing 32	1.49	8.54	9.39	1.05E-05	0.000754882
58	Gbp2	guanylate binding protein 2	1.48	11.56	7.68	4.79E-05	0.001904754
59	Caln1	calneuron 1	1.48	8.43	6.18	0.000228083	0.005366295
60	Gpr34	G protein-coupled receptor 34	1.48	8.77	6.37	0.000182843	0.004607992
61	Serpina3g	serine (or cysteine) peptidase inhibitor, clade A, member 3G	1.47	12.85	8.47	2.30E-05	0.001206517
62	Rbm47	RNA binding motif protein 47	1.45	9.83	11.02	3.04E-06	0.000376611
63	Fgd2	FYVE, RhoGEF and PH domain containing 2	1.45	11.91	5.76	0.000368771	0.007141322
64	Fgd6	FYVE, RhoGEF and PH domain containing 6	1.44	9.42	8.42	2.41E-05	0.001236456
65	Neurod4	neurogenic differentiation 4	1.43	8.19	7.07	8.74E-05	0.002845238
66	Rassf4	Ras association (RalGDS/AF-6) domain family member 4	1.42	10.14	7.45	5.94E-05	0.002191762
67	Rhbdf1	rhomboid family 1 (Drosophila)	1.42	10.30	8.74	1.81E-05	0.001056366
68	Gstt3	glutathione S-transferase, theta 3	1.40	9.14	6.47	0.000164429	0.004282628
69	Pafah1b3	platelet-activating factor acetylhydrolase, isoform 1b, subunit 3	1.40	10.94	5.50	0.000501117	0.00873186
70	Asph	aspartate-beta-hydroxylase	1.37	8.04	5.64	0.000426167	0.007789294
71	Nid1	nidogen 1	1.37	8.57	7.77	4.37E-05	0.001817842
72	Sgk3	serum/glucocorticoid regulated kinase 3	1.36	9.50	10.11	5.97E-06	0.000539159
73	Gas7	growth arrest specific 7	1.35	8.81	6.41	0.000176165	0.004524439
74	NA	NA	1.34	10.20	11.26	2.56E-06	0.000352915
75	Gm14137	predicted gene 14137	1.34	8.18	17.51	7.51E-08	7.20E-05
76	Prps2	phosphoribosyl pyrophosphate synthetase 2	1.33	9.69	8.55	2.13E-05	0.001175007
77	Sox5	SRY-box containing gene 5	1.32	8.42	11.10	2.87E-06	0.000365287
78	Hsp90ab1	heat shock protein 90 alpha (cytosolic), class B member 1	1.30	11.59	7.12	8.32E-05	0.002740232
79	Eps15	epidermal growth factor receptor pathway substrate 15	1.30	9.95	9.51	9.50E-06	0.000711445
80	Cybasc3	cytochrome b, ascorbate dependent 3	1.29	10.90	11.68	1.92E-06	0.000303211
81	Fbxw13	F-box and WD-40 domain protein 13	1.29	8.28	9.97	6.61E-06	0.000572834
82	Recql5	RecQ protein-like 5	1.28	9.46	7.40	6.26E-05	0.002267958
83	Kcnk5	potassium channel, subfamily K, member 5	1.28	10.16	10.71	3.81E-06	0.00039567
84	Ptpn22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	1.27	11.57	8.75	1.80E-05	0.001056366

85	Pdia4	protein disulfide isomerase associated 4	1.27	12.33	7.50	5.68E-05	0.002143803
86	Ccnd2	cyclin D2	1.27	10.35	7.56	5.37E-05	0.002064785
87	Plac1l	placenta-specific 1-like	1.27	8.63	7.49	5.74E-05	0.002156914
88	Gpr137b	G protein-coupled receptor 137B	1.26	8.68	8.07	3.31E-05	0.001539334
89	R74862	expressed sequence R74862	1.26	8.57	8.55	2.13E-05	0.001175007
90	Cfp	complement factor properdin	1.25	13.35	8.51	2.22E-05	0.001185616
91	Plp2	proteolipid protein 2	1.24	10.88	5.00	0.000937488	0.013156142
92	Blm	Bloom syndrome, RecQ helicase-like	1.23	10.01	10.89	3.33E-06	0.000389533
93	Slc29a3	solute carrier family 29 (nucleoside transporters), member 3	1.23	9.84	11.50	2.17E-06	0.000322971
94	Neo1	neogenin	1.23	8.29	5.97	0.00028734	0.006114237
95	Gpr34	G protein-coupled receptor 34	1.23	8.84	3.81	0.004793627	0.038587517
96	Lysmd2	LysM, putative peptidoglycan-binding, domain containing 2	1.22	8.80	9.68	8.32E-06	0.000643485
97	Optn	optineurin	1.22	8.75	8.54	2.16E-05	0.001177646
98	D10Wsu102e	DNA segment, Chr 10, Wayne State University 102, expressed	1.22	9.14	10.67	3.91E-06	0.000397578
99	Igsf9	immunoglobulin superfamily, member 9	1.22	8.76	8.60	2.06E-05	0.001143744
100	Hmgn3	high mobility group nucleosomal binding domain 3	1.21	11.16	4.63	0.001537694	0.018154491
101	Man1a	mannosidase 1, alpha	1.21	9.81	7.55	5.42E-05	0.002069558
102	Robo1	roundabout homolog 1 (Drosophila)	1.21	8.23	5.95	0.000295706	0.006190469
103	Hmgn3	high mobility group nucleosomal binding domain 3	1.21	11.28	3.69	0.005709615	0.043452096
104	Apoe	apolipoprotein E	1.20	9.14	5.38	0.000582464	0.009505843
105	Oosp1	oocyte secreted protein 1	1.20	10.03	3.57	0.006900257	0.049987767
106	Nsf	N-ethylmaleimide sensitive fusion protein	1.20	10.71	10.25	5.36E-06	0.0005051
107	Rap1gap2	RAP1 GTPase activating protein 2	1.19	9.90	5.20	0.000724814	0.011044987
108	Camk2d	calcium/calmodulin-dependent protein kinase II, delta	1.19	9.22	8.84	1.66E-05	0.001025724
109	Il10	interleukin 10	1.19	8.19	11.29	2.51E-06	0.000352016
110	Ticam2	toll-like receptor adaptor molecule 2	1.19	8.59	14.17	4.14E-07	0.000126365
111	NA	NA	1.18	8.92	7.95	3.69E-05	0.001646747
112	Sgk3	serum/glucocorticoid regulated kinase 3	1.18	9.77	14.40	3.65E-07	0.000126365
113	Tcstv1	2-cell-stage, variable group, member 1	1.18	8.27	4.56	0.001684377	0.019177893
114	Rilpl2	Rab interacting lysosomal protein-like 2	1.18	10.26	4.87	0.001106368	0.014432138
115	Dnase1l3	deoxyribonuclease 1-like 3	1.17	9.18	7.37	6.46E-05	0.002318592
116	Suclg2	succinate-Coenzyme A ligase, GDP-forming, beta subunit	1.17	10.23	3.86	0.004475495	0.036934103
117	NA	NA	1.17	9.63	4.38	0.002134617	0.022487262
118	Ivns1abp	influenza virus NS1A binding protein	1.16	10.99	5.23	0.000701269	0.010805477
119	Rtn4ip1	reticulon 4 interacting protein 1	1.16	8.44	8.02	3.46E-05	0.001575493
120	Gpm6a	glycoprotein m6a	1.16	8.19	13.33	6.77E-07	0.000167465
121	Tmem154	transmembrane protein 154	1.15	10.03	16.07	1.50E-07	8.74E-05
122	Cd59b	CD59b antigen	1.15	8.70	5.23	0.0007025	0.010805477
123	NA	NA	1.14	8.20	11.11	2.86E-06	0.000365287
124	Tbc1d5	TBC1 domain family, member 5	1.13	10.54	7.82	4.17E-05	0.00179651
125	Lrrc8a	leucine rich repeat containing 8A	1.13	8.66	5.80	0.000350171	0.006880043
126	Optn	optineurin	1.12	8.65	7.60	5.16E-05	0.002011249
127	Timd2	T cell immunoglobulin and mucin domain containing 2	1.12	8.33	8.74	1.82E-05	0.001056366
128	Cobll1	Cobl-like 1	1.11	9.58	7.97	3.61E-05	0.001626306

129	Gbp3	guanylate binding protein 3	1.10	11.00	7.46	5.93E-05	0.002191762
130	Ftsjd2	FtsJ methyltransferase domain containing 2	1.10	12.78	7.16	7.94E-05	0.002664622
131	Kctd12	potassium channel tetramerisation domain containing 12	1.10	9.91	7.79	4.30E-05	0.00181213
132	Chrnb1	cholinergic receptor, nicotinic, beta polypeptide 1 (muscle)	1.09	8.73	7.03	9.11E-05	0.002946967
133	Psmd14	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	1.09	10.25	8.53	2.17E-05	0.001177646
134	Irak2	interleukin-1 receptor-associated kinase 2	1.09	10.08	12.03	1.52E-06	0.000258979
135	Fcho2	FCH domain only 2	1.08	11.25	9.05	1.40E-05	0.000906822
136	Tnfsf4	tumor necrosis factor (ligand) superfamily, member 4	1.07	8.38	5.71	0.000389426	0.007374903
137	Hsp90ab1	heat shock protein 90 alpha (cytosolic), class B member 1	1.07	11.52	6.39	0.000179522	0.004550005
138	Fut8	fucosyltransferase 8	1.06	8.78	7.94	3.74E-05	0.001653635
139	Ryk	receptor-like tyrosine kinase	1.06	9.24	7.63	5.01E-05	0.001984949
140	Myadm	myeloid-associated differentiation marker	1.05	10.34	6.12	0.000242092	0.005562299
141	Tgif1	TGFB-induced factor homeobox 1	1.05	9.59	7.81	4.20E-05	0.001800796
142	Cobll1	Cobl-like 1	1.05	9.11	10.81	3.53E-06	0.00039538
143	Il15	interleukin 15	1.05	8.97	3.89	0.004302602	0.036006133
144	Ints4	integrator complex subunit 4	1.04	11.45	13.91	4.82E-07	0.000130244
145	Camk2n1	calcium/calmodulin-dependent protein kinase II inhibitor 1	1.04	8.74	4.50	0.001832528	0.02028317
146	Fert2	fer (fms/fps related) protein kinase, testis specific 2	1.04	8.04	13.68	5.50E-07	0.000144109
147	Gbp2	guanylate binding protein 2	1.03	8.47	7.78	4.32E-05	0.00181213
148	Aifm2	apoptosis-inducing factor, mitochondrion-associated 2	1.03	8.85	8.25	2.80E-05	0.00138606
149	F9	coagulation factor IX	1.03	8.67	9.68	8.30E-06	0.000643485
150	Ppfibp2	PTPRF interacting protein, binding protein 2 (liprin beta 2)	1.03	8.92	7.87	3.98E-05	0.001737923
151	Marcks	myristoylated alanine rich protein kinase C substrate	1.03	11.22	6.39	0.000179412	0.004550005
152	Sh3bgrl2	SH3 domain binding glutamic acid-rich protein like 2	1.03	8.48	4.36	0.002212342	0.023065033
153	Rab31	RAB31, member RAS oncogene family	1.02	10.80	4.78	0.001244962	0.015627613
154	Unc119	unc-119 homolog (C. elegans)	1.02	9.44	5.65	0.000422166	0.007726939
155	Cbfa2t3	core-binding factor, runt domain, alpha subunit 2, translocated to, 3 (human)	1.01	9.74	6.72	0.000125996	0.003600003
156	Ly96	lymphocyte antigen 96	1.01	9.80	5.08	0.000853268	0.012383256
157	NA	NA	1.01	10.56	8.60	2.06E-05	0.001143744
158	Irak2	interleukin-1 receptor-associated kinase 2	1.01	9.42	12.27	1.30E-06	0.000236954
159	Cyp51	cytochrome P450, family 51	1.01	9.19	5.56	0.000465437	0.008365629
160	Cyb5r2	cytochrome b5 reductase 2	1.00	8.00	4.16	0.002895417	0.027587193
161	Tmem38b	transmembrane protein 38B	-1.00	8.78	-8.49	2.25E-05	0.001195989
162	Sit1	suppression inducing transmembrane adaptor 1	-1.01	8.65	-8.52	2.20E-05	0.001183798
163	Fam117a	family with sequence similarity 117, member A	-1.02	10.61	-4.07	0.003303844	0.030200374
164	Abhd8	abhydrolase domain containing 8	-1.02	8.87	-9.44	1.01E-05	0.00074318
165	Rgcc	regulator of cell cycle	-1.02	9.66	-4.43	0.001991212	0.021459569
166	P2ry6	pyrimidinergic receptor P2Y, G-protein coupled, 6	-1.03	8.68	-6.88	0.000106007	0.003202773
167	Cyp27a1	cytochrome P450, family 27, subfamily a, polypeptide 1	-1.03	8.45	-6.08	0.000253691	0.005670363
168	Fcgrt	Fc receptor, IgG, alpha chain transporter	-1.03	9.14	-4.21	0.002699974	0.026333605
169	Rnf144a	ring finger protein 144A	-1.03	8.65	-6.75	0.000121862	0.003541023
170	Gm5483	predicted gene 5483	-1.04	8.51	-4.12	0.003099077	0.028885777
171	Gata3	GATA binding protein 3	-1.04	8.38	-12.33	1.26E-06	0.00023571
172	Ddit4	DNA-damage-inducible transcript 4	-1.04	10.01	-5.42	0.000556829	0.009225337

173	Mgst1	microsomal glutathione S-transferase 1	-1.05	8.54	-3.57	0.006872263	0.049887542
174	Nrp1	neuropilin 1	-1.05	8.57	-6.12	0.000243368	0.005575765
175	Frat2	frequently rearranged in advanced T cell lymphomas 2	-1.05	8.43	-11.89	1.67E-06	0.000270244
176	Brwd1	bromodomain and WD repeat domain containing 1	-1.05	9.29	-3.86	0.004465454	0.036920991
177	P2ry14	purinergic receptor P2Y, G-protein coupled, 14	-1.05	8.76	-9.39	1.05E-05	0.000754882
178	Gmfg	glia maturation factor, gamma	-1.06	11.65	-4.91	0.001051831	0.013994156
179	Akap12	A kinase (PRKA) anchor protein (gravin) 12	-1.06	8.18	-7.97	3.64E-05	0.001633462
180	Gpr83	G protein-coupled receptor 83	-1.06	8.47	-9.72	8.06E-06	0.000643485
181	Cd27	CD27 antigen	-1.06	8.66	-12.95	8.50E-07	0.000191956
182	Apol7c	apolipoprotein L 7c	-1.06	8.64	-6.23	0.000214469	0.005176085
183	Socs3	suppressor of cytokine signaling 3	-1.06	10.41	-6.32	0.00019337	0.004772354
184	Serpinb6a	serine (or cysteine) peptidase inhibitor, clade B, member 6a	-1.07	9.46	-8.26	2.78E-05	0.00138606
185	Hpgd	hydroxyprostaglandin dehydrogenase 15 (NAD)	-1.07	8.13	-5.49	0.000510547	0.008795466
186	Xdh	xanthine dehydrogenase	-1.07	8.70	-9.00	1.45E-05	0.000923744
187	Xcl1	chemokine (C motif) ligand 1	-1.07	8.29	-13.93	4.75E-07	0.000130244
188	Zap70	zeta-chain (TCR) associated protein kinase	-1.08	8.76	-7.71	4.62E-05	0.001888593
189	Lrig1	leucine-rich repeats and immunoglobulin-like domains 1	-1.08	8.39	-10.76	3.66E-06	0.00039567
190	Klhl6	kelch-like 6	-1.08	12.72	-4.80	0.001218916	0.015444675
191	Pilrb1	paired immunoglobin-like type 2 receptor beta 1	-1.08	8.38	-9.71	8.15E-06	0.000643485
192	Klra7	killer cell lectin-like receptor, subfamily A, member 7	-1.09	8.24	-6.95	9.86E-05	0.003077668
193	Stat4	signal transducer and activator of transcription 4	-1.09	8.58	-7.78	4.35E-05	0.001817277
194	Nsg2	neuron specific gene family member 2	-1.09	8.21	-5.72	0.000388558	0.007374903
195	Sepp1	selenoprotein P, plasma, 1	-1.09	9.20	-7.46	5.92E-05	0.002191762
196	Prkcq	protein kinase C, theta	-1.09	8.51	-9.26	1.17E-05	0.000820031
197	Gstk1	glutathione S-transferase kappa 1	-1.09	8.52	-10.55	4.26E-06	0.000429447
198	Arrdc4	arrestin domain containing 4	-1.09	8.62	-9.97	6.64E-06	0.000572834
199	Stard10	START domain containing 10	-1.10	9.46	-5.06	0.000867359	0.012512813
200	Fam189b	family with sequence similarity 189, member B	-1.10	8.64	-5.67	0.000407926	0.007601808
201	Acss2	acyl-CoA synthetase short-chain family member 2	-1.10	9.26	-4.55	0.001709577	0.019280562
202	Tnfaip8l2	tumor necrosis factor, alpha-induced protein 8-like 2	-1.10	9.45	-14.09	4.34E-07	0.000126365
203	Rab3d	RAB3D, member RAS oncogene family	-1.10	9.20	-6.95	9.83E-05	0.003077254
204	Lpl	lipoprotein lipase	-1.10	8.47	-7.03	9.09E-05	0.002946967
205	Smad1	SMAD family member 1	-1.10	8.66	-6.20	0.000221278	0.005282699
206	Cdk5r1	cyclin-dependent kinase 5, regulatory subunit 1 (p35)	-1.11	9.32	-4.76	0.001283768	0.015886479
207	Apobr	apolipoprotein B receptor	-1.11	8.51	-6.82	0.00011301	0.003305794
208	Nrn1	neuritin 1	-1.11	8.48	-5.92	0.000306885	0.006333433
209	Cd14	CD14 antigen	-1.12	8.42	-7.59	5.21E-05	0.002014325
210	Clec4b1	C-type lectin domain family 4, member b1	-1.12	8.45	-7.25	7.28E-05	0.00250334
211	Smad1	SMAD family member 1	-1.13	8.63	-8.82	1.70E-05	0.001044514
212	Slc11a1	solute carrier family 11 (proton-coupled divalent metal ion transporters), membe	-1.13	8.94	-5.50	0.000504008	0.008759984
213	Prkd3	protein kinase D3	-1.13	8.79	-5.06	0.000872332	0.012548754
214	Krt10	keratin 10	-1.13	8.55	-14.30	3.85E-07	0.000126365
215	Vopp1	vesicular, overexpressed in cancer, prosurvival protein 1	-1.13	9.30	-5.65	0.000419539	0.007700368
216	Nkg7	natural killer cell group 7 sequence	-1.14	11.33	-4.16	0.002918159	0.027732032

217	Tcf7	transcription factor 7, T cell specific	-1.14	8.18	-5.88	0.000321124	0.00652455
218	Klk1	kallikrein 1	-1.14	8.12	-4.59	0.001608869	0.018579935
219	Hcst	hematopoietic cell signal transducer	-1.15	10.76	-5.30	0.00064044	0.010185633
220	Lck	lymphocyte protein tyrosine kinase	-1.15	11.14	-5.56	0.000467913	0.008365629
221	LOC547323	uncharacterized LOC547323	-1.15	8.57	-12.71	9.89E-07	0.000212413
222	NA	NA	-1.16	8.67	-10.00	6.49E-06	0.000571
223	Tmem66	transmembrane protein 66	-1.16	9.48	-7.35	6.56E-05	0.002342512
224	Pygl	liver glycogen phosphorylase	-1.17	9.54	-3.58	0.006723284	0.049085591
225	Cst3	cystatin C	-1.17	12.82	-6.51	0.000156878	0.004170149
226	Kmo	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	-1.17	8.51	-7.15	8.07E-05	0.002684506
227	Ear10	eosinophil-associated, ribonuclease A family, member 10	-1.18	8.48	-7.67	4.80E-05	0.001904754
228	Hist1h1c	histone cluster 1, H1c	-1.18	10.21	-10.20	5.56E-06	0.000513086
229	Acpl2	acid phosphatase-like 2	-1.18	8.76	-7.89	3.91E-05	0.001713178
230	Pi16	peptidase inhibitor 16	-1.19	8.55	-6.92	0.000102002	0.003130533
231	NA	NA	-1.20	8.87	-15.10	2.49E-07	0.000112734
232	Prkd3	protein kinase D3	-1.21	9.48	-4.03	0.003499375	0.031290611
233	Nrp1	neuropilin 1	-1.21	8.85	-5.06	0.000867924	0.012512813
234	Anxa1	annexin A1	-1.21	8.46	-4.22	0.002662353	0.026134882
235	Il27ra	interleukin 27 receptor, alpha	-1.22	11.04	-9.74	7.94E-06	0.000643485
236	Selplg	selectin, platelet (p-selectin) ligand	-1.22	10.59	-7.42	6.13E-05	0.002226548
237	Lpar6	lysophosphatidic acid receptor 6	-1.22	10.65	-12.22	1.35E-06	0.000242138
238	Ppic	peptidylprolyl isomerase C	-1.23	8.33	-4.05	0.003389681	0.030741707
239	Sh2d2a	SH2 domain protein 2A	-1.23	10.75	-5.55	0.000476	0.008464011
240	Ear12	eosinophil-associated, ribonuclease A family, member 12	-1.23	8.60	-10.75	3.69E-06	0.00039567
241	Hvcn1	hydrogen voltage-gated channel 1	-1.24	12.02	-5.22	0.000712795	0.010897018
242	Sun2	Sad1 and UNC84 domain containing 2	-1.25	11.74	-10.93	3.24E-06	0.000384201
243	Fyb	FYN binding protein	-1.25	9.43	-9.69	8.24E-06	0.000643485
244	Fxyd5	FXYD domain-containing ion transport regulator 5	-1.25	10.04	-7.25	7.25E-05	0.00250152
245	Lrrk2	leucine-rich repeat kinase 2	-1.26	9.33	-3.86	0.004483801	0.036956105
246	Neurl3	neuralized homolog 3 homolog (Drosophila)	-1.27	8.57	-9.06	1.38E-05	0.000901477
247	NA	NA	-1.28	9.30	-14.09	4.34E-07	0.000126365
248	Sit1	suppression inducing transmembrane adaptor 1	-1.28	8.95	-10.25	5.34E-06	0.0005051
249	Foxp3	forkhead box P3	-1.28	8.88	-11.28	2.52E-06	0.000352016
250	Klf13	Kruppel-like factor 13	-1.29	10.80	-7.22	7.52E-05	0.002573373
251	Cd160	CD160 antigen	-1.29	8.72	-8.53	2.17E-05	0.001177646
252	Fas	Fas (TNF receptor superfamily member 6)	-1.29	8.97	-9.43	1.02E-05	0.00074318
253	Ifngr1	interferon gamma receptor 1	-1.29	9.89	-7.94	3.72E-05	0.001650647
254	Fam78a	family with sequence similarity 78, member A	-1.30	11.06	-6.07	0.000258382	0.005742531
255	Osm	oncostatin M	-1.30	9.04	-17.16	8.87E-08	7.20E-05
256	Asb2	ankyrin repeat and SOCS box-containing 2	-1.30	9.77	-7.78	4.33E-05	0.00181213
257	Tyrobp	TYRO protein tyrosine kinase binding protein	-1.31	9.68	-8.30	2.69E-05	0.001355341
258	Casp1	caspase 1	-1.32	11.79	-5.17	0.000758776	0.01139033
259	Bcl7a	B cell CLL/lymphoma 7A	-1.32	9.92	-9.88	7.12E-06	0.000602323
260	Cd247	CD247 antigen	-1.32	9.05	-12.17	1.39E-06	0.0002457

261	Zfp36	zinc finger protein 36	-1.33	11.52	-10.17	5.67E-06	0.000519334
262	Myo1f	myosin IF	-1.33	9.89	-6.64	0.000137512	0.003809939
263	Gpr68	G protein-coupled receptor 68	-1.34	8.89	-12.97	8.39E-07	0.000191956
264	Ephx1	epoxide hydrolase 1, microsomal	-1.34	10.81	-8.48	2.28E-05	0.001199946
265	Tacstd2	tumor-associated calcium signal transducer 2	-1.35	8.54	-15.12	2.46E-07	0.000112734
266	Itgad	integrin, alpha D	-1.35	8.45	-6.40	0.000178721	0.004550005
267	Tcf7	transcription factor 7, T cell specific	-1.36	8.57	-6.99	9.46E-05	0.003022162
268	Rgs10	regulator of G-protein signalling 10	-1.36	9.95	-13.03	8.11E-07	0.000189903
269	Sep9	septin 9	-1.37	8.95	-8.86	1.63E-05	0.001013828
270	Cd8b1	CD8 antigen, beta chain 1	-1.37	9.16	-3.66	0.005982465	0.045057589
271	Sema4a	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and s	-1.37	10.37	-9.41	1.04E-05	0.000750419
272	Csf3r	colony stimulating factor 3 receptor (granulocyte)	-1.37	8.57	-10.39	4.81E-06	0.00047416
273	F13a1	coagulation factor XIII, A1 subunit	-1.37	8.55	-6.71	0.000126752	0.003600003
274	Tmem51	transmembrane protein 51	-1.38	9.54	-5.82	0.000341886	0.006791276
275	Gpr171	G protein-coupled receptor 171	-1.38	9.82	-17.78	6.65E-08	7.20E-05
276	Glipr2	GLI pathogenesis-related 2	-1.38	10.42	-8.98	1.48E-05	0.000927095
277	Csrp2	cysteine and glycine-rich protein 2	-1.39	8.74	-14.65	3.17E-07	0.000126365
278	Dusp2	dual specificity phosphatase 2	-1.39	9.31	-9.72	8.02E-06	0.000643485
279	Sostdc1	sclerostin domain containing 1	-1.41	8.64	-4.41	0.002053933	0.021870864
280	Ccr6	chemokine (C-C motif) receptor 6	-1.42	9.02	-6.59	0.000144894	0.003946706
281	Igfbp4	insulin-like growth factor binding protein 4	-1.42	8.50	-5.22	0.000711049	0.010897018
282	Sort1	sortilin 1	-1.42	9.14	-9.58	8.99E-06	0.000680709
283	Fpr2	formyl peptide receptor 2	-1.43	11.21	-4.16	0.002929021	0.027754753
284	Fxyd5	FXYD domain-containing ion transport regulator 5	-1.44	11.88	-7.42	6.12E-05	0.002226548
285	Klra4	killer cell lectin-like receptor, subfamily A, member 4	-1.45	8.77	-14.12	4.26E-07	0.000126365
286	Cmc1	COX assembly mitochondrial protein 1	-1.45	10.24	-8.41	2.43E-05	0.001241645
287	Myl4	myosin, light polypeptide 4	-1.45	10.47	-6.37	0.000183946	0.004612847
288	Dennd3	DENN/MADD domain containing 3	-1.46	10.21	-3.97	0.003801274	0.033188335
289	Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1	-1.46	9.49	-14.72	3.06E-07	0.000126365
290	Csf3r	colony stimulating factor 3 receptor (granulocyte)	-1.47	8.43	-17.60	7.20E-08	7.20E-05
291	F2r	coagulation factor II (thrombin) receptor	-1.48	10.49	-7.14	8.10E-05	0.002688196
292	Fxyd5	FXYD domain-containing ion transport regulator 5	-1.48	10.46	-9.09	1.34E-05	0.000886427
293	Klre1	killer cell lectin-like receptor family E member 1	-1.48	8.64	-9.52	9.47E-06	0.000711445
294	Trib2	tribbles homolog 2 (Drosophila)	-1.50	9.82	-8.80	1.72E-05	0.001046831
295	Neurl3	neuralized homolog 3 homolog (Drosophila)	-1.50	8.63	-8.74	1.81E-05	0.001056366
296	Ctnna1	catenin (cadherin associated protein), alpha 1	-1.51	9.75	-15.87	1.67E-07	8.74E-05
297	Bcl11b	B cell leukemia/lymphoma 11B	-1.52	10.28	-5.81	0.000345715	0.006826447
298	Tmem108	transmembrane protein 108	-1.52	8.48	-10.23	5.44E-06	0.000509109
299	Cyp27a1	cytochrome P450, family 27, subfamily a, polypeptide 1	-1.52	9.00	-10.72	3.77E-06	0.00039567
300	Csf1r	colony stimulating factor 1 receptor	-1.52	9.68	-4.30	0.002397836	0.024405413
301	Gpc1	glypican 1	-1.52	9.28	-10.92	3.25E-06	0.000384201
302	Fcer2a	Fc receptor, IgE, low affinity II, alpha polypeptide	-1.55	8.61	-7.02	9.20E-05	0.002950886
303	Gcnt2	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	-1.55	9.28	-9.91	6.92E-06	0.000592709
304	Zap70	zeta-chain (TCR) associated protein kinase	-1.56	10.13	-12.46	1.15E-06	0.000226433

305	Chst1	carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	-1.56	9.18	-4.94	0.001016143	0.013686077
306	Hvcn1	hydrogen voltage-gated channel 1	-1.56	11.55	-9.26	1.16E-05	0.000820031
307	Cdc42ep3	CDC42 effector protein (Rho GTPase binding) 3	-1.57	9.86	-10.46	4.57E-06	0.000453629
308	Cxcr3	chemokine (C-X-C motif) receptor 3	-1.59	9.84	-8.07	3.31E-05	0.001539334
309	Leprotl1	leptin receptor overlapping transcript-like 1	-1.61	10.19	-12.28	1.30E-06	0.000236954
310	Mmp9	matrix metallopeptidase 9	-1.61	9.03	-4.50	0.001814258	0.020131968
311	Gpr114	G protein-coupled receptor 114	-1.61	9.43	-7.29	6.97E-05	0.002424401
312	Ffar2	free fatty acid receptor 2	-1.61	9.67	-4.83	0.001168508	0.015027774
313	Pacsin1	protein kinase C and casein kinase substrate in neurons 1	-1.62	9.02	-7.53	5.52E-05	0.002097544
314	Lyz1	lysozyme 1	-1.62	14.55	-9.75	7.87E-06	0.000643485
315	Clec7a	C-type lectin domain family 7, member a	-1.62	8.95	-14.82	2.89E-07	0.000126365
316	Clec4d	C-type lectin domain family 4, member d	-1.64	8.80	-7.18	7.80E-05	0.002639989
317	Ngfrap1	nerve growth factor receptor (TNFRSF16) associated protein 1	-1.64	9.86	-8.08	3.27E-05	0.001539334
318	Rab32	RAB32, member RAS oncogene family	-1.64	9.66	-10.97	3.15E-06	0.000382174
319	Mt1	metallothionein 1	-1.65	10.74	-4.34	0.002276487	0.023602341
320	Cyp27a1	cytochrome P450, family 27, subfamily a, polypeptide 1	-1.65	8.74	-9.01	1.44E-05	0.00092333
321	Sgk1	serum/glucocorticoid regulated kinase 1	-1.65	10.28	-7.75	4.46E-05	0.001845096
322	Lgmn	legumain	-1.65	10.49	-7.38	6.39E-05	0.002306549
323	Itk	IL2 inducible T cell kinase	-1.66	9.64	-7.55	5.41E-05	0.002069558
324	Tmem66	transmembrane protein 66	-1.66	11.10	-6.92	0.000101909	0.003130533
325	Sort1	sortilin 1	-1.66	9.30	-10.09	6.06E-06	0.000540025
326	Tmem66	transmembrane protein 66	-1.67	11.01	-4.80	0.001212497	0.015397073
327	Arap3	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3	-1.67	9.00	-8.54	2.17E-05	0.001177646
328	Ramp1	receptor (calcitonin) activity modifying protein 1	-1.69	8.75	-7.93	3.77E-05	0.001662267
329	St6galnac2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosamini	-1.69	9.92	-13.38	6.54E-07	0.000164898
330	Pglyrp1	peptidoglycan recognition protein 1	-1.69	10.42	-4.91	0.001058114	0.014063476
331	Serpinb1a	serine (or cysteine) peptidase inhibitor, clade B, member 1a	-1.70	9.57	-9.08	1.35E-05	0.000886427
332	Ifitm3	interferon induced transmembrane protein 3	-1.72	11.17	-6.49	0.000160495	0.004214998
333	Hp	haptoglobin	-1.72	9.14	-4.28	0.002457471	0.024849662
334	Lmo2	LIM domain only 2	-1.72	11.85	-14.32	3.80E-07	0.000126365
335	Cd3e	CD3 antigen, epsilon polypeptide	-1.74	10.70	-10.78	3.61E-06	0.00039567
336	Tgfbi	transforming growth factor, beta induced	-1.74	9.71	-11.93	1.62E-06	0.000270244
337	Xlr4a	X-linked lymphocyte-regulated 4A	-1.74	11.46	-12.47	1.15E-06	0.000226433
338	Dok2	docking protein 2	-1.79	9.77	-7.16	7.95E-05	0.002664622
339	Zfp608	zinc finger protein 608	-1.79	8.70	-9.44	1.01E-05	0.00074318
340	Cd6	CD6 antigen	-1.80	9.99	-13.13	7.62E-07	0.00018156
341	NA	NA	-1.80	9.99	-8.21	2.90E-05	0.001422116
342	Tnfrsf4	tumor necrosis factor receptor superfamily, member 4	-1.81	10.02	-7.35	6.59E-05	0.002347302
343	Zyx	zyxin	-1.81	12.54	-12.12	1.44E-06	0.000248712
344	Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1	-1.82	9.90	-18.01	5.98E-08	7.20E-05
345	Dusp2	dual specificity phosphatase 2	-1.82	10.38	-11.63	2.00E-06	0.000307633
346	Dgka	diacylglycerol kinase, alpha	-1.82	10.89	-7.18	7.82E-05	0.002642851
347	Lat	linker for activation of T cells	-1.83	10.61	-16.23	1.39E-07	8.74E-05
348	Tiam1	T cell lymphoma invasion and metastasis 1	-1.83	9.29	-10.33	5.03E-06	0.000491624

349	Trf	transferrin	-1.85	9.00	-6.84	0.000111008	0.003276496
350	NA	NA	-1.87	8.91	-8.06	3.35E-05	0.001544265
351	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	-1.90	10.08	-9.22	1.21E-05	0.000832985
352	Bcl6	B cell leukemia/lymphoma 6	-1.91	10.44	-8.08	3.29E-05	0.001539334
353	Dgka	diacylglycerol kinase, alpha	-1.91	11.54	-6.59	0.000144058	0.003943894
354	Il18r1	interleukin 18 receptor 1	-1.93	9.89	-17.37	8.04E-08	7.20E-05
355	C1qc	complement component 1, q subcomponent, C chain	-1.93	9.22	-11.44	2.27E-06	0.000333942
356	Fcgr3	Fc receptor, IgG, low affinity III	-1.93	9.07	-5.93	0.000302045	0.006253232
357	Cd6	CD6 antigen	-1.94	10.43	-11.40	2.32E-06	0.000338092
358	Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1	-1.96	10.48	-23.34	7.23E-09	1.90E-05
359	Il7r	interleukin 7 receptor	-1.96	10.42	-9.99	6.53E-06	0.000571
360	Cd3g	CD3 antigen, gamma polypeptide	-1.98	11.13	-11.02	3.05E-06	0.000376611
361	Pxdc1	PX domain containing 1	-1.98	9.32	-9.14	1.29E-05	0.000864652
362	Tmem176b	transmembrane protein 176B	-2.00	9.68	-12.43	1.17E-06	0.000226433
363	Cd8b1	CD8 antigen, beta chain 1	-2.02	12.18	-5.97	0.000289485	0.006114237
364	Igfbp4	insulin-like growth factor binding protein 4	-2.04	8.88	-7.02	9.21E-05	0.002950886
365	Cd6	CD6 antigen	-2.05	10.96	-9.43	1.02E-05	0.00074318
366	Emb	embigin	-2.06	9.96	-15.10	2.49E-07	0.000112734
367	Ear4	eosinophil-associated, ribonuclease A family, member 4	-2.07	9.25	-8.97	1.48E-05	0.000927095
368	Fcer2a	Fc receptor, IgE, low affinity II, alpha polypeptide	-2.07	9.04	-9.09	1.35E-05	0.000886427
369	Cd27	CD27 antigen	-2.08	11.11	-17.27	8.41E-08	7.20E-05
370	Klk8	kallikrein related-peptidase 8	-2.09	9.80	-8.60	2.05E-05	0.001143744
371	Ifitm6	interferon induced transmembrane protein 6	-2.10	9.77	-5.07	0.000859724	0.012443619
372	C1qb	complement component 1, q subcomponent, beta polypeptide	-2.10	9.58	-11.12	2.82E-06	0.000365287
373	Lyz2	lysozyme 2	-2.12	12.10	-15.93	1.62E-07	8.74E-05
374	Ly6c1	lymphocyte antigen 6 complex, locus C1	-2.14	10.49	-6.17	0.000228945	0.005376928
375	Lgals3	lectin, galactose binding, soluble 3	-2.15	11.95	-11.17	2.73E-06	0.000364587
376	Stat4	signal transducer and activator of transcription 4	-2.17	10.16	-10.82	3.52E-06	0.00039538
377	B3gnt8	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8	-2.17	10.18	-14.13	4.23E-07	0.000126365
378	Cd3d	CD3 antigen, delta polypeptide	-2.18	10.96	-13.89	4.87E-07	0.000130244
379	Sirpb1a	signal-regulatory protein beta 1A	-2.19	9.88	-16.08	1.50E-07	8.74E-05
380	Ccr6	chemokine (C-C motif) receptor 6	-2.19	10.34	-5.75	0.000374546	0.007176066
381	Il4i1	interleukin 4 induced 1	-2.22	11.72	-7.69	4.75E-05	0.00190209
382	Thy1	thymus cell antigen 1, theta	-2.24	10.76	-10.21	5.49E-06	0.000510469
383	Ifitm2	interferon induced transmembrane protein 2	-2.25	10.80	-10.10	6.01E-06	0.000539159
384	Ccl9	chemokine (C-C motif) ligand 9	-2.27	9.39	-13.17	7.42E-07	0.000180048
385	Dpp4	dipeptidylpeptidase 4	-2.28	10.18	-11.69	1.91E-06	0.000303211
386	Ctsw	cathepsin W	-2.28	10.26	-14.53	3.40E-07	0.000126365
387	Klrd1	killer cell lectin-like receptor, subfamily D, member 1	-2.29	9.84	-10.70	3.83E-06	0.00039567
388	Il1b	interleukin 1 beta	-2.31	9.62	-12.15	1.41E-06	0.000246908
389	Axl	AXL receptor tyrosine kinase	-2.33	9.65	-9.10	1.33E-05	0.000885286
390	Lyz2	lysozyme 2	-2.33	13.02	-10.27	5.28E-06	0.0005051
391	Hp	haptoglobin	-2.34	9.83	-5.82	0.000344727	0.006824249
392	S100a9	S100 calcium binding protein A9 (calgranulin B)	-2.35	12.85	-4.01	0.003592089	0.031897675

393	Ear2	eosinophil-associated, ribonuclease A family, member 2	-2.44	9.87	-10.97	3.14E-06	0.000382174
394	Prg2	proteoglycan 2, bone marrow	-2.50	9.43	-26.42	2.61E-09	1.14E-05
395	Vcam1	vascular cell adhesion molecule 1	-2.53	9.44	-10.93	3.24E-06	0.000384201
396	Alox5ap	arachidonate 5-lipoxygenase activating protein	-2.53	10.63	-12.50	1.12E-06	0.000226433
397	Chchd10	coiled-coil-helix-coiled-coil-helix domain containing 10	-2.60	11.67	-23.74	6.28E-09	1.90E-05
398	Chi3l3	chitinase 3-like 3	-2.63	10.46	-3.92	0.004099802	0.034940263
399	Fcna	ficolin A	-2.74	9.51	-14.52	3.40E-07	0.000126365
400	Fcer2a	Fc receptor, IgE, low affinity II, alpha polypeptide	-2.90	9.99	-9.64	8.61E-06	0.000659489
401	Satb1	special AT-rich sequence binding protein 1	-2.92	11.26	-9.26	1.17E-05	0.000820031
402	Slc40a1	solute carrier family 40 (iron-regulated transporter), member 1	-2.98	10.90	-16.03	1.54E-07	8.74E-05
403	Vpreb3	pre-B lymphocyte gene 3	-3.59	10.07	-26.45	2.58E-09	1.14E-05
404	Slpi	secretory leukocyte peptidase inhibitor	-3.67	11.58	-6.89	0.000105045	0.003186622

Column headings:

LogFC: Log2-fold of change between TRAF3-/- B lymphomas and LMC spleens

AveExpr: average expression level for both conditions (LMC and TRAF3-/-)

t: the t-statistic

P. Value: the p value based on the t-statistic

Adj. P. Val.: the p value adjusted for multiple measurements (essentially the false discovery rate)

The mRNA expression profiles of splenocytes from LMC and tumor-bearing B-TRAF3-/- mice (mouse ID: 6983-2, 7041-10, and 7060-8) were analyzed by a microarray analysis. cRNA was hybridized to Illumina Sentrix Mouse Whole Genome 24K Microarray (Illumina). We determined two group comparisons of the normalized data for triplicate samples using paired t tests and false discovery rate. Genes listed include 160 up-regulated and 244 down-regulated genes (fold of change: >2).

Supplementary Table 2. The MCC-interactome in human MM cells identified by affinity purification followed by LC-MS/MS

#	Accession	Description	SwissProt ID	Average spectral count difference (hMCC-SBP-6xHis - FLAG-hMCC)		Previously known MCC-interactor
				Mitochondria	Whole lysates	
NA	P23508	Colorectal mutant cancer protein	CRCM_HUMAN	415	1223	Not applicable
1	P23508-2	Isoform 2 of Colorectal mutant cancer protein	CRCM_HUMAN	395.5	1172.5	No
2	P09874	Poly [ADP-ribose] polymerase 1	PARP1_HUMAN	72	43.5	No
3	O15020-2	Isoform 2 of Spectrin beta chain, non-erythrocytic 2	SPTN2_HUMAN	34	3	No
4	P40939	Trifunctional enzyme subunit alpha, mitochondrial	ECHA_HUMAN	31	24	No
5	P05141	ADP/ATP translocase 2	ADT2_HUMAN	24	33.5	No
6	P12236	ADP/ATP translocase 3	ADT3_HUMAN	20.5	27.5	No
7	P06576	ATP synthase subunit beta, mitochondrial	ATPB_HUMAN	18.5	19.5	No
8	Q99623	Prohibitin-2	PHB2_HUMAN	17	23.5	Yes (Ewing, 2007)
9	Q13576	Ras GTPase-activating-like protein IQGAP2	IQGA2_HUMAN	16	50	No
10	P12235	ADP/ATP translocase 1	ADT1_HUMAN	15.5	24	No
11	Q9Y277	Voltage-dependent anion-selective channel protein 3	VDAC3_HUMAN	15.5	2.5	No
12	Q02880-2	Isoform Beta-1 of DNA topoisomerase 2-beta	TOP2B_HUMAN	14.5	12.5	No
13	O60264	SWI/SNF-related matrix-associated actin-dependent regulator	SMCA5_HUMAN	13.5	2	No
14	P35232	Prohibitin	PHB_HUMAN	13	3.5	No
15	O00571	ATP-dependent RNA helicase DDX3X	DDX3X_HUMAN	12	17.5	No
16	P13674	Prolyl 4-hydroxylase subunit alpha-1	P4HA1_HUMAN	12	9	No
17	Q9Y2X3	Nucleolar protein 58	NOP58_HUMAN	11.5	10.5	No
18	P13674-2	Isoform 2 of Prolyl 4-hydroxylase subunit alpha-1	P4HA1_HUMAN	11	8.5	No
19	P16403	Histone H1.2	H12_HUMAN	9.5	8.5	Yes (Sigglekow, 2012)
20	P13796	Plastin-2	PLSL_HUMAN	9.5	7.5	No
21	P42166	Lamina-associated polypeptide 2, isoform alpha	LAP2A_HUMAN	9	19.5	No
22	P46940	Ras GTPase-activating-like protein IQGAP1	IQGA1_HUMAN	8	31	No
23	P47756-2	Isoform 2 of F-actin-capping protein subunit beta	CAPZB_HUMAN	7.5	6.5	Yes (Ewing, 2007)
24	P06493	Cyclin-dependent kinase 1	CDK1_HUMAN	7	8	No
25	P12956	X-ray repair cross-complementing protein 6	XRCC6_HUMAN	7	6.5	No
26	P35251-2	Isoform 2 of Replication factor C subunit 1	RFC1_HUMAN	7	3.5	No
27	Q14974	Importin subunit beta-1	IMB1_HUMAN	6.5	18	No
28	P78371	T-complex protein 1 subunit beta	TCPB_HUMAN	6.5	12	No
29	Q9Y5B9	FACT complex subunit SPT16	SP16H_HUMAN	6.5	6	Yes (Ewing, 2007)
30	Q86UE4	Protein LYRIC	LYRIC_HUMAN	6.5	3.5	No
31	Q9BVP2-2	Isoform 2 of Guanine nucleotide-binding protein-like 3	GNL3_HUMAN	6.5	1	No
32	Q5T4S7-3	Isoform 3 of E3 ubiquitin-protein ligase UBR4	UBR4_HUMAN	6	69	No
33	Q96ER9	Coiled-coil domain-containing protein 51	CCD51_HUMAN	6	17	No
34	P31689	DnaJ homolog subfamily A member 1	DNJA1_HUMAN	6	14.5	No
35	P63092	Guanine nucleotide-binding protein G(s) subunit alpha isoforms	GNAS2_HUMAN	6	5.5	No
36	P53621	Coatomer subunit alpha	COPA_HUMAN	6	5	No
37	P22732	Solute carrier family 2, facilitated glucose transporter member 5	GTR5_HUMAN	6	4	No

38	P63092-2	Isoform Gnas-2 of Guanine nucleotide-binding protein G(s) sub	GNAS2_HUMAN	5.5	5.5	No
39	P63092-3	Isoform 3 of Guanine nucleotide-binding protein G(s) subunit al	GNAS2_HUMAN	5.5	5	No
40	Q14839	Chromodomain-helicase-DNA-binding protein 4	CHD4_HUMAN	5.5	4.5	No
41	Q8NI36	WD repeat-containing protein 36	WDR36_HUMAN	5.5	4	No
42	P17858	6-phosphofructokinase, liver type	K6PL_HUMAN	5	6.5	No
43	P30041	Peroxiredoxin-6	PRDX6_HUMAN	5	6	No
44	Q9P035	Very-long-chain (3R)-3-hydroxyacyl-[acyl-carrier protein] dehyd	HACD3_HUMAN	5	6	No
45	P18754	Regulator of chromosome condensation	RCC1_HUMAN	5	4.5	No
46	Q9BSD7	Cancer-related nucleoside-triphosphatase	NTPCR_HUMAN	5	4	No
47	P62873	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta	GBB1_HUMAN	5	3.5	Yes (Ewing, 2007)
48	O94874	E3 UFM1-protein ligase 1	UFL1_HUMAN	5	3	No
49	O95299	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subun	NDUAA_HUMAN	5	3	No
50	Q9UQE7	Structural maintenance of chromosomes protein 3	SMC3_HUMAN	4.5	24	Yes (Ewing, 2007)
51	P62136	Serine/threonine-protein phosphatase PP1-alpha catalytic subu	PP1A_HUMAN	4.5	8.5	No
52	Q14157	Ubiquitin-associated protein 2-like	UBP2L_HUMAN	4.5	7	No
53	O75534-2	Isoform Short of Cold shock domain-containing protein E1	CSDE1_HUMAN	4.5	5	Yes (Ewing, 2007)
54	Q00013	55 kDa erythrocyte membrane protein	EM55_HUMAN	4.5	2.5	No
55	P17480-2	Isoform UBF2 of Nucleolar transcription factor 1	UBF1_HUMAN	4.5	2.5	No
56	Q01813	6-phosphofructokinase type C	K6PP_HUMAN	4.5	2	Yes (Ewing, 2007)
57	Q9UIG0-2	Isoform 2 of Tyrosine-protein kinase BAZ1B	BAZ1B_HUMAN	4.5	2	No
58	P33991	DNA replication licensing factor MCM4	MCM4_HUMAN	4	27	Yes (Ewing, 2007)
59	Q92973-2	Isoform 2 of Transportin-1	TNPO1_HUMAN	4	15.5	No
60	Q08945	FACT complex subunit SSRP1	SSRP1_HUMAN	4	9.5	Yes (Ewing, 2007)
61	Q13242	Serine/arginine-rich splicing factor 9	SRSF9_HUMAN	4	5	No
62	P23528	Cofilin-1	COF1_HUMAN	4	4	No
63	Q92922	SWI/SNF complex subunit SMARCC1	SMRC1_HUMAN	4	3.5	No
64	Q14699	Raftlin	RFTN1_HUMAN	4	3	No
65	O94826	Mitochondrial import receptor subunit TOM70	TOM70_HUMAN	4	2.5	No
66	P51531-2	Isoform Short of Probable global transcription activator SNF2L2	SMCA2_HUMAN	4	2.5	No
67	Q9UQ80	Proliferation-associated protein 2G4	PA2G4_HUMAN	4	2	No
68	O43396	Thioredoxin-like protein 1	TXNL1_HUMAN	3.5	15.5	No
69	O60884	DnaJ homolog subfamily A member 2	DNJA2_HUMAN	3.5	10	No
70	P36873	Serine/threonine-protein phosphatase PP1-gamma catalytic su	PP1G_HUMAN	3.5	8.5	No
71	P53985	Monocarboxylate transporter 1	MOT1_HUMAN	3.5	8.5	No
72	P40938	Replication factor C subunit 3	RFC3_HUMAN	3.5	4.5	No
73	Q53HL2	Borealin	BOREA_HUMAN	3.5	4	No
74	P21912	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mito	DHSB_HUMAN	3.5	3.5	Yes (Ewing, 2007)
75	Q86WU2-2	Isoform 2 of Probable D-lactate dehydrogenase, mitochondrial	LDHD_HUMAN	3.5	3.5	No
76	Q96JB5	CDK5 regulatory subunit-associated protein 3	CK5P3_HUMAN	3.5	3	No
77	O95563	Mitochondrial pyruvate carrier 2	MPC2_HUMAN	3.5	2.5	No
78	Q96QK1	Vacuolar protein sorting-associated protein 35	VPS35_HUMAN	3.5	2.5	Yes (Ewing, 2007)
79	Q9BZQ8	Protein Niban	NIBAN_HUMAN	3.5	1.5	No
80	O14773-2	Isoform 2 of Tripeptidyl-peptidase 1	TPP1_HUMAN	3.5	1.5	No
81	Q92945	Far upstream element-binding protein 2	FUBP2_HUMAN	3	7.5	No

82	Q9P0L0	Vesicle-associated membrane protein-associated protein A	VAPA_HUMAN	3	6	No
83	Q15181	Inorganic pyrophosphatase	IPYR_HUMAN	3	5.5	No
84	Q12788	Transducin beta-like protein 3	TBL3_HUMAN	3	5	No
85	O95573	Long-chain-fatty-acid--CoA ligase 3	ACSL3_HUMAN	3	4.5	No
86	Q02978	Mitochondrial 2-oxoglutarate/malate carrier protein	M2OM_HUMAN	3	3	No
87	Q9UKM7	Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-ma	MA1B1_HUMAN	3	3	No
88	Q7Z3B4	Nucleoporin p54	NUP54_HUMAN	3	2	No
89	P42224	Signal transducer and activator of transcription 1-alpha/beta	STAT1_HUMAN	3	2	No
90	Q99733	Nucleosome assembly protein 1-like 4	NP1L4_HUMAN	3	2	No
91	P35998	26S protease regulatory subunit 7	PRS7_HUMAN	2.5	10	No
92	P12004	Proliferating cell nuclear antigen	PCNA_HUMAN	2.5	8.5	Yes (Ewing, 2007)
93	O00299	Chloride intracellular channel protein 1	CLIC1_HUMAN	2.5	8.5	Yes (Ewing, 2007)
94	O75083	WD repeat-containing protein 1	WDR1_HUMAN	2.5	5.5	No
95	Q9Y3F4	Serine-threonine kinase receptor-associated protein	STRAP_HUMAN	2.5	5	No
96	P43358	Melanoma-associated antigen 4	MAGA4_HUMAN	2.5	5	No
97	Q08752	Peptidyl-prolyl cis-trans isomerase D	PPID_HUMAN	2.5	5	No
98	O43175	D-3-phosphoglycerate dehydrogenase	SERA_HUMAN	2.5	4.5	No
99	Q9GZS3	WD repeat-containing protein 61	WDR61_HUMAN	2.5	4	No
100	P53701	Cytochrome c-type heme lyase	CCHL_HUMAN	2.5	4	No
101	Q14258	E3 ubiquitin/ISG15 ligase TRIM25	TRI25_HUMAN	2.5	3.5	No
102	P35249	Replication factor C subunit 4	RFC4_HUMAN	2.5	3	No
103	P51159	Ras-related protein Rab-27A	RB27A_HUMAN	2.5	3	No
104	P07741	Adenine phosphoribosyltransferase]	APT_HUMAN	2.5	2.5	Yes (Ewing, 2007)
105	Q96HE7	ERO1-like protein alpha	ERO1A_HUMAN	2.5	2.5	No
106	Q8WYP5	Protein ELYS	ELYS_HUMAN	2.5	2.5	No
107	Q9H0U3	Magnesium transporter protein 1	MAGT1_HUMAN	2.5	1.5	No
108	O15400-2	Isoform 2 of Syntaxin-7	STX7_HUMAN	2.5	1	No
109	Q9Y5X1	Sorting nexin-9	SNX9_HUMAN	2.5	1	No
110	Q969V3-2	Isoform 2 of Nicalin	NCLN_HUMAN	2.5	1	No
111	Q9BQG0	Myb-binding protein 1A	MBB1A_HUMAN	2.5	1	No
112	P38117	Electron transfer flavoprotein subunit beta	ETFB_HUMAN	2	14	No
113	Q9BYG3	MKI67 FHA domain-interacting nucleolar phosphoprotein	MK67I_HUMAN	2	9	No
114	Q9NX58	Cell growth-regulating nucleolar protein	LYAR_HUMAN	2	9	No
115	Q9BPW8	Protein NipSnap homolog 1	NIPS1_HUMAN	2	8	No
116	Q8IYB3	Serine/arginine repetitive matrix protein 1	SRRM1_HUMAN	2	8	No
117	P69849	Nodal modulator 3	NOMO3_HUMAN	2	7.5	No
118	Q14203-5	Isoform 5 of Dynactin subunit 1	DCTN1_HUMAN	2	6.5	No
119	Q6DD88	Atlastin-3	ATLA3_HUMAN	2	6.5	No
120	P53004	Biliverdin reductase A	BIEA_HUMAN	2	6.5	No
121	O60763	General vesicular transport factor p115	USO1_HUMAN	2	5	No
122	P13010	X-ray repair cross-complementing protein 5	XRCC5_HUMAN	2	5	No
123	Q16836	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	HCDH_HUMAN	2	4.5	Yes (Ewing, 2007)
124	Q09028-3	Isoform 3 of Histone-binding protein RBBP4	RBBP4_HUMAN	2	4	No
125	Q15126	Phosphomevalonate kinase	PMVK_HUMAN	2	3.5	No

126	P18669	Phosphoglycerate mutase 1	PGAM1_HUMAN	2	3.5	No
127	P23258	Tubulin gamma-1 chain	TBG1_HUMAN	2	3.5	No
128	Q9HC21	Mitochondrial thiamine pyrophosphate carrier	TPC_HUMAN	2	3.5	No
129	P61160	Actin-related protein 2	ARP2_HUMAN	2	3	No
130	Q12769	Nuclear pore complex protein Nup160	NU160_HUMAN	2	2.5	No
131	Q96HY6	DDRGK domain-containing protein 1	DDRGK_HUMAN	2	2	No
132	P48960-2	Isoform 2 of CD97 antigen	CD97_HUMAN	2	2	No
133	Q13610	Periodic tryptophan protein 1 homolog	PWP1_HUMAN	2	1.5	No
134	Q9H7Z7	Prostaglandin E synthase 2	PGES2_HUMAN	2	1.5	No
135	Q14008-2	Isoform 2 of Cytoskeleton-associated protein 5	CKAP5_HUMAN	2	1	No
136	Q14C86-4	Isoform 4 of GTPase-activating protein and VPS9 domain-cont	GAPD1_HUMAN	1.5	29.5	No
137	P62937	Peptidyl-prolyl cis-trans isomerase A	PPIA_HUMAN	1.5	14.5	No
138	Q99798	Aconitate hydratase, mitochondrial	ACON_HUMAN	1.5	8.5	No
139	O43291	Kunitz-type protease inhibitor 2	SPIT2_HUMAN	1.5	6	No
140	Q02790	Peptidyl-prolyl cis-trans isomerase FKBP4	FKBP4_HUMAN	1.5	5	No
141	Q15397	Pumilio domain-containing protein KIAA0020	K0020_HUMAN	1.5	5	No
142	Q96SB4	SRSF protein kinase 1	SRPK1_HUMAN	1.5	5	No
143	P46977	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase	STT3A_HUMAN	1.5	4.5	No
144	P07686	Beta-hexosaminidase subunit beta	HEXB_HUMAN	1.5	4	No
145	Q6P1M0	Long-chain fatty acid transport protein 4	S27A4_HUMAN	1.5	3.5	No
146	P11532-3	Isoform 2 of Dystrophin	DMD_HUMAN	1.5	2.5	No
147	Q9NQS7-2	Isoform 2 of Inner centromere protein	INCE_HUMAN	1.5	2.5	No
148	O96008	Mitochondrial import receptor subunit TOM40 homolog	TOM40_HUMAN	1.5	2	No
149	O60488-2	Isoform Short of Long-chain-fatty-acid--CoA ligase 4	ACSL4_HUMAN	1.5	2	No
150	Q9UL25	Ras-related protein Rab-21	RAB21_HUMAN	1.5	2	Yes (Ewing, 2007)
151	Q9Y2W1	Thyroid hormone receptor-associated protein 3	TR150_HUMAN	1	25	No
152	Q9Y3T9	Nucleolar complex protein 2 homolog	NOC2L_HUMAN	1	7	No
153	P07195	L-lactate dehydrogenase B chain	LDHB_HUMAN	1	6.5	No
154	Q13243	Serine/arginine-rich splicing factor 5	SRSF5_HUMAN	1	6.5	No
155	P22061	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	PIMT_HUMAN	1	5.5	Yes (Ewing, 2007)
156	Q5JTV8	Torsin-1A-interacting protein 1 OS=Homo sapiens GN=TOR1A	TOIP1_HUMAN	1	5.5	No
157	O75494-5	Isoform 5 of Serine/arginine-rich splicing factor 10	SRS10_HUMAN	1	5.5	No
158	P35914	Hydroxymethylglutaryl-CoA lyase, mitochondrial	HMGCL_HUMAN	1	5	No
159	Q9H9B4	Sideroflexin-1	SFXN1_HUMAN	1	4.5	Yes (Ewing, 2007)
160	O00487	26S proteasome non-ATPase regulatory subunit 14	PSDE_HUMAN	1	4.5	Yes (Ewing, 2007)
161	O43684-2	Isoform 2 of Mitotic checkpoint protein BUB3	BUB3_HUMAN	1	4	No
162	Q9UNQ2	Probable dimethyladenosine transferase	DIM1_HUMAN	1	4	No
163	Q8ND30	Liprin-beta-2	LIPB2_HUMAN	1	4	No
164	Q96F07-2	Isoform 2 of Cytoplasmic FMR1-interacting protein 2	CYFP2_HUMAN	1	4	No
165	Q9NTK5	Obg-like ATPase 1	OLA1_HUMAN	1	4	No
166	P62879	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta	GBB2_HUMAN	1	3.5	No
167	Q9UKG1	DCC-interacting protein 13-alpha	DP13A_HUMAN	1	3.5	No
168	Q9Y5K5-2	Isoform 2 of Ubiquitin carboxyl-terminal hydrolase isozyme L5	UCHL5_HUMAN	1	3	No
169	P61964	WD repeat-containing protein 5	WDR5_HUMAN	1	3	Yes (Ewing, 2007)

170	Q8NE71-2	Isoform 2 of ATP-binding cassette sub-family F member 1	ABCF1_HUMAN	1	3	No
171	Q9BQ75-2	Isoform 2 of Protein CMSS1	CMS1_HUMAN	1	2.5	No
172	Q14764	Major vault protein	MVP_HUMAN	1	2.5	No
173	Q96N66-2	Isoform 2 of Lysophospholipid acyltransferase 7	MBOA7_HUMAN	1	2.5	No
174	O75477	Erlin-1	ERLN1_HUMAN	1	2.5	No
175	Q9Y679-2	Isoform Short of Ancient ubiquitous protein 1	AUP1_HUMAN	1	2	No
176	Q15149-4	Isoform 4 of Plectin	PLEC_HUMAN	61.5	0	No
177	P33527-4	Isoform 4 of Multidrug resistance-associated protein 1	MRP1_HUMAN	9.5	0	No
178	Q8N884	Cyclic GMP-AMP synthase	CGAS_HUMAN	9	0.5	No
179	P43121	Cell surface glycoprotein MUC18	MUC18_HUMAN	6.5	0	No
180	P31946-2	Isoform Short of 14-3-3 protein beta/alpha	1433B_HUMAN	6	0	No
181	O00161-2	Isoform SNAP-23b of Synaptosomal-associated protein 23	SNP23_HUMAN	5.5	0.5	No
182	Q9BUR5	Apolipoprotein O	APOO_HUMAN	5.5	0.5	No
183	P46459	Vesicle-fusing ATPase	NSF_HUMAN	5	0	No
184	Q04917	14-3-3 protein eta	1433F_HUMAN	4.5	0.5	No
185	Q7Z403	Transmembrane channel-like protein 6	TMC6_HUMAN	4.5	0	No
186	Q92508	Piezo-type mechanosensitive ion channel component 1	PIEZ1_HUMAN	4.5	0	No
187	Q8NC56	LEM domain-containing protein 2	LEMD2_HUMAN	4	0.5	No
188	P31947-2	Isoform 2 of 14-3-3 protein sigma	1433S_HUMAN	4	0	No
189	Q9C0B5-2	Isoform 2 of Palmitoyltransferase ZDHHC5	ZDHC5_HUMAN	3.5	0	No
190	Q8IV63-3	Isoform 3 of Inactive serine/threonine-protein kinase VRK3	VRK3_HUMAN	3.5	0	No
191	Q8NBJ5	Procollagen galactosyltransferase 1	GT251_HUMAN	3.5	0	No
192	O96000	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit	NDUBA_HUMAN	3	0.5	No
193	Q9Y5Y6	Suppressor of tumorigenicity 14 protein	ST14_HUMAN	3	0	No
194	Q96S97	Myeloid-associated differentiation marker	MYADM_HUMAN	3	0	No
195	Q92485	Acid sphingomyelinase-like phosphodiesterase 3b	ASM3B_HUMAN	3	0	No
196	Q13185	Chromobox protein homolog 3	CBX3_HUMAN	3	0	No
197	Q9H078-2	Isoform 2 of Caseinolytic peptidase B protein homolog	CLPB_HUMAN	2.5	0.5	No
198	Q8NBI5	Solute carrier family 43 member 3	S43A3_HUMAN	2.5	0	No
199	O94776	Metastasis-associated protein MTA2	MTA2_HUMAN	2.5	0	No
200	Q13425-2	Isoform 2 of Beta-2-syntrophin	SNTB2_HUMAN	2.5	0	No
201	Q13951-2	Isoform 2 of Core-binding factor subunit beta	PEBB_HUMAN	2.5	0	No
202	Q99595	Mitochondrial import inner membrane translocase subunit Tim1	TI17A_HUMAN	2.5	0	No
203	Q8TB52	F-box only protein 30	FBX30_HUMAN	2	0	No
204	Q6PJF5-2	Isoform 2 of Inactive rhomboid protein 2	RHDF2_HUMAN	2	0	No
205	Q9H4I3	TraB domain-containing protein	TRABD_HUMAN	2	0	No
206	Q15599-2	Isoform 2 of Na(+)/H(+) exchange regulatory cofactor NHE-RF2	NHRF2_HUMAN	2	0	No
207	Q9H061	Transmembrane protein 126A	T126A_HUMAN	2	0	No
208	P12270	Nucleoprotein TPR	TPR_HUMAN	0	22	No
209	Q9H307	Pinin	PININ_HUMAN	0	18.5	No
210	Q9NYF8-2	Isoform 2 of Bcl-2-associated transcription factor 1	BCLF1_HUMAN	0	18	No
211	P49674	Casein kinase I isoform epsilon	KC1E_HUMAN	0	15.5	Yes (Ewing, 2007)
212	Q9UQ35	Serine/arginine repetitive matrix protein 2	SRRM2_HUMAN	0	15.5	No
213	Q14980-2	Isoform 2 of Nuclear mitotic apparatus protein 1	NUMA1_HUMAN	0.5	14.5	No

214	Q86V48-2	Isoform 2 of Leucine zipper protein 1	LUZP1_HUMAN	0	14.5	No
215	P48730-2	Isoform 2 of Casein kinase I isoform delta	KC1D_HUMAN	0	14.5	Yes (Ewing, 2007)
216	Q9UKV3	Apoptotic chromatin condensation inducer in the nucleus	ACINU_HUMAN	0.5	14	No
217	Q14978-3	Isoform 3 of Nucleolar and coiled-body phosphoprotein 1	NOLC1_HUMAN	0	13	No
218	Q14978	Nucleolar and coiled-body phosphoprotein 1	NOLC1_HUMAN	0	13	No
219	P02788-2	Isoform DeltaLf of Lactotransferrin	TRFL_HUMAN	0	11.5	No
220	P27816-6	Isoform 6 of Microtubule-associated protein 4	MAP4_HUMAN	0.5	10	No
221	Q8N163	DBIRD complex subunit KIAA1967	K1967_HUMAN	0	10	No
222	O75828	Carbonyl reductase [NADPH] 3	CBR3_HUMAN	0	9.5	No
223	P05109	Protein S100-A8	S10A8_HUMAN	0.5	9	No
224	Q9H2U2	Inorganic pyrophosphatase 2, mitochondrial	IPYR2_HUMAN	0.5	8.5	No
225	O75691	Small subunit processome component 20 homolog	UTP20_HUMAN	0	8.5	No
226	P62191	26S protease regulatory subunit 4	PRS4_HUMAN	0	8	No
227	Q96R06	Sperm-associated antigen 5	SPAG5_HUMAN	0	8	No
228	Q15154	Pericentriolar material 1 protein	PCM1_HUMAN	0	8	No
229	Q9BZQ6	ER degradation-enhancing alpha-mannosidase-like protein 3	EDEM3_HUMAN	0.5	7.5	No
230	Q13595	Transformer-2 protein homolog alpha	TRA2A_HUMAN	0.5	7	No
231	Q9NZ01	Very-long-chain enoyl-CoA reductase	TECR_HUMAN	0	7	No
232	Q9NY61	Protein AATF	AATF_HUMAN	0	7	No
233	P46109	Crk-like protein	CRKL_HUMAN	0	6.5	No
234	O75663	TIP41-like protein	TIPRL_HUMAN	0	6.5	No
235	Q06323	Proteasome activator complex subunit 1	PSME1_HUMAN	0	6.5	No
236	P29590-14	Isoform PML-14 of Protein PML	PML_HUMAN	0	6.5	No
237	Q9P0J7	E3 ubiquitin-protein ligase KCMF1	KCMF1_HUMAN	0	6.5	No
238	Q8IZT6-2	Isoform 2 of Abnormal spindle-like microcephaly-associated pr	ASPM_HUMAN	0	6.5	No
239	P49821-2	Isoform 2 of NADH dehydrogenase [ubiquinone] flavoprotein 1,	NDUV1_HUMAN	0.5	6	No
240	P45974-2	Isoform Short of Ubiquitin carboxyl-terminal hydrolase 5	UBP5_HUMAN	0.5	6	No
241	Q5SRE5-2	Isoform 2 of Nucleoporin NUP188 homolog	NU188_HUMAN	0.5	6	No
242	P51114	Fragile X mental retardation syndrome-related protein 1	FXR1_HUMAN	0	6	No
243	Q9H9E3	Conserved oligomeric Golgi complex subunit 4	COG4_HUMAN	0	6	No
244	P29353-2	Isoform p52Shc of SHC-transforming protein 1	SHC1_HUMAN	-0.5	6	No
245	Q92597	Protein NDRG1	NDRG1_HUMAN	-0.5	6	No
246	Q9BRK5	45 kDa calcium-binding protein	CAB45_HUMAN	0.5	5.5	No
247	P50897	Palmitoyl-protein thioesterase 1	PPT1_HUMAN	0.5	5.5	No
248	Q6NVY1	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial	HIBCH_HUMAN	0.5	5.5	No
249	O00170	AH receptor-interacting protein	AIP_HUMAN	0	5.5	No
250	Q9NVN8	Guanine nucleotide-binding protein-like 3-like protein	GNL3L_HUMAN	0	5.5	No
251	Q00534	Cyclin-dependent kinase 6	CDK6_HUMAN	0	5.5	No
252	Q96FW1	Ubiquitin thioesterase OTUB1	OTUB1_HUMAN	0	5	Yes (Ewing, 2007)
253	Q7LBC6	Lysine-specific demethylase 3B	KDM3B_HUMAN	0	5	No
254	Q5VT06	Centrosome-associated protein 350	CE350_HUMAN	0	5	No
255	Q8TC07-2	Isoform 2 of TBC1 domain family member 15	TBC15_HUMAN	0	5	Yes (Ewing, 2007)
256	P54819-5	Isoform 5 of Adenylate kinase 2, mitochondrial	KAD2_HUMAN	0.5	4.5	Yes (Ewing, 2007)
257	Q6FI81-3	Isoform 3 of Anamorsin	CPIN1_HUMAN	0.5	4.5	No

258	Q8WUM0	Nuclear pore complex protein Nup133	NU133_HUMAN	0.5	4.5	No
259	O75832	26S proteasome non-ATPase regulatory subunit 10	PSD10_HUMAN	0	4.5	No
260	O00233	26S proteasome non-ATPase regulatory subunit 9	PSMD9_HUMAN	0	4.5	No
261	O60711	Leupaxin	LPXN_HUMAN	0	4.5	No
262	P61289	Proteasome activator complex subunit 3	PSME3_HUMAN	0	4.5	Yes (Ewing, 2007)
263	P55735-2	Isoform 2 of Protein SEC13 homolog	SEC13_HUMAN	0	4.5	Yes (Ewing, 2007)
264	Q8IYS1	Peptidase M20 domain-containing protein 2	P20D2_HUMAN	0	4.5	No
265	Q8IUF8-4	Isoform 4 of Bifunctional lysine-specific demethylase and histid	MINA_HUMAN	0	4.5	No
266	Q9UHD8-7	Isoform 7 of Septin-9	SEPT9_HUMAN	0	4.5	No
267	Q9ULX6	A-kinase anchor protein 8-like	AKP8L_HUMAN	0	4.5	No
268	Q8N3U4	Cohesin subunit SA-2	STAG2_HUMAN	0	4.5	No
269	P59045-3	Isoform 3 of NACHT, LRR and PYD domains-containing protein	NAL11_HUMAN	0	4.5	No
270	O75330-4	Isoform 4 of Hyaluronan mediated motility receptor	HMMR_HUMAN	0	4.5	No
271	Q6RFH5	WD repeat-containing protein 74	WDR74_HUMAN	0.5	4	No
272	O75569-3	Isoform 3 of Interferon-inducible double stranded RNA-depende	PRKRA_HUMAN	0.5	4	No
273	Q9Y5Y2	Cytosolic Fe-S cluster assembly factor NUBP2	NUBP2_HUMAN	0.5	4	No
274	Q9Y3C1	Nucleolar protein 16	NOP16_HUMAN	0.5	4	No
275	Q8NEJ9-2	Isoform 2 of Neuroguidin	NGDN_HUMAN	0.5	4	No
276	Q92785	Zinc finger protein ubi-d4	REQU_HUMAN	0.5	4	No
277	Q9GZP4-2	Isoform 2 of PITH domain-containing protein 1	PITH1_HUMAN	0	4	No
278	P61758	Prefoldin subunit 3	PFD3_HUMAN	0	4	No
279	Q9UBV8	Peflin	PEF1_HUMAN	0	4	No
280	P68400	Casein kinase II subunit alpha	CSK21_HUMAN	0	4	No
281	Q9H501	ESF1 homolog	ESF1_HUMAN	0	4	No
282	Q7Z2W4	Zinc finger CCCH-type antiviral protein 1	ZCCHV_HUMAN	0	4	No
283	Q15628	Tumor necrosis factor receptor type 1-associated DEATH dom	TRADD_HUMAN	0.5	3.5	No
284	Q86YT6	E3 ubiquitin-protein ligase MIB1	MIB1_HUMAN	0.5	3.5	No
285	P25787	Proteasome subunit alpha type-2	PSA2_HUMAN	0	3.5	No
286	P09525	Annexin A4	ANXA4_HUMAN	0	3.5	No
287	P40925	Malate dehydrogenase, cytoplasmic	MDHC_HUMAN	0	3.5	No
288	Q16740	Putative ATP-dependent Clp protease proteolytic subunit, mitoc	CLPP_HUMAN	0	3.5	No
289	O95861	3'(2'),5'-bisphosphate nucleotidase 1	BPNT1_HUMAN	0	3.5	No
290	P23526-2	Isoform 2 of Adenosylhomocysteinase	SAHH_HUMAN	0	3.5	Yes (Ewing, 2007)
291	Q15054	DNA polymerase delta subunit 3	DPOD3_HUMAN	0	3.5	No
292	O15355	Protein phosphatase 1G	PPM1G_HUMAN	0	3.5	No
293	Q9UJU6	Drebrin-like protein	DBNL_HUMAN	0	3.5	No
294	Q8IWC1-2	Isoform 2 of MAP7 domain-containing protein 3	MA7D3_HUMAN	0	3.5	No
295	Q9H3P7	Golgi resident protein GCP60	GCP60_HUMAN	0	3.5	No
296	O00267-2	Isoform 2 of Transcription elongation factor SPT5	SPT5H_HUMAN	0	3.5	No
297	Q6ZU80	Centrosomal protein of 128 kDa	CE128_HUMAN	0	3.5	No
298	Q8IWJ2	GRIP and coiled-coil domain-containing protein 2	GCC2_HUMAN	0	3.5	No
299	Q9H9E3-3	Isoform 3 of Conserved oligomeric Golgi complex subunit 4	COG4_HUMAN	0	3.5	No
300	P36957	Dihydrolipoyllysine-residue succinyltransferase component of 2	ODO2_HUMAN	0.5	3	No
301	P41227-2	Isoform 2 of N-alpha-acetyltransferase 10	NAA10_HUMAN	0	3	Yes (Ewing, 2007)

302	Q96J01	THO complex subunit 3	THOC3_HUMAN	0	3	No
303	Q96P16	Regulation of nuclear pre-mRNA domain-containing protein 1A	RPR1A_HUMAN	0	3	No
304	P35520	Cystathionine beta-synthase	CBS_HUMAN	0	3	No
305	Q9UJU6-2	Isoform 2 of Drebrin-like protein	DBNL_HUMAN	0	3	No
306	P23458	Tyrosine-protein kinase JAK1	JAK1_HUMAN	0	3	No
307	Q9HA64	Ketosamine-3-kinase	KT3K_HUMAN	0	3	No
308	Q6PJT7-4	Isoform 4 of Zinc finger CCCH domain-containing protein 14	ZC3HE_HUMAN	0	3	No
309	Q86VM9	Zinc finger CCCH domain-containing protein 18	ZCH18_HUMAN	0	3	No
310	Q9Y2X9	Zinc finger protein 281	ZN281_HUMAN	0	3	No
311	Q9BUQ8	Probable ATP-dependent RNA helicase DDX23	DDX23_HUMAN	0	3	No
312	O14976	Cyclin-G-associated kinase	GAK_HUMAN	0	3	No
313	Q53H96	Pyrroline-5-carboxylate reductase 3	P5CR3_HUMAN	0	3	No
314	P52732	Kinesin-like protein KIF11	KIF11_HUMAN	0	3	No
315	Q96KA5-2	Isoform 2 of Cleft lip and palate transmembrane protein 1-like p	CLP1L_HUMAN	0	3	No
316	P55769	NHP2-like protein 1	NH2L1_HUMAN	0.5	2.5	No
317	Q15691	Microtubule-associated protein RP/EB family member 1	MARE1_HUMAN	0.5	2.5	Yes (Ewing, 2007)
318	Q15653-2	Isoform 2 of NF-kappa-B inhibitor beta	IKBB_HUMAN	0	2.5	Yes (Bouwmeester, 2004
319	Q9NUQ9	Protein FAM49B	FA49B_HUMAN	0	2.5	No
320	P62993	Growth factor receptor-bound protein 2	GRB2_HUMAN	0	2.5	Yes (Ewing, 2007)
321	Q96EY8	Cob(I)yrinic acid a,c-diamide adenosyltransferase, mitochondria	MMAB_HUMAN	0	2.5	No
322	P51553-2	Isoform 2 of Isocitrate dehydrogenase [NAD] subunit gamma, n	IDH3G_HUMAN	0	2.5	No
323	Q9BTE7	DCN1-like protein 5	DCNL5_HUMAN	0	2.5	No
324	Q16637-4	Isoform SMN-delta57 of Survival motor neuron protein	SMN_HUMAN	0	2.5	No
325	Q9H8H0	Nucleolar protein 11	NOL11_HUMAN	0	2.5	No
326	Q9BV38	WD repeat-containing protein 18	WDR18_HUMAN	0	2.5	No
327	P08758	Annexin A5	ANXA5_HUMAN	0	2.5	No
328	Q6JBY9	CapZ-interacting protein	CPZIP_HUMAN	0	2.5	No
329	Q9Y4R8	Telomere length regulation protein TEL2 homolog	TELO2_HUMAN	0	2.5	No
330	Q92540-2	Isoform 2 of Protein SMG7	SMG7_HUMAN	0	2.5	No
331	Q76FK4-4	Isoform 4 of Nucleolar protein 8	NOL8_HUMAN	0	2.5	No
332	Q9UJC3	Protein Hook homolog 1	HOOK1_HUMAN	0	2.5	No
333	P11802	Cyclin-dependent kinase 4	CDK4_HUMAN	0	2.5	Yes (Ewing, 2007)
334	Q00535-2	Isoform 2 of Cyclin-dependent kinase 5	CDK5_HUMAN	0	2.5	No
335	P04083	Annexin A1	ANXA1_HUMAN	0	2.5	Yes (Sigglekow, 2012)
336	P43487	Ran-specific GTPase-activating protein	RANG_HUMAN	0.5	2	Yes (Ewing, 2007)
337	P54920	Alpha-soluble NSF attachment protein	SNAA_HUMAN	0.5	2	No
338	Q6UXN9	WD repeat-containing protein 82	WDR82_HUMAN	0.5	2	No
339	Q14790-8	Isoform 8 of Caspase-8	CASP8_HUMAN	0.5	2	No
340	Q96T51	RUN and FYVE domain-containing protein 1	RUFY1_HUMAN	0.5	2	No
341	Q86Y82	Syntaxin-12	STX12_HUMAN	0.5	2	No
342	Q9NXW2	DnaJ homolog subfamily B member 12	DJB12_HUMAN	0.5	2	No
343	P16591-3	Isoform 3 of Tyrosine-protein kinase Fer	FER_HUMAN	0.5	2	No
344	Q9BVC4	Target of rapamycin complex subunit LST8	LST8_HUMAN	0.5	2	No
345	Q8IZP0-10	Isoform 10 of Abl interactor 1	ABI1_HUMAN	0	2	No

346	P48556	26S proteasome non-ATPase regulatory subunit 8	PSMD8_HUMAN	0	2	Yes (Ewing, 2007)
347	Q9H6Y2	WD repeat-containing protein 55	WDR55_HUMAN	0	2	No
348	Q9NQT4	Exosome complex component RRP46	EXOS5_HUMAN	0	2	No
349	Q15003	Condensin complex subunit 2	CND2_HUMAN	0	2	No
350	P38432	Coilin	COIL_HUMAN	0	2	No
351	Q86X83	COMM domain-containing protein 2	COMD2_HUMAN	0	2	No
352	Q6IQ49-2	Isoform 2 of Protein SDE2 homolog	SDE2_HUMAN	0	2	No
353	P42574	Caspase-3	CASP3_HUMAN	0	2	No
354	Q3KQU3-2	Isoform 2 of MAP7 domain-containing protein 1	MA7D1_HUMAN	0	2	No
355	P39687	Acidic leucine-rich nuclear phosphoprotein 32 family member A	AN32A_HUMAN	0	2	No
356	P78417-3	Isoform 3 of Glutathione S-transferase omega-1	GSTO1_HUMAN	0	2	No
357	O00273	DNA fragmentation factor subunit alpha	DFFA_HUMAN	0	2	Yes (Ewing, 2007)
358	Q7Z460-2	Isoform 2 of CLIP-associating protein 1	CLAP1_HUMAN	0	2	No
359	Q5JSZ5	Protein PRRC2B	PRC2B_HUMAN	0	2	No
360	O15118	Niemann-Pick C1 protein	NPC1_HUMAN	0	2	No
361	Q9NWH9	SAFB-like transcription modulator	SLTM_HUMAN	0	2	No
362	Q9Y263	Phospholipase A-2-activating protein	PLAP_HUMAN	0	2	No
363	P15927	Replication protein A 32 kDa subunit	RFA2_HUMAN	0	2	No
364	Q9Y697-2	Isoform Cytoplasmic of Cysteine desulfurase, mitochondrial	NFS1_HUMAN	0	2	No
365	Q9H3N1	Thioredoxin-related transmembrane protein 1	TMX1_HUMAN	0	2	Yes (Ewing, 2007)

The human MM cell line 8226 cells were transduced with pUB-hMCC-SBP-6xHis or pUB-FLAG-hMCC. Immunoprecipitates of hMCC-SBP-6xHis by streptavidin-sepharose beads from whole cell lysates and purified mitochondria of 8226 cells were analyzed by high resolution LC-MS/MS, respectively. Immunoprecipitates of FLAG-hMCC by streptavidin-sepharose beads were used as negative control in these experiments. LC-MS/MS data were searched against the human IPI and UniProt databases using the Mascot and Proteome Discoverer search engines. Protein assignments were considered highly confident using a stringent false discovery rate threshold of <1%, as estimated by reversed database searching, and requiring that ≥2 peptides per protein be unambiguously identified. Rough relative protein amounts were estimated using spectra counting values, and requiring that ≥2 of average spectra count difference between hMCC-SBP-6xHis and FLAG-hMCC (negative control) of two experiments. Proteins that were previously identified as MCC-interactors in CRCs or 293T cells are indicated. SBP: streptavidin binding peptide tag

References:

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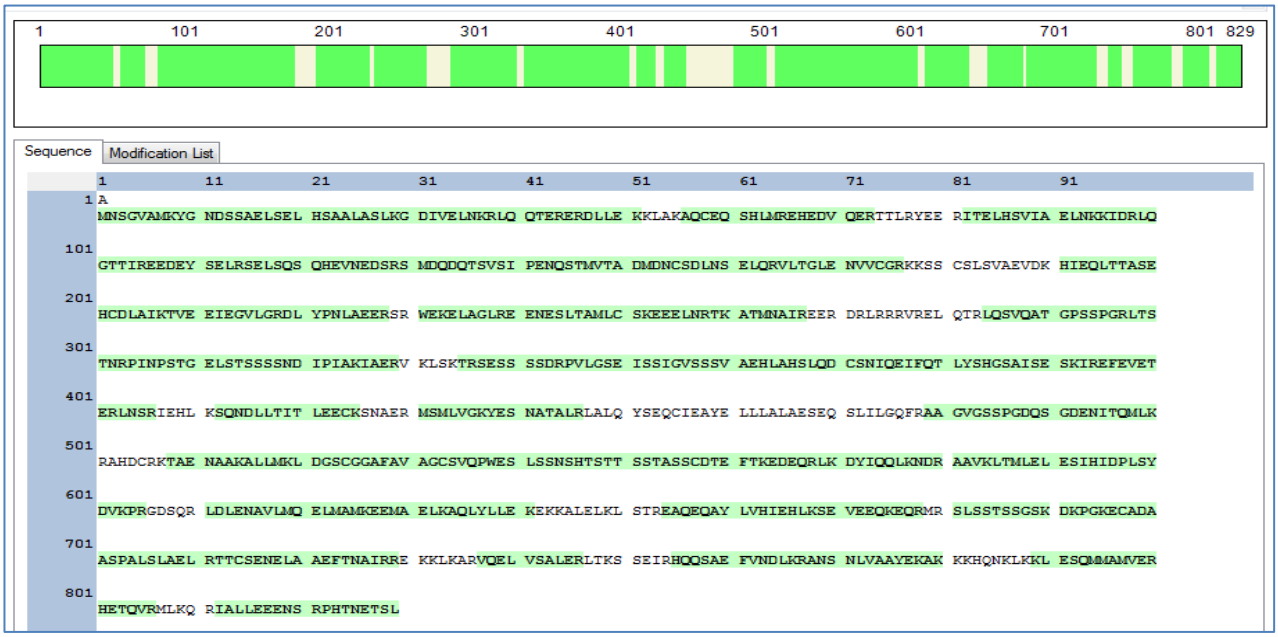
Supplementary Figure 1

A MCC isoform 1: 82.63% coverage



B MCC isoform 2: 74.29% coverage

Supplementary Figure 1. The MCC isoform 2 was identified as an MCC-interacting protein by affinity purification and LC-MS/MS. The two isoforms of MCC proteins differ at their extreme N-terminus due to alternative promoter usage. Both pUB-FLAG-hMCC and pUB-hMCC-SBP-6xHis are cloned from MCC isoform 1 (829 aa). (**A**) Schematic diagram of peptide sequences of MCC isoform 1 identified by LC-MS/MS. (**B**) Schematic diagram of peptide sequences of MCC isoform 2 (1019 aa) identified by LC-MS/MS. The peptide sequences detected by LC-MS/MS are highlighted in green color. The unique region of MCC isofrom 2 is marked with a dashed black box.

Cancer
Science & Therapy

Moore et al., J Cancer Sci Ther 2015, 7:2
http://dx.doi.org/10.4172/1948-5956.1000327

Review Article Open Access

Targeting TRAF3 Downstream Signaling Pathways in B cell Neoplasms

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Abstract

B cell neoplasms comprise >50% of blood cancers. However, many types of B cell malignancies remain incurable. Identification and validation of novel genetic risk factors and oncogenic signaling pathways are imperative for the development of new therapeutic strategies. We and others recently identified TRAF3, a cytoplasmic adaptor protein, as a novel tumor suppressor in B lymphocytes. We found that TRAF3 inactivation results in prolonged survival of mature B cells, which eventually leads to spontaneous development of B lymphomas in mice. Corroborating our findings, TRAF3 deletions and inactivating mutations frequently occur in human B cell chronic lymphocytic leukemia, splenic marginal zone lymphoma, mantle cell lymphoma, multiple myeloma, Waldenström's macroglobulinemia, and Hodgkin lymphoma. In this context, we have been investigating TRAF3 signaling mechanisms in B cells, and are developing new therapeutic strategies to target TRAF3 downstream signaling pathways in B cell neoplasms. Here we discuss our new translational data that demonstrate the therapeutic potential of targeting TRAF3 downstream signaling pathways in B lymphoma and multiple myeloma.

Keywords: TRAF3; B lymphoma; Multiple myeloma; NF-κB2; AD 198; MCC; Sox5

Abbreviations: AD 198: N-benzyladriamycin-14-valerate; BAFF: B Cell Activating Factor; BAFF-R: BAFF Receptor; B-CLL: B Cell Chronic Lymphocytic Leukemia; BCR: B Cell Receptor; BL: Burkitt's Lymphoma; B-TRAF3$^{-/-}$: B Cell-specific TRAF3-deficient; CBL: Centroblastic Lymphoma; cIAP1/2: Cellular Inhibitor of Apoptosis Proteins ½; CRC: Colorectal Cancer; DLBCL: Diffuse Large B-cell Lymphoma; ER: Endoplasmic Reticulum; ERK: Extracellular Signal-regulated Kinase; JNK: c-Jun N-terminal Kinase; LPS: Lipopolysaccharides; LC-MS/MS: Liquid Chromatography-Mass Spectrometry/Mass Spectrometry; MCC: Mutated in Colorectal Cancer; MCL: Mantle Cell Lymphoma; MM: Multiple Myeloma; MZL: Splenic Marginal Zone Lymphoma; NF-κB: Nuclear Factor κ Light Chain Enhancer of Activated B Cells; NIK: NF-κB Inducing Kinase; PARP1: Poly [ADP-ribose] Polymerase 1; PEL: Primary Effusion Lymphoma; PEP005: Ingenol-3-angelate; PHB2: Prohibitin-2; shRNA: Short Hairpin RNA; TRAF3: Tumor Necrosis Factor Receptor (TNF-R)-associated Factor 3; WM: Waldenström's Macroglobulinemia

TRAF3: A New Tumor Suppressor Gene in B Lymphocytes

B cell neoplasms comprise >50% of blood cancers. Despite recent advances in treatment, many types of human B cell neoplasms remain incurable, highlighting a clear need for new therapeutic strategies [1-3]. Identification and validation of new genetic risk factors and critical oncogenic pathways are imperative for a better understanding of B cell malignant transformation and for the development of new therapeutic strategies [4-6]. Recent studies from our laboratory and others have identified TRAF3, a crucial regulator of B cell survival, as a novel tumor suppressor in B lymphocytes [7-17].

TRAF3 is a member of the tumor necrosis factor receptor (TNF-R)-associated factor (TRAF) family (TRAF1-7) of cytoplasmic adaptor proteins [18]. TRAF3 is used for signaling by receptors of the TNF-R superfamily, pattern recognition receptors (PRRs), and a number of viral proteins such as the Epstein-Barr virus encoded oncoprotein LMP1 [18]. Consistent with the shared usage of TRAF3 by so many receptors, mice made genetically deficient in TRAF3 exhibit global defects and die by 10 days after birth [19]. To circumvent experimental limitations imposed by the early mortality of TRAF3$^{-/-}$ mice and, more specifically, to explore the *in vivo* functions of TRAF3 in B lymphocytes of adult mice, we recently generated and characterized B cell-specific TRAF3-deficient (B-TRAF3$^{-/-}$; TRAF3$^{flox/flox}$CD19$^{+/cre}$) mice [7,8]. We found that specific deletion of TRAF3 in B lymphocytes results in marked peripheral B cell hyperplasia, due to remarkably prolonged survival of mature B cells independent of the B cell survival factor BAFF. This eventually leads to spontaneous development of splenic marginal zone lymphomas (MZL) or B1 lymphomas in mice by 18 months of age [7,8]. B lymphomas spontaneously developed in B-TRAF3$^{-/-}$ mice are easily transplantable to immunodeficient NOD SCID recipient mice, demonstrating their malignant nature [8]. Corroborating our findings, biallelic deletions and inactivating mutations of the *Traf3* gene have been identified in human patients with a variety of B cell neoplasms, including multiple myeloma (MM), splenic marginal zone lymphoma (MZL), B cell chronic lymphocytic leukemia (B-CLL), mantle cell lymphoma (MCL), Waldenström's macroglobulinemia (WM), and Hodgkin lymphoma [10-17]. Taken together, the findings obtained from both the B-TRAF3$^{-/-}$ mice and human patients provide conclusive evidence that *Traf3* is a tumor suppressor gene in B lymphocytes.

TRAF3 Downstream Signaling Pathways

In pursuing the signaling pathways downstream of TRAF3 inactivation, we found that both TRAF3$^{-/-}$ premalignant B cells and B

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Received December 29, 2014; **Accepted** February 23, 2015; **Published** February 25, 2015

Citation: Moore CR, Edwards SKE, Xie P (2015) Targeting TRAF3 Downstream Signaling Pathways in B cell Neoplasms. J Cancer Sci Ther 7: 067-074. doi:10.4172/1948-5956.1000327

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lymphomas exhibit constitutive NF-κB2 activation but reduced PKCδ nuclear levels [7,8]. The proximal signaling events of how TRAF3 inhibits the activation of noncanonical NF-κB, NF-κB2, have been explicitly elucidated in the literature (Figure 1) [20-24]. It was found that in the absence of stimulation, TRAF3 and TRAF2 assemble a regulatory complex with cIAP1/2 and NIK. Assembly of this complex requires direct binding between TRAF3 and NIK, and also direct association between TRAF2 and cIAP1/2. TRAF3 and TRAF2 heteromeric interaction bring all 4 proteins into the complex. In this complex, the E3 ubiquitin ligase cIAP1/2 induces K48-linked polyubiquitination of NIK and targets NIK for proteasome-mediated degradation, thereby inhibiting NF-κB2 activation. Therefore, in the absence of stimulation, TRAF3 promotes cellular apoptosis in resting B cells by targeting NIK for degradation and inhibiting NF-κB2 activation (Figure 1A) [20-24].

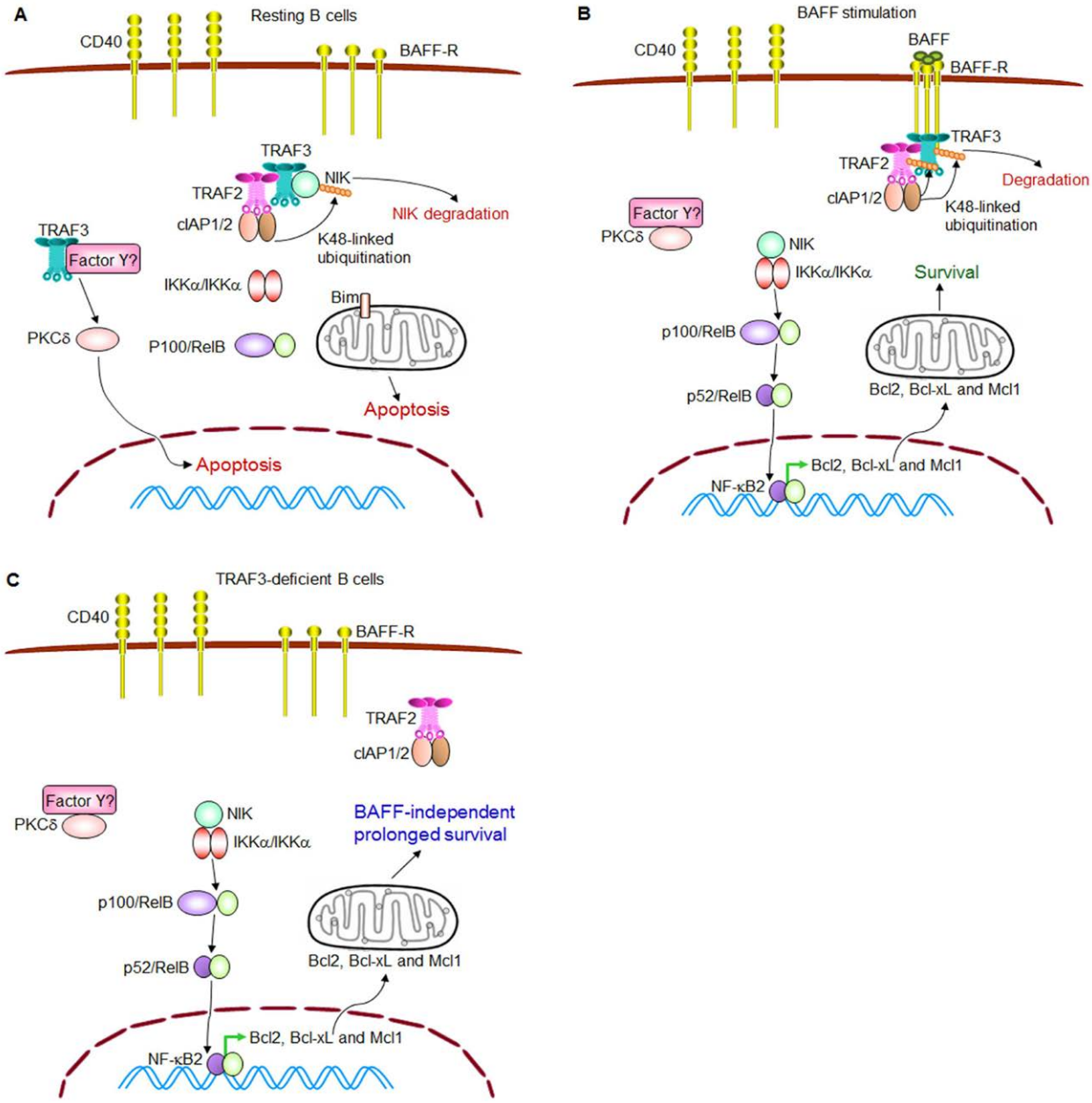

Figure 1: TRAF3 and BAFF signaling pathways in regulating B cell survival. (**A**) TRAF3 promotes apoptosis in resting B cells. In the absence of stimulation, TRAF3 inhibits NF-κB2 activation while facilitating PKCδ nuclear translocation to promote B cell apoptosis. TRAF3 and TRAF2 constitutively form a complex with cIAP1/2 and NIK, targeting NIK for K48-linked polyubiquitination and degradation, thereby inhibiting NF-κB2 activation in B cells. How TRAF3 facilitates PKCδ nuclear translocation remains to be determined (depicted as through binding to an unknown protein or multi-protein complex Factor Y in the figure). (**B**) BAFF stimulates B cell survival. Upon ligand engagement, trimerized BAFF-R recruits TRAF3 and the associated TRAF2-cIAP1/2 complex to membrane rafts, and thus releases NIK, allowing NIK accumulation and NF-κB2 activation. Meanwhile, Factor Y is also released from TRAF3 and may sequester PKCδ in the cytosol. NF-κB2 activation together with reduced nuclear level of PKCδ functions to induce the expression of anti-apoptotic proteins, and thus promotes B cell survival. (**C**) TRAF3 deficiency causes BAFF-independent B cell survival. Similar to BAFF stimulation, deletion of TRAF3 from B cells (caused by either biallelic deletions or inactivating mutations of the *Traf3* gene) also releases NIK from the TRAF2-cIAP1/2 complex, causing constitutive NF-κB2 activation. In the absence of TRAF3, Factor Y may also sequester PKCδ in the cytosol. Therefore, constitutive NF-κB2 activation together with reduced nuclear level of PKCδ leads to BAFF-independent, prolonged survival of TRAF3$^{-/-}$ B cells.

In response to stimulation by the B cell survival factor BAFF or the co-stimulatory ligand CD154, trimerized BAFF receptor (BAFF-R) or CD40 recruits the translocation of cytoplasmic TRAF3 and TRAF2 to the receptor signaling complex in sphingolipid-enriched membrane rafts (Figure 1B). This releases NIK from the TRAF3-TRAF2-cIAP1/2 complex, allowing NIK accumulation, which induces the phosphorylation of IKKα and then the processing of NF-κB2 from the inactive precursor p100 into the active p52. Processed p52 and RelB dimers subsequently translocate into the nucleus to induce the transcription of anti-apoptotic proteins of the Bcl2 family, including Bcl2, Bcl-xL and Mcl1, thereby promoting B cell survival (Figure 1B) [18,25]. Similar to BAFF or CD40 stimulation, deletion of TRAF3 from B cells, caused by either biallelic deletions or inactivating mutations of the *Traf3* gene, also releases NIK from the TRAF2-cIAP1/2 complex and thus allows NIK accumulation, leading to constitutive NF-κB2 activation and BAFF-independent survival of B cells (Figure 1C). However, how TRAF3 promotes PKCδ nuclear translocation remains to be determined.

Targeting NF-κB2 Activation in TRAF3$^{-/-}$ B Cell Neoplasms

Based on the above findings about TRAF3 downstream signaling pathways, we first tested whether constitutive NF-κB2 activation can serve as a therapeutic target in B cell neoplasms with TRAF3 deletions or inactivating mutations. To test this, we employed genetic and pharmacological means to target the NF-κB2 activation (Figure 2). Using lentiviral shRNA vectors, we demonstrated that NF-κB2 shRNA 653 and 1226 knock down both p100 and p52 NF-κB2 proteins by ~95% and ~ 75% reductions, respectively. Interestingly, both NF-κB2 shRNA 653 and 1226 inhibit the proliferation and induce apoptosis in TRAF3$^{-/-}$ mouse B lymphoma cells [8]. Importantly, the potency of the two shRNAs in knocking down NF-κB2 protein levels correlates with their ability in inhibiting the proliferation and inducing apoptosis in TRAF3$^{-/-}$ B lymphoma cells [8]. These data suggest that constitutive NF-κB2 activation is one major oncogenic pathway in TRAF3$^{-/-}$ B cells, which could be therapeutically targeted.

In an effort to test pharmacological means to target the NF-κB2 activation, we examined the effects of an inhibitor of NF-κB2, oridonin, and an inhibitor of IKKβ, BMS-345541, on primary TRAF3$^{-/-}$ B lymphoma cells [8]. The effects of these agents were compared with the activities of drugs used clinically to treat B lymphomas or leukemias, including vincristine, all *trans*-retinoic acid (ATRA), doxorubicin, and cyclophosphamide. We found that oridonin exhibits potent dose-dependent tumoricidal activity on primary TRAF3$^{-/-}$ B lymphoma cells, while BMS-345541 and the four clinical drugs are inactive [8]. We also verified the therapeutic effects of oridonin using three TRAF3$^{-/-}$ B lymphoma cell lines (27-9, 105-8, and 115-6) derived from B-TRAF3$^{-/-}$ mice [8,26] and three human patient-derived MM cell lines (8226, KMS11, and LP1) containing TRAF3 deletions or mutations (Edwards and Xie, unpublished data). Using transplanted TRAF3$^{-/-}$ mouse B lymphoma models, we further demonstrated that oridonin exhibits potent anti-tumor activity in whole animals and is able to prolong the survival of the tumor-bearing mice [26]. Together, our *in vitro* and *in vivo* data provided the preclinical evidence for the therapeutic potential of oridonin in the treatment of B cell neoplasms with TRAF3 deletions or inactivating mutations.

To understand the mechanisms of oridonin, we determined its

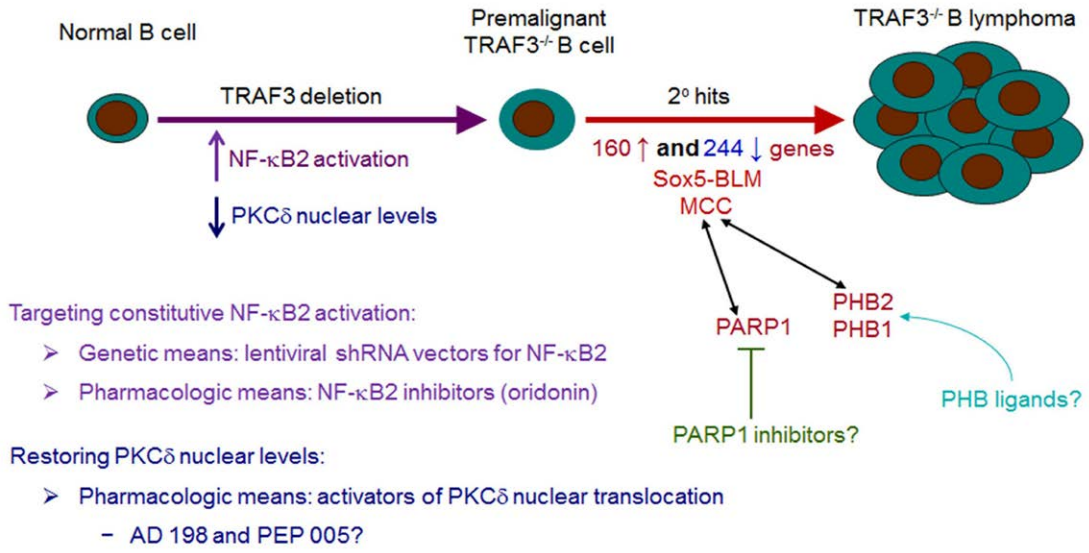

Figure 2: Targeting TRAF3 downstream signaling pathways in B cell neoplasms. To test whether constitutive NF-κB2 activation can serve as a therapeutic target in B cell neoplasms, we employed genetic and pharmacological means to target the NF-κB2 pathway. Our results demonstrated that lentiviral shRNA vector-mediated knockdown of NF-κB2 robustly inhibits the proliferation and induces apoptosis in TRAF3$^{-/-}$ B lymphoma cells. We also found that oridonin, an inhibitor of NF-κB2, exhibits potent anti-tumor activities on TRAF3$^{-/-}$ B lymphomas and human MM. To restore PKCδ nuclear levels, we tested two pharmacological activators of PKCδ nuclear translocation, AD 198 and PEP005. We found that AD 198, but not PEP005, potently kills malignant B cells. However, AD 198 does not induce PKCδ nuclear translocation but specifically targets c-Myc in malignant B cells. Therefore, further studies (using genetic means or more specific PKCδ activators) are required to evaluate the therapeutic potential of activating PKCδ nuclear translocation in B cell neoplasms. Interestingly, TRAF3$^{-/-}$ B cells purified from young B-TRAF3$^{-/-}$ mice exhibit prolonged survival but do not proliferate autonomously, and are therefore premalignant B cells. This suggests that additional oncogenic alterations are required for B lymphomagenesis. To identify such secondary oncogenic alterations, we performed a microarray analysis and identified 160 up-regulated genes and 244 down-regulated genes in TRAF3$^{-/-}$ B lymphomas. Among these, we further studied a novel oncogene, *MCC*, and a new isoform of Sox5, Sox5-BLM, expressed in TRAF3$^{-/-}$ B lymphomas. Since our proteomic data identified PARP1 and PHB2/PHB1 as two signaling hubs of MCC in human MM cells, we are currently testing the therapeutic efficacy of PARP1 inhibitors and PHB ligands in B cell neoplasms with TRAF3 inactivation or aberrant *MCC* expression.

effects on nuclear levels of NF-κB2 and NF-κB1 subunits in TRAF3$^{-/-}$ B lymphoma cells. Our results showed that oridonin predominantly inhibits the activation of NF-κB2, but also moderately reduces the activation of NF-κB1 in TRAF3$^{-/-}$ B lymphoma cells [8]. In contrast, nuclear translocation of PKCδ, or activation of ERK, p38, JNK, and AKT is unaffected by oridonin [8]. Thus, the potent tumoricidal effects of oridonin can be ascribed to its activity in inhibiting the activation of NF-κB2 (primarily) and also NF-κB1 (moderately). Our findings suggest that oridonin or closely related NF-κB2 inhibitors should be considered as new candidates for the treatment of B cell neoplasms characterized by genetic/epigenetic inactivation of TRAF3 or TRAF3-dependent signaling pathways.

Targeting PKCδ Nuclear Translocation in TRAF3$^{-/-}$ B Cell Neoplasms

Available evidence suggests that the second signaling pathway downstream of TRAF3 inactivation, the reduced PKCδ nuclear translocation, also contributes to prolonged B cell survival. First, the splenic B cell numbers of PKCδ$^{-/-}$ mice are greatly increased [27,28], similar to that observed in B-TRAF3$^{-/-}$ mice [7,9] and BAFF- or NF-κB2 transgenic mice [29,30]. Second, the principal B cell survival factor, BAFF, also decreases PKCδ nuclear levels in splenic B cells [31]. In light of the above evidence, we next sought to evaluate the therapeutic potential of PKCδ activation in B cell neoplasms with TRAF3 deletions or inactivating mutations (Figure 2). To restore PKCδ nuclear levels, we tested two pharmacological activators of PKCδ nuclear translocation, N-benzyladriamycin-14-valerate (AD 198) and ingenol-3-angelate (PEP005) [32-37]. We found that AD 198 potently kills TRAF3$^{-/-}$ mouse B lymphoma and human MM cell lines with TRAF3 deletions or mutations [26]. In contrast, PEP005 displays contradictory anti- or pro-tumor activities on different malignant B cell lines [26].

Our detailed mechanistic investigation revealed that AD 198 and PEP005 act through distinct biochemical mechanisms in malignant B cells [26]. Interestingly, although PKCδ was identified as the principal target of AD 198 in other cancer cells [32,33], AD 198-induced apoptosis of malignant B cells is mediated through PKCδ-independent mechanisms. We found that AD 198 does not induce PKCδ nuclear translocation in both TRAF3$^{-/-}$ mouse B lymphoma and human MM cells [26]. In contrast, PEP005 promotes the rapid translocation of PKCδ from the cytosol to the nuclei and membranes (including mitochondria) in TRAF3$^{-/-}$ malignant B cells [26]. Interestingly, PEP005 also activates multiple additional signaling pathways in these cells, including PKCα, PKCε, NF-κB1, ERK, JNK, and Akt [26].

In cancer, PKCα and PKCε are generally linked to proliferation or survival and thus considered as oncogenes. In contrast, PKCδ has a pro-apoptotic function in a variety of cancer cells [34,36,38]. Activation of PKC isoforms signals further downstream events, such as the activation of p38, ERK, JNK or NF-κB1 in melanoma, myeloid leukemia and colon cancer cells [37-41], which are all observed in malignant B cells [26]. Therefore, the ultimate effect of cell proliferation or apoptosis induction by PEP005 depends on the balance between the levels and activities of pro-apoptotic (PKCδ) and anti-apoptotic (PKCα and PKCε) isoforms of PKC as well as their crosstalk with different signaling pathways (MAPKs, NF-κB1, and Akt) in each malignant B cell line. Indeed, we detected varying levels of different PKC isoforms (α, δ and ε) in different TRAF3$^{-/-}$ mouse B lymphoma and human MM cell lines, and this may contribute to the observed divergent responses of these cells to PEP005 [26]. Our findings provide new insights into the complexity of the signaling pathways controlled by PEP005 in malignant B cells. However, further studies (using genetic means or more specific PKCδ activators) are required to establish the possibility of activating PKCδ nuclear translocation as a therapeutic avenue for B cell neoplasms with TRAF3 deletions or inactivating mutations.

AD198 Specifically Targets c-Myc in B Cell Neoplasms

In pursuing the therapeutic mechanisms of AD 198, we found that it specifically targets c-Myc in TRAF3$^{-/-}$ mouse B lymphoma and human MM cells in dose-dependent and time-dependent manner [26]. Both the mRNA and protein levels of c-Myc are drastically and rapidly suppressed by AD 198 [26]. AD 198 inhibits c-Myc protein levels as early as 1 hour after treatment in TRAF3$^{-/-}$ mouse B lymphoma and human MM cell lines [26]. The c-Myc protein is a central regulator of B cell survival and proliferation, and has a short half-life (about 20 - 30 minutes) [42,43]. The promoter regions of both human and mouse *c-Myc* genes contain binding sites for AP-1, a transcription factor directly activated by ERK, p38 and JNK signaling pathways [44-46]. AP-1 is also indirectly inhibited by Akt activity [44]. Interestingly, we found that AD 198 inhibits ERK, p38 and JNK activation, but promotes Akt activation in TRAF3$^{-/-}$ malignant B cells [26]. In this context, our results suggest that AD 198 targets c-Myc by inhibiting c-Myc transcription in malignant B cells, which is mediated through inhibition of ERK, p38 and JNK pathways as well as activation of the Akt pathway [26]. However, we could not exclude additional mechanisms, especially considering that AD 198 inhibits *E. coli* RNA polymerase or chicken leukemic RNA polymerase activity through drug-template interaction or enzyme inactivation, respectively [47]. Regardless of the exact mechanisms, our results showed that AD 198 potently kills TRAF3$^{-/-}$ mouse B lymphoma and human MM cells by targeting c-Myc.

Given that elevated expression of c-Myc is ubiquitously associated with numerous B cell malignancies [48], we extended the investigation of AD 198 to TRAF3-sufficient B lymphoma cell lines. We found that AD198 also potently inhibits the proliferation/survival and suppresses c-Myc expression in six TRAF3-sufficient mouse and human B lymphoma cell lines [26]. Thus, AD 198 also has therapeutic potential and targets c-Myc in TRAF3-sufficient B lymphomas. To further understand whether c-Myc suppression is the therapeutic mechanism of AD 198 in malignant B cells, we performed c-Myc reconstitution experiments. We generated a lentiviral expression vector of FLAG-tagged human c-Myc, pUB-FLAG-c-Myc-Thy1.1, in which constitutive expression of FLAG-c-Myc is driven by the ubiquitin promoter (pUB). Following treatment with AD 198, although endogenous c-Myc protein levels are strikingly decreased, the transduced FLAG-c-Myc protein levels are not suppressed by AD 198 in human MM cells [26]. The observation that expression of the transduced FLAG-c-Myc (driven by the ubiquitin promoter) is not suppressed by AD 198 indicates that AD 198 targets the transcription of endogenous c-Myc via its effects on the c-Myc promoter. In support of the major role of c-Myc down-regulation, we found that lentiviral vector-mediated constitutive expression of FLAG-c-Myc confers robust resistance to the anti-tumor effects of AD 198 in human MM cells [26]. Together, our results indicate that c-Myc suppression is a major contributing factor to the anti-tumor effects of AD 198 in both TRAF3$^{-/-}$ and TRAF3-sufficient B cell neoplasms.

Using transplanted TRAF3$^{-/-}$ mouse B lymphoma models, we also demonstrated that AD 198 has potent anti-tumor activities in whole animals. It has been previously shown that AD 198 does not exhibit significant organ toxicities at therapeutic doses, and is cardioprotective in rodent models [40,49-51]. Indeed, we found that in NOD SCID mice

transplanted with TRAF3$^{-/-}$ mouse B lymphomas, administration of AD 198 drastically extends the survival of mice and inhibits the growth and metastasis of B lymphomas [26]. In fact, AD 198 demonstrates a higher *in vivo* potency than oridonin, an inhibitor of NF-κB2 and NF-κB1 pathways [26]. Our findings thus support further clinical studies of AD 198 as an anti-cancer agent for B cell neoplasms involving TRAF3 inactivation or Myc up-regulation.

Secondary Oncogenic Hits in TRAF3$^{-/-}$ Mouse B Lymphomas

We noticed that TRAF3$^{-/-}$ B cells purified from young B-TRAF3$^{-/-}$ mice exhibit prolonged survival but do not proliferate autonomously [7], and are therefore premalignant B cells. Consistent with this, no B lymphoma development is observed in B-TRAF3$^{-/-}$ mice younger than 9 months old [8]. The long latency of B lymphoma development observed in B-TRAF3$^{-/-}$ mice suggests that TRAF3 inactivation and its downstream signaling pathways are not sufficient and that additional oncogenic alterations are required for B lymphomagenesis (Figure 2). Although TRAF3 deletions or mutations exist in human patients with B cell neoplasms, it is not known whether TRAF3 inactivation is the primary or secondary oncogenic event in human samples. Therefore, B-TRAF3$^{-/-}$ mice offer the unique advantage to identify secondary oncogenic pathways that drive B lymphomagenesis in the context of TRAF3 inactivation.

To identify such secondary oncogenic alterations and to discover new therapeutic targets for the treatment of B cell neoplasms, we performed global gene expression profiling of TRAF3$^{-/-}$ mouse B lymphomas by transcriptomic microarray analysis [52,53]. Our results of the microarray analysis identified 160 up-regulated genes and 244 down-regulated genes in TRAF3$^{-/-}$ B lymphomas as compared to TRAF3-sufficient littermate control spleens (cut-off fold of changes: 2-fold up or down, $p < 0.05$) (NCBI GEO accession number: GSE48818) [52,53]. From the 160 up-regulated genes, we selected 13 genes for further verification by quantitative real time PCR using TaqMan gene expression assay kits. Our data verified the mRNA up-regulation of the 13 genes examined, including *MCC*, *Sox5*, *Diras2*, *Tbc1d9*, *Ccbp2*, *Btbd14a*, *Sema7a*, *Twsg1*, *Ppap2b*, *TCF4*, *Tnfrsf19*, *Zcwpw1,* and *Abca3* [52,54]. Striking up-regulation of these transcripts was verified in the three splenic B lymphoma samples used for the microarray analysis, and also confirmed in three additional splenic B lymphomas as well as ascites from two cases [52,54]. Thus, these 13 genes are recurrently up-regulated in B lymphomas spontaneously developed in different individual B-TRAF3$^{-/-}$ mice, and need to be further investigated as candidates of diagnostic markers or therapeutic targets in B cell neoplasms. We have further studied two of the up-regulated genes, *MCC* and *Sox5*.

MCC: A Novel Oncogene in B Lymphocytes

Mutated in colorectal cancer (*MCC*) was originally identified as a tumor suppressor gene in colorectal cancer (CRC). Our unexpected finding that MCC is strikingly up-regulated in TRAF3$^{-/-}$ mouse B lymphomas prompted us to further investigate the expression and function of MCC in B cell neoplasms. We demonstrated high levels of aberrant *MCC* expression in six human MM cell lines with TRAF3 deletions or relevant mutations [52]. *MCC* expression is also significantly elevated in a variety of primary human B cell malignancies, including primary effusion lymphoma (PEL), centroblastic lymphoma (CBL), diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma (BL), and MM [55-58]. However, expression of the transcript and protein of *MCC* is not detected in normal B cells or premalignant TRAF3$^{-/-}$ B cells, even after treatment with a variety of B cell stimuli, including CD40, B cell receptor (BCR), LPS (a TLR4 agonist), and CpG (a TLR9 agonist) [52]. These results suggest that aberrant *MCC* expression is specifically associated with B cell neoplasms.

We next investigated the functions of MCC in malignant B cells. In contrast to the cell cycle blocking and proliferation inhibitory effects of MCC overexpression reported in fibroblasts and CRCs [59-62], we observed that overexpression of MCC does not affect cell cycle progression, cell proliferation, or cell survival in human MM cells [52]. These results thus argue against a negative role for MCC in the survival or proliferation of malignant B cells. Furthermore, we found that lentiviral shRNA vector-mediated knockdown of MCC induces apoptosis and inhibits proliferation in human MM cell lines with TRAF3 deletions or mutations [52]. Interestingly, MCC shRNA 1332 that results in a greater decrease in MCC protein level is also most effective at inducing apoptosis and inhibiting proliferation in human MM cells [52]. Together, our results demonstrate that MCC plays a positive role and is required for the survival and proliferation in human MM cells, indicating that *MCC* acts as an oncogene in B lymphocytes. Our knockdown studies also suggest that MCC could serve as a therapeutic target in B cell neoplasms.

To elucidate where MCC exerts its oncogenic roles in malignant B cells, we examined the subcellular localization of MCC using a biochemical fractionation method. Our results revealed that MCC protein is primarily localized in mitochondria, but also detectable in the ER, cytosol and nucleus in human MM cells [52]. To understand the mechanisms of MCC, we investigated MCC downstream signaling pathways using complementary overexpression and knockdown approaches. We found that MCC inhibits cleavage of caspases 8 and 3, down-regulates the cell cycle inhibitor p27, and up-regulates Mcl1, c-Myc and cyclin B1 as well as ERK phosphorylation in human MM cells [52]. Furthermore, we identified 365 proteins of the MCC-interactome in human MM cells using affinity purification followed by LC-MS/MS [52]. Among these, PARP1 and prohibitin-2 (PHB2) were recognized as two signaling hubs of the MCC interaction network in human MM cells [52]. PARP1 and PHB2/PHB1 have been previously shown to directly or indirectly interact with and/or regulate all MCC downstream targets identified in our study, including ERK, c-Myc, p27, cyclin B1, Mcl-1, caspase 8, and caspase 3 [63-66]. Taken together, our results indicate that MCC promotes cellular survival and proliferation by interacting with and modulating the interaction network centered at PARP1 and PHB2/PHB1 in malignant B cells. Given the preclinical evidence that both PARP1 and PHB2/PHB1 are excellent therapeutic targets in human cancers [63,66], our findings also implicate potential applications of PARP inhibitors and PHB ligands in the treatment of B cell neoplasms involving TRAF3 inactivation or aberrant *MCC* expression (Figure 2).

A Novel Isoform of Sox5, Sox5-BLM, is Expressed in TRAF3$^{-/-}$ B Lymphomas

Sox5 is a member of the Sox family of transcription factors. Corroborating our finding that Sox5 is strikingly up-regulated in TRAF3$^{-/-}$ mouse B lymphomas [54], Sox5 up-regulation has also been documented in DLBCL developed in Brd2 transgenic mice (GEO accession number: GSE6136) [67] and MM developed in XBP-1 transgenic mice (GEO accession number: GSE6980) [68]. Importantly, up-regulation of SOX5 mRNA has also been identified in human memory B cells [69], in clonal B cells of patients with hepatitis C virus (HCV)-associated B cell lymphoproliferative disorders mixed

cryoglobulinemia [70], and in human follicular lymphoma (FL) [71,72] and hairy cell leukemia (a sub-type of chronic lymphoid leukemia of B lymphocytes) [55]. Interestingly, up-regulation of SOX5 expression in a case of human FL is caused by a chromosomal translocation t(X;12), which fuses the promoter region of the G-protein coupled purinergic receptor *P2Y8* gene with the *SOX5* coding sequence [71]. In contrast, Sox5 protein expression is not detectable in normal B cells or premalignant TRAF3$^{-/-}$ B cells even after treatment with B cell stimuli [54], suggesting that up-regulation of Sox5 protein is selectively associated with B cell malignant transformation.

Our detailed cloning and sequencing studies revealed that the Sox5 expressed in TRAF3$^{-/-}$ mouse B lymphomas represents a novel isoform of Sox5, Sox5-BLM (GenBank accession number: KF793916), which contains a 35 amino acid (aa) deletion in the N-terminal region in front of the leucine zipper domain [54]. Our biochemical fractionation results demonstrated that Sox5-BLM is primarily localized in the nucleus of malignant B cells [54]. Furthermore, we found that Sox5-BLM regulates cell cycle progression by modulating p27 and β-catenin protein levels in transduced human MM cells [54]. However, whether SOX5 can serve as a therapeutic target in human B cell neoplasms involving TRAF3 inactivation or aberrant *SOX5* expression awaits further investigation.

Conclusions

In summary, our study identified a variety of candidate diagnostic markers for B cell malignancies, including TRAF3 inactivation, NF-κB2 constitutive activation, reduced PKCδ nuclear levels, and aberrant expression of MCC or SOX5. Our study also discovered a number of candidate therapeutic targets for the treatment of B cell neoplasms, including NF-κB2, PKCδ, MCC, PARP1, and PHB2. We have tested several drugs targeting NF-κB2 and PKCδ, and found that both oridonin and AD198 exhibit potent anti-tumor activities on B cell neoplasms with TRAF3 deletions or inactivating mutations. Oridonin acts by inhibiting the activation of NF-κB2 (primarily) and also NF-κB1 (moderately). Although we originally tested AD 198 as an activator of PKCδ, our results revealed that AD 198 does not affect PKCδ but specifically targets c-Myc in malignant B cells. Since our new proteomic data identified PARP1 and PHB2/PHB1 as two signaling hubs of the novel oncoprotein MCC in human MM cells, we are currently testing the therapeutic efficacy of PARP1 inhibitors and PHB ligands in B cell neoplasms with TRAF3 inactivation or aberrant *MCC* expression. Collectively, our findings indicate that restoration of TRAF3 downstream signaling pathways represents an important line of new therapeutic avenues for B cell neoplasms.

Acknowledgements

This study was supported by the National Institutes of Health grant R01CA158402 (P. Xie), the Department of Defense grant W81XWH-13-1-0242 (P. Xie), and a Research Development Award of Cancer Institute of New Jersey through Grant Number P30CA072720 from the National Cancer Institute (P. Xie).

Competing interests

The authors declare that they have no potential conflicts of interest.

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Citation: Moore CR, Edwards SKE, Xie P (2015) Targeting TRAF3 Downstream Signaling Pathways in B cell Neoplasms. J Cancer Sci Ther 7: 067-074. doi:10.4172/1948-5956.1000327

Macrophage 2015; 2: e1009. doi: 10.14800/Macrophage.1009; © 2015 by Almin I. Lalani, *et al*.
http://www.smartscitech.com/index.php/Macrophage

REVIEW

TRAF3: a novel tumor suppressor gene in macrophages

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Received: September 09, 2015
Published online: September 30, 2015

Tumor necrosis factor receptor-associated factor 3 (TRAF3), a member of the TRAF family of cytoplasmic adaptor proteins with E3 ligase activity, is ubiquitously expressed in various cell types of the immune system. It is shared for signaling by a variety of adaptive and innate immune receptors as well as cytokine receptors. Previous studies examining conditional TRAF3-deficient mouse models that have the *Traf3* gene specifically deleted in B lymphocytes or T lymphocytes have revealed the diverse and critical *in vivo* functions of TRAF3 in adaptive immunity. Although *in vitro* evidence points to a pivotal and indispensable role for TRAF3 in type I interferon production induced by pattern recognition receptors in macrophages and dendritic cells, the *in vivo* functions of TRAF3 in the innate immune system had long remained unclear. Three laboratories have recently addressed this gap in knowledge by investigating myeloid cell-specific TRAF3-deficient (genotype: $TRAF3^{flox/flox}LysM^{+/Cre}$) mice. The new evidence together demonstrates that specific ablation of TRAF3 in myeloid cells leads to inflammatory diseases, altered progression of diabetes, and spontaneous development of different types of tumors and infections in mice. These new findings indicate that TRAF3 acts as an anti-inflammatory factor and is required for optimal innate immunity in myeloid cells. Strikingly, the new evidence also identifies TRAF3 as a novel tumor suppressor gene in macrophages and other myeloid cells. In this review, we discuss and summarize the new findings and current knowledge about the multi-faceted regulatory roles and complex signaling mechanisms of myeloid cell TRAF3 in inflammation, innate immunity, and tumor development.

To cite this article: Almin I. Lalani, *et al*. TRAF3: a novel tumor suppressor gene in macrophages. Macrophage 2015; 2: e1009. doi: 10.14800/ Macrophage.1009.

Introduction

Tumor necrosis factor receptor-associated factor 3 (TRAF3), a member of the TRAF family of cytoplasmic adaptor proteins with E3 ligase activity, is ubiquitously expressed in various cell types of the immune system [1, 2]. It is broadly employed in signaling by a variety of adaptive and innate immune receptors as well as cytokine receptors [1, 2]. TRAF3 binds directly to almost all members of the tumor necrosis factor receptor (TNF-R) superfamily that do not contain death domains, including CD40, BAFF-R, TACI, BCMA, LT-βR, CD27, CD30, RANK, HVEM, EDAR, XEDAR, 4-1BB, OX-40, and GITR [1]. TRAF3 is indirectly recruited to the signaling complexes of pattern recognition receptors (PRRs) of the innate immune system through interactions with additional adaptor proteins, including MyD88 and TRIF for Toll-like receptors (TLRs), RIP2 for NOD-like receptors (NLRs), and MAVS for RIG-I-like receptors (RLRs) [1, 3-5]. TRAF3 also directly or indirectly regulates signaling by cytokine receptors, including receptors for M-CSF, GM-CSF, IL-2, IL-15, and IL-17 [1, 6, 7]. Consistent with the shared usage of TRAF3 by such a variety of immune receptors, increasing evidence from studies of conditional TRAF3-deficient mouse models demonstrates the

diverse and critical *in vivo* functions of TRAF3 in B and T lymphocytes of the adaptive immune system [1, 7].

We and Gardam *et al*. previously reported that B cell-specific TRAF3-deficient (B-TRAF3$^{-/-}$; TRAF3$^{flox/flox}$CD19$^{+/Cre}$) mice exhibit severe peripheral B cell hyperplasia and autoimmunity, due to vastly prolonged B cell survival and constitutive activation of the NIK-NF-κB2 pathway [8, 9]. These mice eventually develop splenic marginal zone lymphomas (MZL) or B1 lymphomas by 18 months of age [10]. We also found that TRAF3$^{-/-}$ B cells display enhanced production of cytokines and type I interferons (IFN) as well as elevated Ig isotype switching in response to signaling by TLR3, 4, 7/8, and 9 [11]. These observations indicate that TRAF3 is a critical regulator of peripheral B cell homeostasis and autoimmunity, and serves as an important tumor suppressor in B lymphocytes.

In contrast, T cell-specific TRAF3-deficient (T-TRAF3$^{-/-}$; TRAF3$^{flox/flox}$CD4-Cre) mice exhibit normal homeostasis of CD4 and CD8 T cells, but are defective in T cell-dependent IgG1 responses and in T cell-mediated immunity to infection with *Listeria monocytogenes* [12]. The defects in T cell-mediated immune responses are caused by compromised T cell receptor (TCR)/CD28 signaling in both TRAF3$^{-/-}$ CD4 and CD8 T cells [12]. Interestingly, T-TRAF3$^{-/-}$ mice contain markedly increased frequency and numbers of regulatory T cells (Treg) cells [12], but decreased numbers of CD8 central memory T (Tcm) cells [13] and invariant natural killer T (iNKT) cells [14]. It was revealed that TRAF3 inhibits IL-2 signaling by facilitating the recruitment of the tyrosine phosphatase TCPTP to the IL-2 receptor complex to dephosphorylate Jak1, Jak3 and STAT5, thereby restraining thymic Treg development [15]. On the other hand, TRAF3 is required for TCR-induced expression of T-bet and CD122, two molecules required for IL-15 signaling, and as a consequence, IL-15-mediated homeostasis of CD8 Tcm cells and development of iNKT cells are impaired in T-TRAF3$^{-/-}$ mice [13, 14]. Furthermore, Treg cell-specific TRAF3-deficient (Treg-TRAF3$^{-/-}$; TRAF3$^{flox/flox}$Foxp3-Cre) mice exhibit heightened formation of germinal centers (GCs) and increased production of high-affinity IgG antibodies, resulting from decreased numbers of follicular Treg cells (T_{FR} cells) and increased numbers of follicular T helper cells (T_{FH} cells) [16]. It is found that TRAF3 signaling in Treg cells is required to maintain high level expression of the inducible co-stimulator (ICOS), which in turn is essential for T_{FR} cell generation in GCs and inhibition of antibody responses [16]. Both T-TRAF3$^{-/-}$ and Treg-TRAF3$^{-/-}$ mice have increased numbers of CD4 effector/memory T cells, suggesting that TRAF3$^{-/-}$ Treg cells might have defects in suppression of Th1 responses [7]. Collectively, the findings obtained from B-TRAF3$^{-/-}$, T-TRAF3$^{-/-}$, and Treg-TRAF3$^{-/-}$ mice indicate that TRAF3 is a highly versatile regulator of different lymphocyte subpopulations in the adaptive immune system and thus adaptive immune responses.

Different from adaptive immune responses, inflammation and innate immunity are mainly mediated by myeloid cells, including granulocytes, monocytes, macrophages and dendritic cells (DCs) [17]. These cells constitutively or inducibly express a number of receptors of the TNF-R, TLR, NLR, and RLR families as well as cytokine receptors, whose signals are regulated by TRAF3 [1, 2, 6, 7, 17, 18]. In particular, macrophages represent a major type of innate immune cells that initiate inflammatory responses and host defense against infections by producing pro-inflammatory cytokines and type I IFN [18]. Although *in vitro* evidence indicates that TRAF3 regulates pro-inflammatory cytokine and type I IFN production in macrophages and DCs [19, 20] almost a decade ago, the *in vivo* functions of TRAF3 in the innate immune system had remained elusive. Now three laboratories have addressed this gap in knowledge by investigating myeloid cell-specific TRAF3-deficient (M-TRAF3$^{-/-}$; TRAF3$^{flox/flox}$LysM$^{+/Cre}$) mice [21-23]. It is shown that young adult M-TRAF3$^{-/-}$ mice have normal sized lymphoid organs and also normal frequency and numbers of myeloid cell populations in various hematopoietic compartments [21]. This indicates that specific ablation of TRAF3 in myeloid cells neither affects the development nor alters the homeostasis of myeloid cells in young adult mice [21]. However, evidence from all three groups indeed demonstrates that TRAF3 is a crucial intrinsic regulator of myeloid cell functions [21-23]. Here we review the new findings about the multi-faceted regulatory roles of myeloid cell TRAF3 in inflammation, innate immunity, and tumor development, which identify TRAF3 as a novel tumor suppressor in macrophages.

TRAF3-mediated regulation of inflammatory responses in macrophages

The intensity and duration of macrophage-mediated inflammatory responses need to be tightly controlled to avoid tissue damage and inflammatory diseases [24]. Previous *in vitro* evidence suggests that TRAF3 acts as an anti-inflammatory factor in macrophages, as TLR-induced pro-inflammatory cytokine production is enhanced by TRAF3 deficiency in bone marrow-derived macrophages (BMDMs) and DCs [19, 20]. Consistent with the *in vitro* observations, M-TRAF3$^{-/-}$ mice display elevated serum levels of the pro-inflammatory cytokines IL-6 and IL-12 but decreased serum levels of the anti-inflammatory cytokine IL-10 in response to *i.p.* injection with LPS (an agonist for TLR4) or polyI:C (an agonist for TLR3) [21]. Thus, TRAF3 deficiency in myeloid cells appears to favor pro-inflammatory responses following *in vivo* challenge with LPS or polyI:C. In support of this notion, aging M-TRAF3$^{-/-}$ mice (15-22 months old) spontaneously develop chronic inflammation often affecting multiple organs, including the liver, gastrointestinal (GI) tract, lung, kidney, pancreas, and

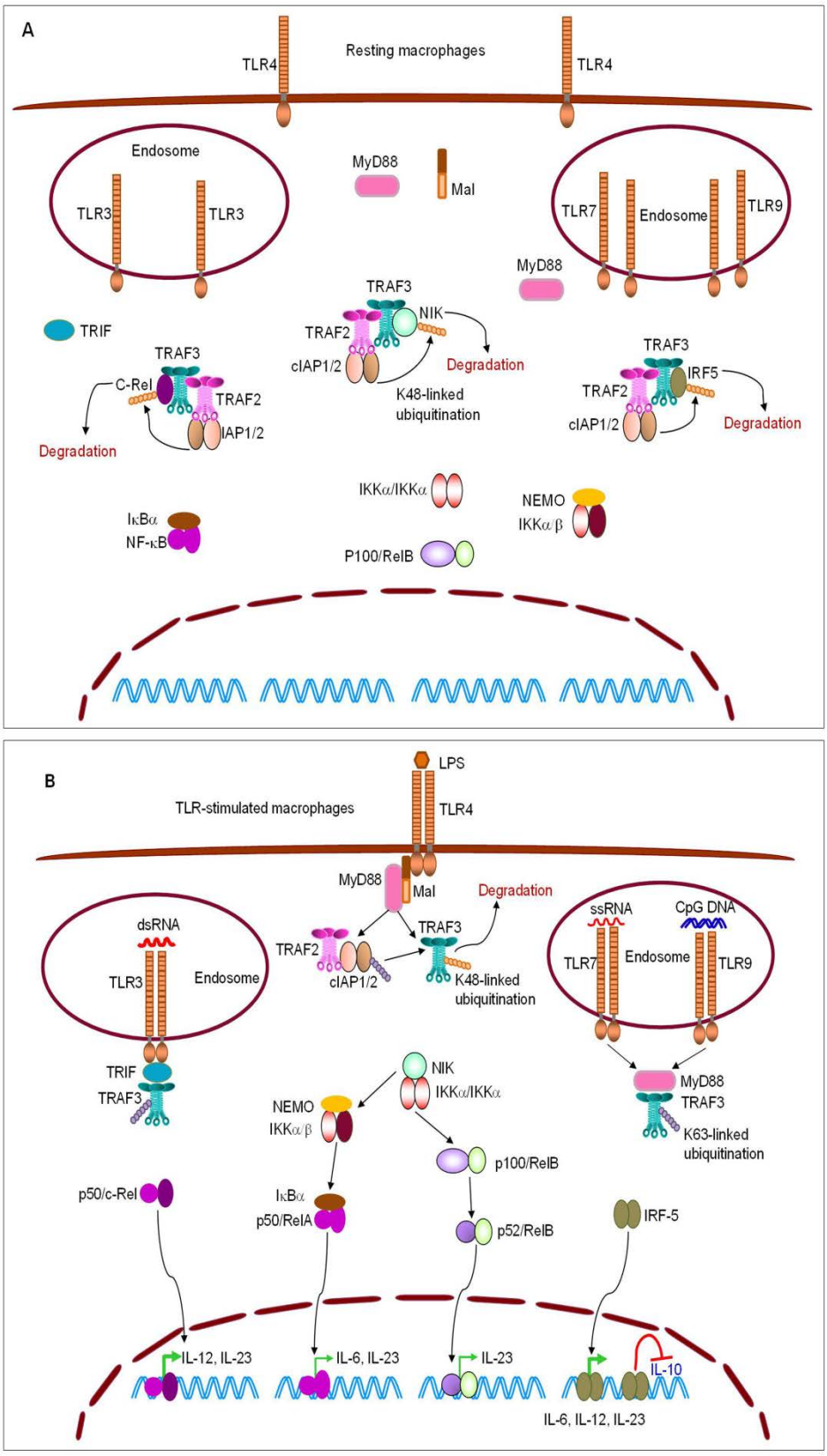

Figure 1. TRAF3-mediated inhibition of proinflammatory cytokine production induced by TLRs in macrophages. (**A**) TRAF3 targets NIK, c-Rel and IRF5 for degradation in resting macrophages. In the absence of stimulation, TRAF3 constitutively binds to NIK, c-Rel, and IRF5, and bring them to the TRAF3-TRAF2-cIAP1/2 complexes. In these complexes, the E3 ligases cIAP1/2 catalyze the K48-linked ubiquitination on NIK, c-Rel, and IRF5, thereby targeting them for proteasome-mediated degradation. Thus, TRAF3 prevents the activation of NF-κB2, NF-κB1, and IRF5 in resting macrophages. (**B**) TRAF3 inhibits TLR-induced proinflammatory cytokine production in macrophages. In response to LPS stimulation, dimerized or oligomerized TLR4 recruits Mal and MyD88, which in turn recruits TRAF3 and the associated TRAF2-cIAP1/2 complex to the receptor signaling complex at the plasma membrane. This allows the activation of cIAP1/2 to target TRAF3 for K48-linked ubiquitination and degradation. Similarly, stimulation of TLR7 by ssRNA or stimulation of TLR9 by CpG DNA also recruits TRAF3 via MyD88, while engagement of TLR3 by dsRNA recruits TRAF3 via TRIF to the receptor signaling complexes at the endosome membrane. Recruitment of TRAF3 by dimerized or oligomerized TLRs via MyD88 or TRIF disrupts the interaction between TRAF3 and NIK, c-Rel, or IRF5. This results in the accumulation of NIK, c-Rel and IRF5, and subsequent activation of NF-κB2 (p52/RelB), p50/c-Rel, and IRF5, which promote the expression of the proinflammatory cytokines IL-6, IL-12, and IL-23 in stimulated macrophages. In addition, nuclear IRF5 also inhibits the expression of the anti-inflammatory cytokine IL-10. Ablation of TRAF3 from macrophages mimics TLR engagement and also releases NIK, c-Rel and IRF5 from the TRAF2-cIAP1/2 complexes, allowing the accumulation of NIK, c-Rel and IRF5. Therefore, TLR agonists induce enhanced production of the proinflammatory cytokines IL-6, IL-12 and IL-23 but decreased production of the anti-inflammatory cytokine IL-10 in TRAF3$^{-/-}$ macrophages. Transcription factors (p50/c-Rel and IRF5) that play major roles in driving the production of IL-6, IL-12 and IL-23 are shown in bold green arrows. TRAF3-independent TLR signaling pathways, including TRAF6- or TRAF2-induced activation of ERK1/2, p38, JNK1/2 and NF-κB1 (p50/RelA), are not depicted in the figures.

heart [21]. Furthermore, in the dextran sulfate sodium (DSS)-induced colitis model, M-TRAF3$^{-/-}$ mice exhibit exacerbated colon inflammation, as demonstrated by reduced survival rate, worsened bodyweight loss, as well as more severe mucosal damage and colon shortening [22]. Indeed, colonic macrophages purified from the DSS-treated M-TRAF3$^{-/-}$ mice express significantly higher levels of proinflammatory cytokines, including IL-6, IL-12 and IL-23

[22]. Taken together, these *in vivo* data demonstrate that TRAF3 normally inhibits inflammation in macrophages and other myeloid cells.

To understand the mechanisms underlying TRAF3-mediated inhibition of proinflammatory cytokine production, several groups have carefully compared the early signaling events of TLR4 engagement in macrophages in the presence or absence of TRAF3. It was previously proposed that TRAF3 inhibits TLR4-induced activation of mitogen-activated protein kinases (MAPKs) in a model whereby TRAF3 degradation is required for the release of the TAK1 signaling complex from the membrane receptor into the cytosol to activate the MAPK signal cascades [2, 25]. However, TLR4-induced activation of MAPKs, including JNK1/2, ERK1/2, and p38, is neither enhanced nor prolonged but appears to be normal in different models of TRAF3-deficient macrophages [19, 21, 22]. These include BMDMs derived from TRAF3$^{-/-}$ chimeric mice, BMDMs and peritoneal exudate macrophages (PEMs) derived from M-TRAF3$^{-/-}$ mice, and BMDMs derived from TRAF3$^{flox/flox}$Cre^{ER} mice and treated with the Cre^{ER} inducer tamoxifen [19, 21, 22]. Therefore, the elevated production of the proinflammatory cytokines IL-6, IL-12 and IL-23 in response to TLR4 stimulation observed in TRAF3-deficient macrophages could not be attributed to the hyperactivation of MAPKs. These data argue against an inhibitory role for TRAF3 in TLR4-induced activation of MAPKs as previously proposed.

In contrast to the unaltered MAPK activation, constitutive activation of the noncanonical NF-κB (NF-κB2) pathway was consistently detected in TRAF3-deficient macrophages and neutrophils as demonstrated by enhanced accumulation of NIK and processing of NF-κB2 (from p100 to p52) as well as increased nuclear levels of the p52-RelB dimer [21, 22]. In fact, constitutive processing of NF-κB2 from the inactive precursor p100 to the active p52 observed in TRAF3-deficient macrophages and neutrophils is as robust as that observed in TRAF3-sufficient counterparts after TLR4 stimulation [21]. Constitutive processing of NF-κB2 results from accumulation of NIK, as NIK was previously demonstrated to be targeted for K48-linked ubiquitination and proteasome-mediated degradation by the TRAF3-TRAF2-cIAP1/2 complex in resting cells [1, 2]. The NIK-NF-κB2 pathway has been shown to mediate inflammation in epithelial cells and hepatocytes [26, 27]. However, Jin *et al*. demonstrated that the enhanced TLR4-induced proinflammatory cytokine production is not reversed by the ablation of NIK, with the exception of IL-23 that is partially downregulated in the NIK-null background [22]. Thus, constitutive activation of the NIK-NF-κB2 pathway only plays a minor role in mediating the hyper-induction of the proinflammatory cytokine IL-23 in TRAF3-deficient macrophages in response to TLR4 stimulation [22] (Fig. 1).

Similar to NIK, two transcription factors essential for the expression of proinflammatory cytokines, the NF-κB member c-Rel [28, 29] and the interferon regulatory factor 5 (IRF5) [30], are also targeted for degradation by TRAF3 in resting macrophages [22]. Both IRF5 and c-Rel have been associated with human inflammatory diseases [22, 28, 30]. As revealed by Jin *et al*., TRAF3 constitutively binds to c-Rel and IRF5, and therefore is responsible for recruiting c-Rel and IRF5 to the TRAF3-TRAF2-cIAP1/2 complexes [22]. In these complexes, the E3 ligases cIAP1/2 catalyze the K48-linked ubiquitination on c-Rel and IRF5, thereby targeting them for proteasome-mediated degradation in resting macrophages [22] (Fig. 1). Indeed, elevated protein levels of c-Rel and IRF5 are observed in both TRAF3- and TRAF2-deficient macrophages in the absence of stimulation [22]. TRAF2$^{-/-}$ macrophages also exhibit hyper-induction of proinflammatory cytokines in response to TLR4 stimulation and M-TRAF2$^{-/-}$ mice are also more susceptible to DSS-induced colon inflammation [22]. In line with these observations, the cIAP inhibitor SMAC mimetics (SM) causes marked accumulation of c-Rel and IRF5, and has proinflammatory actions on macrophages [22, 31]. It is known that c-Rel is specifically required for TLR-stimulated expression of IL-12 and IL-23 [22, 28, 29]. Similarly, IRF5 mediates the expression of multiple proinflammatory cytokines, including IL-6, IL-12, and IL-23, and inhibits the expression of the anti-inflammatory cytokine IL-10 [22, 30] (Fig. 1). Taken together, the above findings support the model that stabilized c-Rel and IRF5 are the major transcription factors that drive the hyper-induction of the proinflammatory cytokines IL-6, IL-12, and IL-23 in TRAF3- or TRAF2-deficient macrophages in response to TLR agonists (Fig. 1).

Interestingly however, Chen *et al*. reported that the anti-inflammatory function of TRAF3 in macrophages is not static, but is dynamically modulated according to the metabolic states [23]. Macrophages in adipose tissue and the liver are the major mediators of metabolic inflammation, promoting insulin resistance and metabolic disease progression in obesity [32]. Obesity is associated with chronic, low-grade inflammation, which contributes to insulin resistance and metabolic disease [32]. Chen *et al*. found that myeloid cell-specific deletion of TRAF3 has opposite effects on inflammation between lean and obese mice [23]. In lean mice, myeloid cell-specific deletion of TRAF3 increases the expression of proinflammatory cytokines in the liver and adipose tissue [23]. In contrast, TRAF3 deficiency in myeloid cells decreases the expression of proinflammatory cytokines in the liver and adipose tissue of obese mice, and largely prevents high-fat diet (HFD)-induced inflammation in these metabolic tissues [23]. Consequently, M-TRAF3$^{-/-}$ mice exhibit significantly attenuated insulin resistance and hepatic steatosis in models of either genetic (ob/ob) or HFD-induced

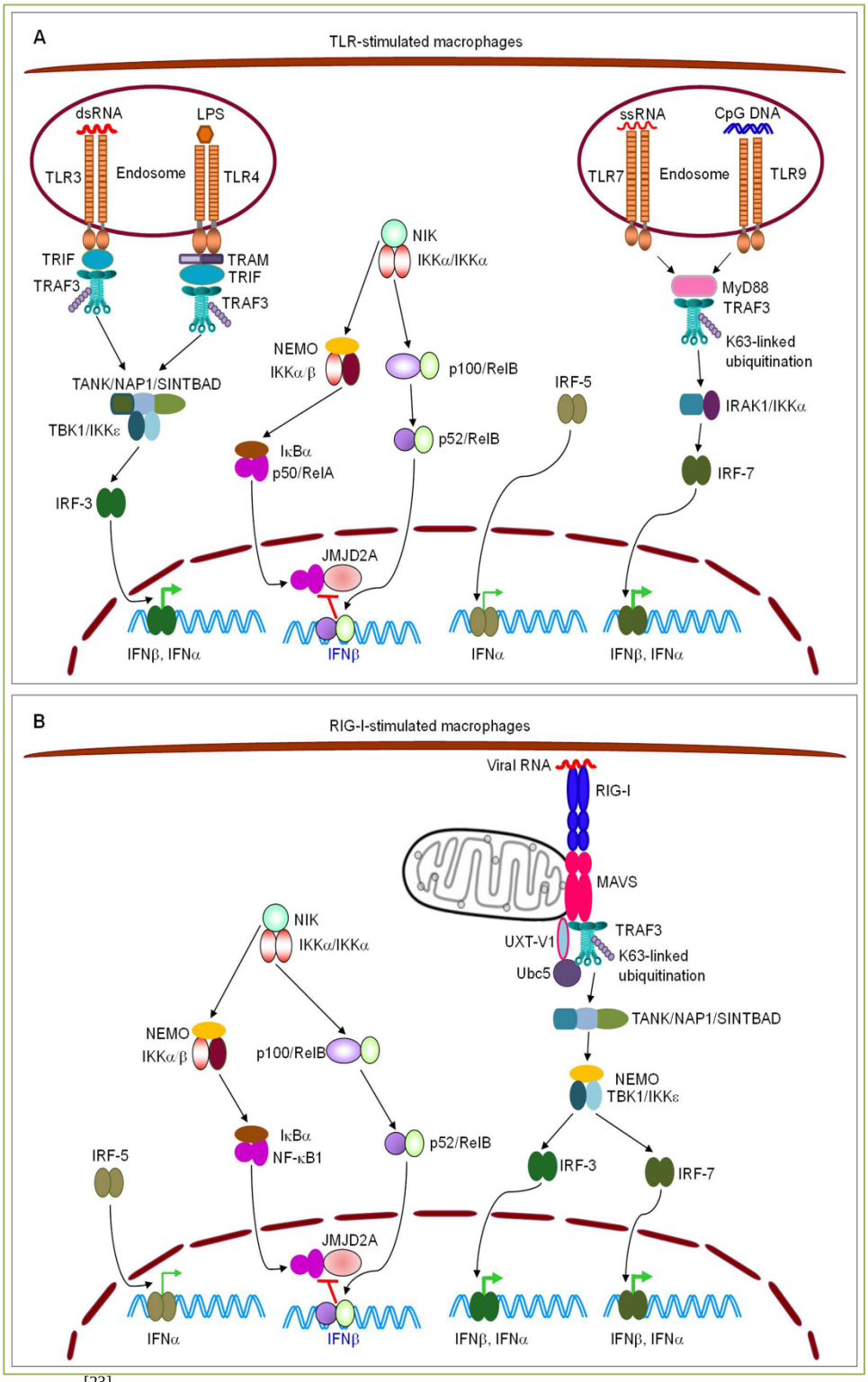

Figure 2. Requirement of TRAF3 in TLR- or RIG-I-induced type I IFN production in macrophages. (**A**) Roles of TRAF3 in type I IFN production induced by TLR3, 4, 7 and 9 signaling. Upon ligand binding in endosomes, TLR3 recruits TRAF3 via TRIF, while TLR7 and TLR9 recruit TRAF3 via MyD88. In response to LPS stimulation, internalized TLR4 also recruits TRAF3 via TRAM-TRIF to the endosome membrane. Recruitment of TRAF3 by endosomal TLRs activates the E3 ligase activity of TRAF3 to catalyze its own K63-linked ubiquitination, and subsequently mediates the activation of TBK1/IKKε in TRIF-dependent signaling or IRAK1/IKKα in MyD88-dependent signaling. This eventually leads to the phosphorylation, dimerization, and nuclear translocation of IRF3 or IRF7 to promote the expression of IFNβ and IFNα in stimulated macrophages. In addition, recruitment of TRAF3 by engaged TLRs via TRIF or MyD88 also allows the accumulation of NIK and IRF5. Activation and nuclear translocation of IRF5 promotes the expression of IFNα, while nuclear NF-κB2 (p52/RelB) inhibits the expression of IFNβ by preventing the binding of NF-κB1 (p50/RelA) and the RelA-interactor histone demethylase JMJD2A to the *Ifnb* promoter region. (**B**) Roles of TRAF3 in type I IFN production induced by RIG-I signaling. Upon viral RNA binding, RIG-I recruits TRAF3 via MAVS to the receptor signaling complex at the mitochondrial membrane. This induces the self-ubiquitination of TRAF3 and subsequent activation of TBK1/IKKε, leading to the activation and nuclear translocation of IRF3 and IRF7 to promote the expression of IFNβ and IFNα in infected macrophages. Similar to TLR signaling, recruitment of TRAF3 by engaged RIG-I via MAVS also allows the accumulation of IRF5 and NIK, which promotes the expression of IFNα and inhibits the expression of IFNβ, respectively. Therefore, TRAF3$^{-/-}$ macrophages are defective in producing type I IFN in response to TLR agonists or viral infections, due to both impaired activation of stimulatory IRFs (IRF3 and IRF7) and constitutive activation of repressive NIK-NF-κB2. TRAF3-independent TLR or RIG-I signaling pathways, including TRAF6- or TRAF2-induced activation of MAPKs and NF-κB1, are not depicted in the figures.

obesity [23]. Chen *et al*. also showed evidence to suggest that in obese state, TRAF3 may promote metabolic inflammation by increasing the expression of proinflammatory cytokines in myeloid cells and by facilitating macrophage infiltration into metabolic tissues [23]. Thus, during metabolic inflammation and obesity progression, TRAF3 functionally switches its activity from anti-inflammatory to pro-inflammatory modes in macrophages, thereby coupling overnutrition to metabolic inflammation, insulin resistance, and metabolic disease [23].

In addition to TLRs, other receptors that directly interact with TRAF3 or indirectly recruit TRAF3 are also likely involved in and contribute to the inflammatory phenotypes observed in M-TRAF3$^{-/-}$ mice, including the spontaneous inflammatory diseases of aging M-TRAF3$^{-/-}$ mice, exacerbated colitis of DSS-treated M-TRAF3$^{-/-}$ mice, and

attenuated metabolic inflammation and hepatic steatosis of obese M-TRAF3$^{-/-}$ mice [21-23]. Under the above disease conditions, cytokines secreted by inflammatory cells, toxic metabolites and damage-associated molecular patterns (DAMPs) released from aberrant or dead cells, and pathogen-associated molecular patterns (PAMPs) derived from commensal microbiota would engage their cognate receptors. These may trigger multiple signaling pathways that converge at TRAF3 to regulate inflammation. For example, markedly elevated serum levels of IL-17, a potent proinflammatory cytokine, were detected in aging M-TRAF3$^{-/-}$ mice with inflammatory diseases [21]. It has been previously shown that TRAF3 inhibits IL-17 receptor signaling by interfering with the IL-17R-Act1-TRAF6 complex formation [1, 33] and that IL-17 receptors are up-regulated in inflammatory macrophages [34]. This suggests that TRAF3 may inhibit IL-17-induced inflammatory responses in macrophages in disease progression. Interestingly, TRAF3 has also been reported to participate in inflammation-related signaling by CD40 [35], lymphotoxin-β receptor **(**LTβR) [36], IL-1R [37], and NLRP12 [38] in macrophages. Therefore, engagement of multiple TRAF3-employing receptors on macrophages likely occurs simultaneously or sequentially in the pathogenesis of inflammatory diseases, which represents an important area for future investigation.

Requirement of TRAF3 in type I IFN production and innate immunity

Innate immunity provides the first line of protection against infectious microorganisms such as bacteria and viruses, which are recognized by pattern recognition receptors (PRRs), including TLRs, NLRs, and RLRs [1, 39, 40]. Detection of microorganisms triggers signaling cascades leading to the production of type I IFN, which is pivotal for the initiation of an anti-microbial state of the host [1, 39, 40]. Previous *in vitro* evidence indicates that TRAF3 is required for the innate anti-viral responses and type I IFN production triggered by TLRs or RLRs in macrophages and DCs [19, 20, 41]. In response to engagement of TLRs or RLRs, TRAF3 serves as a critical link between the upstream adaptor proteins of receptors (TRIF or MyD88 for TLRs; MAVS for RLRs) and downstream activating kinases TBK1-IKKε or IRAK1-IKKα [1, 25, 39] (Fig. 2). Recruitment of TRAF3 by TLR or RLR signaling complexes activates the E3 ligase activity of TRAF3 to catalyze its own K63-linked ubiquitination, and subsequently mediates the activation of TBK1-IKKε or IRAK1-IKKα, which in turn phosphorylate and activate key transcription factors IRF3 and IRF7 to drive type I IFN production [1, 25, 39] (Fig. 2). Consistent with previous observations, TLR4-induced expression of *Ifnb* and *Ifna4* is almost abolished and phosphorylation of IRF3 is markedly reduced in BMDMs and PEMs derived from M-TRAF3$^{-/-}$ mice [21, 22]. However, we noticed that phosphorylation of IRF3 is still significantly induced by TLR4 stimulation in TRAF3$^{-/-}$ BMDMs and PEMs [21], suggesting that reduced IRF3 activation is not the sole contributor of defective type I IFN production observed in these cells and additional mechanism(s) are involved.

Indeed, Jin *et al*. obtained the interesting finding that another signaling pathway affected by TRAF3 deletion, constitutive activation of NIK-IKKα-NF-κB2, also contributes to the defective type I IFN production observed in TRAF3$^{-/-}$ macrophages [6]. A variety of mouse models genetically deficient in this pathway all exhibit hyper-induction of type I IFN in macrophages in response to TLR or RLR ligands and are substantially more resistant to vesicular stomatitis virus (VSV) infection [6]. These mouse models include *Map3k14*$^{-/-}$ (NIK-deficient) mice, *Chuk*$^{-/-}$ (IKKα-deficient) mice, *Nfkb2*^{xdr} (deficient in NF-κB2 expression because of a splicing-disruptive mutation of the *Nfkb2* gene) mice, and *Nfkb2*^{lym1} (deficient in NF-κB2 processing because of a mutation of the *Nfkb2* gene that causes the loss of the C-terminal phosphorylation site on p100) mice [6]. In contrast, macrophages derived from NIKΔT3-transgenic mice overexpressing a stable form of NIK, which lacks its TRAF3-binding motif and thus results in constitutive activation of IKKα-NF-κB2, show remarkably impaired production of type I IFN in response to TLR agonists [6]. Jin *et al*. further revealed that NF-κB2 suppresses TLR or RLR-induced histone modifications at the *Ifnb* promoter, an action that involves attenuated recruitment of the transactivator RelA and the histone demethylase JMJD2A [6]. It is known that JMJD2A, recruited to the *Ifnb* promoter by RelA, induces activating modifications of histone H3 such as trimethylation of H3K4 (H3K4me3) and H3 acetylation (H3Ac), and also decreases the repressive histone modifications such as H3K9me2 and H3K9me3 [6, 42]. Elevated nuclear levels of NF-κB2 (p52-RelB) lead to inhibition of RelA-JMJD2A recruitment to the *Ifnb* promoter, as p52-RelB bind to the *Ifnb* promoter more strongly than RelA [6] (Fig. 2). Consequently, RelA-JMJD2A-mediated activation of chromatin structures at the *Ifnb* promoter is suppressed by constitutive NF-κB2 activation, which is present in TRAF3$^{-/-}$ macrophages [21, 22]. Taken together, TRAF3 promotes TLR- or RLR-induced type I IFN production by facilitating the phosphorylation of IRF3 and IRF7 via its adaptor function and E3 ligase activity, and also by suppressing the inhibitory roles of the NIK-IKKα-NF-κB2 pathway via targeting NIK for degradation (Fig. 2).

In addition to its requirement in type I IFN production, TRAF3 also plays a critical role in mediating the apoptosis-associated specklike protein (ASC)-dependent inflammasome activation in macrophages upon RNA virus infection as demonstrated in a recent study by Guan *et al*. [43]. It is revealed that engagement of RIG-I by RNA viruses

induces the formation of a new MAVS-TRAF3-ASC complex, in which TRAF3 serves as a direct E3 ligase to catalyze K63-linked ubiquitination on Lys174 of ASC [43]. Ubiquitination of ASC by TRAF3 is vital for ASC protein stabilization, speck formation and inflammasome activation, leading to caspase 1 activation and the subsequent processing and secretion of IL-1β and IL-18 [43]. Deficiency in TRAF3 or MAVS impairs ASC ubiquitination and cytosolic speck formation, resulting in reduced inflammasome activation and decreased IL-1β secretion in macrophages upon infection with VSV or influenza virus [43]. Intriguingly, TRAF3 and MAVS appear to be specifically required for inflammasome activation induced by RNA virus infection, but dispensable for inflammasome activation induced by NLRP3 activators such as calcium pyrophosphate dihydrate (CPPD) and monosodium urate (MSU) [43]. Therefore, TRAF3 plays multiple positive roles in innate anti-viral responses mediated by macrophages.

Under normal conditions and in healthy individuals, the magnitude and duration of induction of both type I IFN and pro-inflammatory cytokines are tightly controlled in response to either endogenous ligands or infectious microbes to prevent tissue injury [1, 2, 24, 40]. Imbalanced production of pro-inflammatory cytokines *versus* type I IFNs has been implicated in the pathogenesis of immunological disorders, such as inflammatory diseases, autoimmune diseases, and infectious diseases [1, 2, 24, 40]. The unique positive roles of TRAF3 in type I IFN production but negative roles in proinflammatory cytokine production poise it at the center for fine-tuning of these responses [1, 2]. In support of this notion, the expression, stability, activity, and subcellular localization of TRAF3 are subjected to delicate regulation by a number of cellular proteins, including receptors, K48 or K63-linked E3 ubiquitin ligases, deubiquitinating enzymes (DUBs), kinases, phosphatases and other adaptor proteins [1, 40]. Signaling via TLR2 remarkably up-regulate TRAF3 at both mRNA and protein levels in macrophages [44], while activation of CD40, LTβR, TLR4, estrogen receptor α (ERα), or M-CSFR results in the degradation of TRAF3 proteins [1, 2, 6, 45]. Upon different receptor signaling, E3 ligases that catalyze the ubiquitination of TRAF3 include cIAP1/2, Triad3A, and TRAF3 itself, and DUBs that remove the ubiquitin chains from TRAF3 include DUBA, OTUB1, OTUB2, MCPIP1, and USP25 [1, 46]. In innate immune responses, the kinases c-Src and Syk as well as the protein tyrosine phosphatase nonreceptor type 22 (PTPN22) can directly bind to TRAF3 and modulate its activity [47-49]. Furthermore, numerous adaptor proteins either facilitate or interfere with the complex formation of MAVS-TRAF3-TBK1-IKKε or TRIF-TRAF3-TBK1-IKKε in response to infections. Examples of such adaptor proteins include TANK [50, 51], TRADD [52], UXT-V1 [53], DOK3 [54], A20 [55], TAX1BP1 [55], UBXN1 [56], optineurin [57], TRAM [58], and FLN29 [59]. Therefore, TRAF3 is a central regulatory point for optimal innate immune responses.

Although *in vivo* infection studies on M-TRAF3$^{-/-}$ mice are still lacking, we found that some aging M-TRAF3$^{-/-}$ mice spontaneously develop bacterial or entamoeba infections in the intestine/colon, liver, and lung, which are most likely caused by opportunistic strains of commensal microbiota (termed "pathobionts") [21]. Pathobionts may trigger TRAF3-dependent signaling pathways via TLRs in macrophages, neutrophils and DCs [60-62]. Defective type I IFN production in TRAF3$^{-/-}$ myeloid cells in response to TLR signaling may occasionally allow colonization of commensal bacteria or entamoeba following opportunistic penetration of protective mucosal and epithelial barriers [21]. This indicates that innate immunity is evidently altered by ablation of TRAF3 from myeloid cells. The importance of TRAF3 in innate immunity is also highlighted by the evidence that a variety of viral and bacterial proteins target TRAF3 for inactivation. These include Lb(pro) of foot-and-mouth disease virus, X protein (HBx) of hepatitis B virus, UL36 of herpes simplex virus 1 (HSV-1), Tat protein of HIV-1, Gn protein of NY-1 hantavirus, M protein of severe acute respiratory syndrome coronavirus, and YopJ of the Gram- bacterium Yersinia pestis [1]. All these pathogenic proteins target TRAF3 and thus inhibit IRF3 phosphorylation and type I IFN production [1]. Given the above evidence, future studies are required to determine the *in vivo* responses of M-TRAF3$^{-/-}$ mice to infectious agents, and especially co-infections or sequential infections by different pathogens, such as bacteria and viruses that engage different innate immune receptors.

TRAF3: a novel tumor suppressor gene in macrophages

Of particular interest, we found that the majority of M-TRAF3$^{-/-}$ mice, but none of the TRAF3-sufficient littermate control mice, spontaneously develop tumors that often infiltrate multiple organs at the age of 15 – 22 months [21]. Tumors observed in M-TRAF3$^{-/-}$ mice include histiocytic sarcomas, B cell lymphomas, and hepatocellular adenoma [21]. Among these, histiocytic sarcomas are derived from TRAF3-deficient tissue-resident macrophages [21]. Histiocytic sarcoma (HS) in humans is a rare malignancy with a dismal prognosis, and its pathologic and cytogenetic data are sparse [63-65]. Because the genetic etiology of HS is largely unknown and patients with HS respond poorly to conventional chemotherapy, there is no standard therapy for human HS [63-65]. In M-TRAF3$^{-/-}$ mice with histiocytic sarcomas, whitish nodules of tumors are observed in the liver, and tumor cells are also identified as the major cell type in the spleen and disrupt the splenic architecture [21]. TRAF3$^{-/-}$ histiocytic sarcomas have the typical morphology of HS cells with abundant eosinophilic cytoplasm, and accompanied by frequent mitotic figures and occasional multinucleated giant cells and erythrophagocytosis [21] (Lalani and Xie, unpublished data). Immunophenotypically,

TRAF3$^{-/-}$ HS cells are identified as CD68^{+}MHC class II^{+}CD11b^{low}Gr-1^{low}, and are negative for markers of the B and T lymphoid lineages, including B220, CD19, IgM, CD3, CD4, and CD8 [21] (Lalani and Xie, unpublished data). How TRAF3 deficiency leads to malignant transformation of histiocytes remains to be investigated. We speculate that TRAF3 deficiency may gradually cause prolonged survival or increased proliferation of histiocytes, and eventually result in the development of HS in M-TRAF3$^{-/-}$ mice. Regardless of the detailed mechanisms, the spontaneous malignant transformation of tissue-resident macrophages observed in M-TRAF3$^{-/-}$ mice points to a tumor suppressive role of TRAF3 in macrophages.

Similar to M-TRAF3$^{-/-}$ mice, several other mouse models were previously reported to spontaneously develop histiocytic sarcomas, including p21$^{-/-}$ [66], Cyp1b1$^{-/-}$ [67], p19ARF$^{-/-}$Bax$^{-/-}$ [68], PTEN$^{-/-}$INK4a/ARF$^{-/-}$ [63], Dok1$^{-/-}$Dok2$^{-/-}$Dok3$^{-/-}$ [69], and humanized TLR7/TLR8 transgenic [70] mice, implicating these genes in the pathogenesis of HS. Among these, TRAF3 is functionally linked to TLR7, TLR8, and DOK3 [1, 54]. TRAF3 is recruited to the TLR7 and TLR8 signaling complex through direct interaction with MyD88 [1] (Fig. 2). Snyder *et al*. found that transgenic expression of human TLR7/TLR8 in mice deficient for endogenous TLR7/TLR8 drives proliferative histiocytosis with multisystemic infiltration of histiocytes that efface normal tissue architecture [70]. Compound deletion of MyD88 in humanized TLR7/TLR8 transgenic mice prevents the inflammatory phenotype and the development of HS, suggesting that the illness is caused by constitutive activation of humanized TLR7/TLR8 and exuberant MyD88-mediated signaling [70]. Interestingly, a recent study by Kim *et al*. identified TRAF3 as a new interacting protein for DOK3, a negative regulator of protein tyrosine kinase-mediated signaling [54]. As observed in TRAF3$^{-/-}$ macrophages, DOK3$^{-/-}$ macrophages are also impaired in IRF3 phosphorylation and IFNβ production upon influenza virus infection or polyI:C stimulation [54]. Some DOK3$^{-/-}$ mice exhibit abnormal accumulation of macrophages in the lung, and Dok1$^{-/-}$Dok2$^{-/-}$Dok3$^{-/-}$ mice succumb to spontaneous HS at a high incidence [69]. Taken together, the above findings reinforce the notion that dysregulation of TRAF3-dependent signaling pathways in macrophages contributes to the pathogenesis of histiocytic sarcoma.

We noticed that tumors are not only derived from TRAF3-deficient histiocytes, but also originate from other TRAF3-sufficient cell types that are not affected by LysM-Cre-mediated deletion, including B cells and hepatocytes, in M-TRAF3$^{-/-}$ mice [21]. This is in sharp contrast to that observed in B-TRAF3$^{-/-}$ mice, in which tumor development is limited to TRAF3-deficient B cells but is not observed in other TRAF3-sufficient cell types [10]. These findings suggest that myeloid cell TRAF3 may contribute to tumor surveillance and tumor immunity. Interestingly, B cell lymphomas observed in M-TRAF3$^{-/-}$ mice include diffuse large B cell lymphomas (DLBCLs) and follicular lymphomas (FLs), which are derived from GC or post-GC B cells [21]. During GC reaction, both somatic hypermutation (SHM) and class switch recombination (CSR) of the Ig genes generate double strand DNA breaks (DSBs), which increase the risk of genomic instability in B cells [71-73]. It is conceivable that B cells acquired oncogenic alterations during GC passage may escape the compromised tumor surveillance and develop into lymphomas in M-TRAF3$^{-/-}$ mice. In this regard, defective type I IFN production in TRAF3$^{-/-}$ myeloid cells in response to TLR-MyD88/TRIF or RLR-MAVS signaling, triggered by DAMPs derived from necrotic cancer cells, may result in compromised tumor surveillance and tumor immunity [1, 74-76]. Paradoxically however, TRAF2, another TRAF molecule that has many overlapping functions with TRAF3, was recently reported to play negative roles in myeloid cell-mediated tumor immunity [22]. Jin *et al*. showed that myeloid cell-specific TRAF2-deficient (M-TRAF2$^{-/-}$; TRAF2$^{flox/flox}$LysM$^{+/Cre}$) mice are more potent in controlling the growth of inoculated B16 melanomas than wild type mice [22]. M-TRAF2$^{-/-}$ mice contain increased frequency and numbers of tumor-infiltrating IFN-γ^{+} CD4 and CD8 effector T cells, and TRAF2$^{-/-}$ tumor-associated macrophages exhibit elevated levels of iNOS production [22]. These changes lead to more effective inhibition on the growth of inoculated tumor in M-TRAF2$^{-/-}$ mice [22]. In this context, it would be interesting to examine the roles of myeloid cell TRAF3 in tumor surveillance and tumor immunity using M-TRAF3$^{-/-}$ mice as model systems in future studies.

In addition to its effects on tumor surveillance/immunity, myeloid cell TRAF3 may suppress tumor development through its functions on containing overt inflammation and preventing chronic inflammation. Consistent with this possibility, M-TRAF3$^{-/-}$ mice with spontaneous tumors often also contain chronic inflammation in multiple organs [21]. For example, several M-TRAF3$^{-/-}$ mice with B lymphomas displayed lung, liver or intestine/colon inflammation, and an M-TRAF3$^{-/-}$ mouse with hepatocellular adenoma also had pancreatitis and pericardial inflammation [21] (Lalani and Xie, unpublished data). It is known that chronic inflammation is a strong risk factor for cancer [77-80]. We detected remarkably elevated levels of a number of cytokines and chemokines in M-TRAF3$^{-/-}$ mice with tumors, including CXCL-13, G-CSF, CCL1, IL-16, IL-17, IP-10, MCP-1, MCP-5, CXCL9, TIMP-1, and TREM-1, which have been implicated in the pathogenesis of inflammatory diseases and cancers [81-86]. These proinflammatory cytokines and chemokines may stimulate tumor growth and also promote angiogenesis to accelerate tumor progression, invasion, and metastasis [77-86]. Furthermore, the chronic inflammatory environment of M-TRAF3$^{-/-}$ mice may also induce mutations [77-80] that facilitate malignant transformation of TRAF3-sufficient cells, such as GC B cells and hepatocytes.

Increasing evidence also suggests that myeloid cell TRAF3 may control tumor development by inhibiting the expansion of $CD11b^{+}Gr\text{-}1^{+}$ myeloid derived suppressor cells (MDSCs), which in turn suppress the anti-tumor immunity mounted by natural killer (NK) cells and CD8 cytotoxic T cells. We consistently observed a striking expansion of $CD11b^{+}Gr\text{-}1^{+}$ myeloid cells (most likely MDSCs) in M-TRAF3$^{-/-}$ mice with spontaneous tumors or chronic inflammation [21]. Although the increased population of $CD11b^{+}Gr\text{-}1^{+}$ MDSCs may be a consequence of spontaneous inflammation and tumor development [87-90], emerging new evidence suggests that TRAF3 may regulate the expansion and function of MDSCs. First, Parker *et al*. found that an endogenous agonist of the TRAF3-dependent receptor TLR4, HMGB1, is commonly present in the tumor microenvironment and potently promotes the generation and suppressive activity of MDSCs [91]. Second, Jin *et al*. demonstrated that upon ligand stimulation, TRAF3 is recruited to M-CSFR and GM-CSFR, which are known to play essential roles in regulating the development and differentiation of MDSCs [6, 92, 93]. Signaling by M-CSFR or GM-CSFR induces the degradation of TRAF3 and subsequent accumulation of c-Rel and p52 NF-κB2 [6, 22]. Third, Yu *et al*. reported that c-Rel and p52 NF-κB2 cooperatively bind to the promoter region of the *Csf2* gene to induce the production of GM-CSF in Th17 cells, raising the interesting possibility that this may also occur in certain myeloid cell populations [94]. Increased GM-CSF production has been shown to cause the expansion of MDSCs [95]. Finally, constitutive activation of NF-κB2 has been shown to promote the immuno-suppressive activity of MDSCs by mediating the expression of IDO, an enzyme that catalyzes the degradation of tryptophan through the kynurenine pathway to suppress T cell proliferation and activation [96]. Collectively, the above evidence suggests the importance of TRAF3 in regulating the expansion and function of MDSCs, which are recognized as crucial drivers of tumor progression and chronic inflammation in the tumor microenvironment [87, 88, 90, 97, 98].

In summary, TRAF3 may function as a tumor suppressor in myeloid cells directly by acting in an intrinsic manner to inhibit the malignant transformation of histiocytes, or indirectly by controlling tumor development via multiple mechanisms. These indirect mechanisms of myeloid cell TRAF3-mediated tumor suppression include participating in tumor surveillance and tumor immunity, restricting the magnitude of inflammation and preventing chronic inflammation, and suppressing the expansion and function of MDSCs. All of these potential mechanisms are exciting areas for future exploration.

TRAF3 in human tumors, inflammatory diseases, and infectious diseases

The new findings obtained from *in vivo* studies of M-TRAF3$^{-/-}$ mice by all three laboratories indicate that aberrant functions of TRAF3 in myeloid cells may contribute to the pathogenesis of tumors, inflammatory diseases, metabolic diseases, and infectious diseases [21-23]. However, evidence of TRAF3 mutations or malfunctions in myeloid cells of human patients is still very limited. Previous efforts on human *Traf3* gene mutations have been mainly focused on B cell malignancies considering the instrumental roles of TRAF3 in B cell survival/apoptosis as revealed by investigation of B-TRAF3$^{-/-}$ mice [1, 99]. Somatic biallelic deletions and inactivating mutations of *Traf3* have been detected in a variety of human B cell neoplasms, including multiple myeloma, MZL, B cell chronic lymphocytic leukemia, mantle cell lymphoma, DLBCL, Waldenström's macroglobulinemia, and Hodgkin lymphoma [1, 99-101]. Deletions and missense mutations of *Traf3* have also been reported recently in two types of human carcinomas, the Epstein Barr virus-associated nasopharyngeal carcinoma and papillomavirus-associated head and neck squamous cell carcinomas [102, 103]. Additionally, a genome-wide association study (GWAS) identified *Traf3* as a high-confidence candidate gene associated with multiple sclerosis, a neurological disease with prominent inflammation [104]. However, to date only one publication reports a germline mutation of human *Traf3* that directly affects the function of macrophages and DCs [105]. In this case, a heterozygous *Traf3* autosomal dominant mutation (R118W in the first zinc-finger domain) was found in a young adult with a history of herpes simplex virus-1 encephalitis in childhood. This *Traf3* mutation results in impaired TLR3-induced type I IFN production in macrophages, DCs, and fibroblasts [105]. Together, available evidence suggests that most functions of TRAF3 are conserved between mice and humans.

Given the stringent control of TRAF3 protein levels and activities observed under normal circumstances, altered protein levels or activation of the TRAF3 protein in myeloid cells may also contribute to disease pathogenesis. Indeed, elevated protein levels of TRAF3, TRAF2, and TRAF5 were reported in human patients with inflammatory bowel diseases, and increased TRAF2 was identified as a predictor of relapse in patients with ulcerative colitis [106, 107]. In contrast, expression of TRAF3 is significantly decreased in peripheral blood mononuclear cells of patients chronically infected with hepatitis B virus as compared to that observed in healthy controls [108]. Overall, available information in this area is scattered. Therefore, further efforts are required to systematically examine the existence and frequency of alterations of TRAF3 and other components of TRAF3-dependent signaling pathways at the genetic, epigenetic, and protein as well as activity levels in myeloid cells in human patients with tumors, inflammatory diseases, metabolic diseases, and infectious diseases. Such studies will provide groundwork for therapeutic development of new targeted therapies to manage human diseases and improve

patient outcome.

Conclusions

Substantial progress has recently been made in elucidating the *in vivo* functions of TRAF3 in macrophages and other myeloid cells. Evidence obtained by three laboratories together demonstrates that specific ablation of TRAF3 in myeloid cells leads to inflammatory diseases, altered progression of diabetes, and spontaneous development of different types of tumors and infections in mice. These new findings indicate that myeloid cell TRAF3 acts as an anti-inflammatory factor, and is required to resist infections and control development of hematopoietic and solid tumors. In conclusion, these studies identify TRAF3 as a critical regulator of inflammation and innate immunity, and notably, also a novel tumor suppressor in macrophages. Although information about TRAF3 mutations or malfunctions in human macrophages is limited, available evidence indicates that TRAF3 mutation and aberrant expression exist in myeloid cells of human patients with viral infectious diseases and inflammatory bowel diseases. Therefore, the functions of TRAF3 in myeloid cells appear to be conserved between mice and humans, suggesting that findings obtained from M-TRAF3$^{-/-}$ mice may be extrapolated to human diseases and merit further systematic investigations of TRAF3 in human patients. Furthermore, TRAF3 is now recognized as a converging point of numerous signaling pathways, including the TNF-R superfamily, TLRs, RLRs, and cytokine receptors. It would thus be especially interesting to further decipher how TRAF3 integrates or modulates different signals in situations that simultaneous or sequential engagement of multiple receptors occurs on macrophages and DCs, such as chronic inflammation, co-infections, or tumorigenesis. Deeper mechanistic insights into TRAF3 signaling pathways will be valuable for understanding the molecular pathogenesis of TRAF3-associated diseases, and will provide new opportunities for developing effective therapeutic modalities for chronic inflammation, infection, and cancer.

Acknowledgements

This study was supported by a Pilot Award from the Cancer Institute of New Jersey through Grant Number P30CA072720 from the National Cancer Institute (P. Xie), the Department of Defense grant W81XWH-13-1-0242 (P. Xie), a Busch Biomedical Grant (P. Xie), and the Arthur Herrmann Endowed Cancer Research Fund (P. Xie).

Competing interests statement

The authors declare that they have no competing financial interests.

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