FEBS Letters 589 (2015) 1369–1375

FEBS Letters

journal homepage: www.FEBSLetters.org

The seventh zinc finger motif of A20 is required for the suppression of TNF-α-induced apoptosis

Noritaka Yamaguchi *, Naoto Yamaguchi

Department of Molecular Cell Biology, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 260-8675, Japan

ARTICLE INFO

Article history:
Received 29 January 2015
Revised 11 April 2015
Accepted 14 April 2015
Available online 22 April 2015

Edited by Kazuhiro Iwai

Keywords:
Ubiquitin
Tumor necrosis factor-α
Apoptosis
Nuclear factor-κB

ABSTRACT

The ubiquitin-editing enzyme A20 suppresses nuclear factor-κB (NF-κB) activation and tumor necrosis factor-α (TNF-α)-induced apoptosis in a deubiquitinating and ubiquitin ligase activity-dependent manner. Although recent studies revealed that A20 regulates NF-κB independently of its enzymatic activity through its seventh zinc finger motif (ZnF7), the involvement of ZnF7 in TNF-α-induced apoptosis is not clear. In this study, ZnF7 was found to be important for A20-mediated suppression of TNF-α-induced apoptosis. We also found that the ubiquitin ligases cIAP1/2 are required for A20 to suppress TNF-α-induced apoptosis. Because A20 binds to cIAP1/2 through ZnF7, these results suggest that A20 may control cIAP1/2 when suppressing TNF-α-induced apoptosis.

1. Introduction

TNF-α is a multifunctional cytokine that regulates various cellular events including proliferation, differentiation, and death [1,2]. TNF receptor I (TNF-RI) is the most prevalent receptor for TNF-α. TNF-RI is a member of the death receptor superfamily that activates the transcription factor NF-κB and induces apoptosis [3]. Binding of TNF-α to TNF-RI induces its oligomerization and promotes the formation of a plasma membrane-associated complex [4]. This initial complex is referred to as complex-I and is composed of TRADD, RIP1, TRAF2, cIAP1 and 2 (cIAP1/2), and the linear ubiquitin chain assembly complex (LUBAC) [5]. cIAP1/2 bind to TRAF2 and function as the ubiquitin E3 ligase complex (cIAP1/2:TRAF2) that promotes the synthesis of Lys48- or Lys63-linked polyubiquitin chains [6,7]. LUBAC is composed of HOIL-1, HOIP, and Sharpin and catalyzes the formation of Met1-linked polyubiquitin chains [8–10]. Although RIP1 has serine/threonine kinase activity, it acts as an adaptor protein within complex-I. RIP1 is polyubiquitinated by cIAP1/2:TRAF2 and LUBAC. Polyubiquitin chains of RIP1 serve as scaffolds for downstream signaling molecules such as TAK1 and IKK [11,12]. TAK1 is auto-activated on the ubiquitin chains and induces the phosphorylation and activation of IKK. IKK facilitates the phosphorylation and subsequent degradation of IκBα, thereby allowing NF-κB activation [13].

Binding of TNF-α to TNF-RI also induces the formation of a second protein complex in the cytosol [4]. This complex is referred to as complex-II and is composed of RIP1, RIP3, FADD, and caspase-8. Deubiquitination of RIP1 by deubiquitinating enzymes is required for the formation of complex-II. Upon the formation of complex-II, caspase-8 is activated by auto-cleavage and then induces apoptosis [5]. Because TNF-α-mediated NF-κB activation induces the expression of anti-apoptotic genes and suppresses complex-II-mediated apoptosis, TNF-α-induced apoptosis occurs only in the absence of de novo protein synthesis or in cells lacking crucial NF-κB activators [14,15].

The ubiquitin-editing enzyme A20 is composed of an ovarian tumor (OTU) domain and seven zinc finger motifs (ZnFs). A20 functions as a deubiquitinating enzyme through the OTU domain and as a ubiquitin E3 ligase through the fourth ZnF (ZnF4) [16]. The most important biological role of A20 is terminating NF-κB

Abbreviations: TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; TRADD, TNF receptor-associated death domain; RIP, receptor-interacting protein; TRAF, TNF receptor-associated factor; cIAP, cellular inhibitor of apoptosis; HOIL-1, heme-oxidized iron regulatory protein-2 ubiquitin ligase-1; HOIP, HOIL-1-interacting protein; TAK1, transforming growth factor-β-activated kinase 1; IκB, inhibitor of κB; IKK, IκB kinase; FADD, Fas-associated protein with death domain; TRAIL, TNF-related apoptosis-inducing ligand; IL-1, interleukin-1; PARP, Poly (ADP-ribose) polymerase

Author contributions: No. Y. designed the study, performed the experiments, and wrote the paper. No. Y. and Na. Y. analyzed the data.

* Corresponding author at: Department of Molecular Cell Biology, Graduate School of Pharmaceutical Sciences, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba 260-8675, Japan. Fax: +81 43 226 2869.

E-mail address: yamaguchinoritaka@chiba-u.jp (N. Yamaguchi).

http://dx.doi.org/10.1016/j.febslet.2015.04.022
0014-5793/

activation. Genetic alterations to A20 are associated with autoimmune diseases and B-cell lymphoma [17–19]. To terminate NF-κB activation, A20 promotes the degradation of Lys63-linked polyubiquitin chains on RIP1 and its subsequent proteasomal degradation by adding Lys48-linked polyubiquitin chains [16]. In addition, A20 disrupts the NF-κB-activating ubiquitin ligase complexes through its deubiquitinating activity [20].

A20 is involved in preventing death receptor-induced apoptosis [17]. Previous studies reported that A20 blocks death receptor-induced apoptosis in a manner dependent on its ubiquitin-editing activity. The deubiquitinating activity of A20 reverses the polyubiquitination of caspase-8, which is required for its activation upon stimulation by the death receptor ligand TRAIL [21]. The ubiquitin ligase activity of A20 induces Lys63-linked polyubiquitination of RIP1 upon TRAIL stimulation, and the synthesized polyubiquitin chains bind to caspase-8 and suppress its dimerization and activation [22].

In contrast to these observations, emerging evidence revealed that A20 also functions in an enzymatic activity-independent manner in NF-κB suppression. A20 binds directly to Lys63- and Met1-linked polyubiquitin chains through its seventh ZnF (ZnF7) and suppresses IKK activation [23–25]. Recently, we also found that A20 binds directly to cIAP1/2 through ZnF7 and regulates their roles independently of its enzymatic activity [26]. Although these observations showed that ZnF7 is involved in regulating NF-κB activation, its role in death receptor-mediated apoptosis is not clear. Therefore, the current study was designed to determine the role of ZnF7 in A20-mediated suppression of TNF-α-induced apoptosis.

2. Materials and methods

2.1. Plasmids and antibodies

The plasmids used in this study were described previously [26]. In brief, human and mouse A20 cDNAs were generated by PCR and inserted into pMXs retroviral vectors that were obtained from T. Kitamura (University of Tokyo, Japan). An myc tag was added at the N-terminus of the cDNAs. The antibodies used in this study are shown in Supplementary Table S1.

2.2. Cells and transfection, western blot analyses, and immunoprecipitation

Cell culture conditions were described previously [26]. A20-deficient mouse embryonic fibroblasts (MEFs) were kindly provided by A. Ma (University of California at San Francisco, USA) [17]. Stable cell pools were established as described previously [26]. Recombinant human TNF-α and IL-1α were purchased from Peprotech (Rocky Hill, NJ). MV-1 was kindly provided by Y. Demizu (National Institute of Health Sciences, Japan). Necrostatin-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). z-VAD-fmk was purchased from Peptide Institute (Osaka, Japan). Western blot analyses and immunoprecipitation were performed as described previously [26].

2.3. GST pull-down assay

Human cDNA encoding the TNF-α C-terminus extracellular domain (Val77-Leu233) was generated by PCR with the sense primer 5′-AAAGAATTCGTCAGATCATCTTCTCGAACC-3′ and the antisense primer 5′-TTTGCGGCCGCTCACAGGGCAATGATCCCAAAG-3′. The cDNA generated was digested with *Eco*RI and *Not*I, and cloned into the pGEX-6P-1 vector that was pre-digested with the same enzymes. GST-TNF-α was expressed in *Escherichia coli* DH5α and purified using Glutathione Sepharose 4B (GE Healthcare, Waukesha, WI). Purified GST-TNF-α was dialyzed with phosphate-buffered saline (PBS). Contaminating endotoxins were removed with Detoxi-Gel Endotoxin Removing Columns as per the manufacturer's instructions (Pierce, Rockford, IL). Cells were stimulated with GST-TNF-α, washed with PBS, and then lysed in TNE buffer [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM Na_3VO_4, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride]. The cell lysate was centrifuged to remove cellular debris. The resulting supernatant was mixed with 15 μl of Glutathione Sepharose 4B. After overnight incubation, the beads were washed 3 times with TNE buffer and then suspended in SDS-sample buffer.

2.4. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay

Cells were plated at 1000 cells/well in a 96-well plate and incubated overnight at 37 °C. The cells were then incubated with the reagents indicated in the text for 16 h in triplicate cultures. After this incubation period, each well was washed with PBS and incubated for an additional 4 h in a 1 mg/ml MTT solution (Dojindo, Kumamoto, Japan). The resulting crystals were dissolved in 100 μl of DMSO and the absorbance was measured at 595 nm using an iMark microplate reader (Bio-Rad, Richmond, CA).

3. Results

3.1. ZnF7 is required for A20 to suppress NF-κB upon stimulation with TNF-α and IL-1

Recent studies have revealed that ZnF7 is required for A20 to suppress NF-κB activation by TNF-α [23–25]. We first confirmed the importance of ZnF7 in TNF-α-induced NF-κB activation using A20-deficient MEFs reconstituted with wild-type A20 or its ZnF7 mutant (ZnF7mt). ZnF7mt has alanine substitutions in the conserved cysteine residues of ZnF7 (mouse A20, C764A/C767A; human A20, C779A/C782A) [23]. NF-κB activation was evaluated by determining the phosphorylation of IKK and IκBα. TNF-α-induced NF-κB activation was significantly reduced in cells reconstituted with wild-type A20, but not with ZnF7mt (Fig. 1A). HEK293T cells stably expressing wild-type A20 or ZnF7mt were stimulated with TNF-α. NF-κB activation in these cells was assessed by analyzing the phosphorylation of IκBα. Consistent with previous studies [23], NF-κB activation by TNF-α was significantly suppressed in HEK293T cells stably expressing wild-type A20, but not ZnF7mt (Fig. 1B). We also analyzed the involvement of ZnF7 in NF-κB activation by IL-1α. Similar to stimulation with TNF-α, NF-κB activation induced by IL-1α was significantly suppressed in MEFs reconstituted with wild-type A20, but not with ZnF7mt (Fig. 1C). These results confirmed that ZnF7 is required for A20 to suppress NF-κB activation upon stimulation with IL-1α as well as TNF-α.

3.2. ZnF7 is required for A20 to suppress TNF-α-induced apoptosis

We next investigated the involvement of ZnF7 in the A20-mediated suppression of TNF-α-induced apoptosis. To compare the significance of the enzymatic activities of A20 and ZnF7 in terms of apoptosis, we analyzed the viability of A20-deficient MEFs reconstituted with wild-type A20, its OTU domain mutant (OTUmt), its ZnF4 mutant (ZnF4mt), or the ZnF7mt. The OTUmt lacks deubiquitinating activity due to a mutation in the catalytic cysteine residue (mouse A20, C103A). ZnF4mt lacks ubiquitin E3 ligase activity due to mutations in the conserved cysteine residues (mouse A20, C609A/C612A) [16]. Apoptosis was induced by treatment with

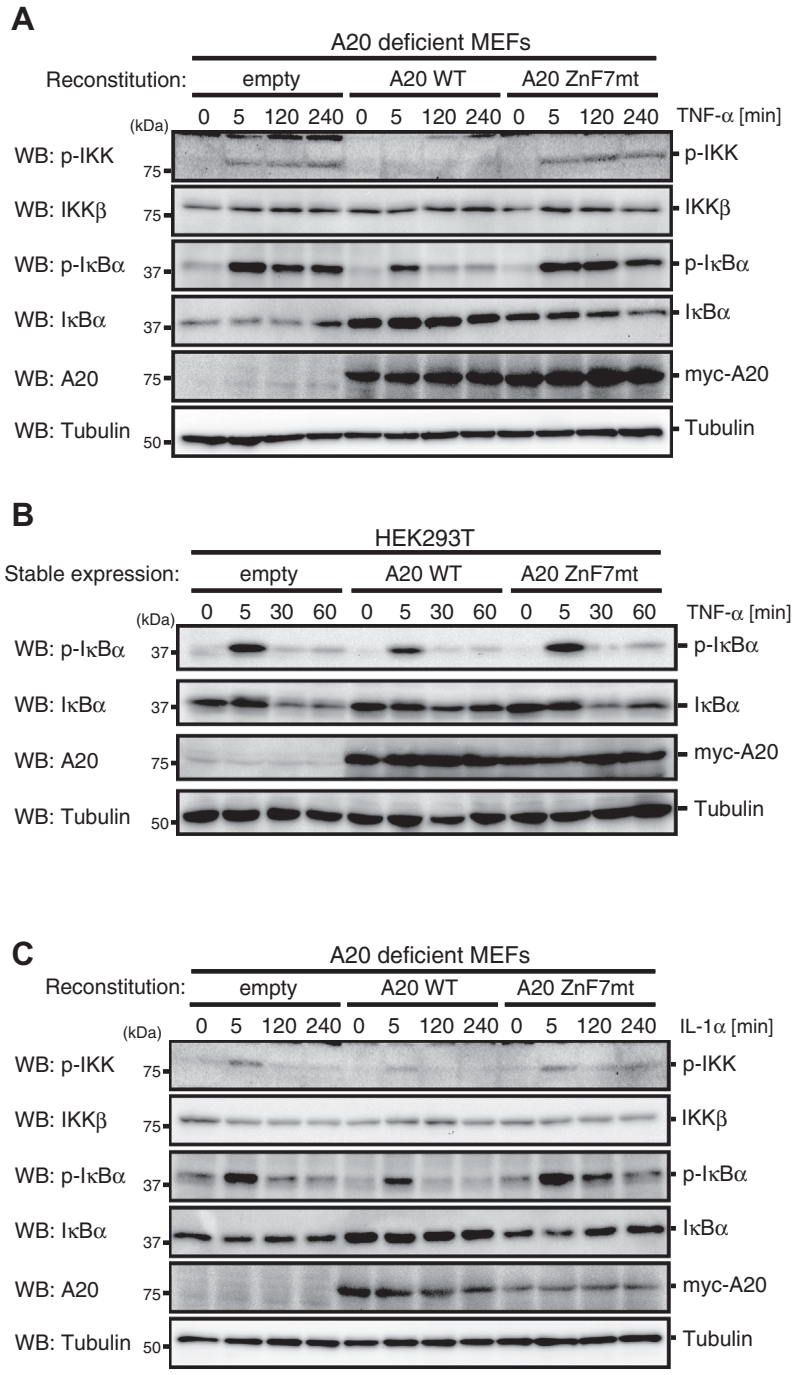

Fig. 1. ZnF7 is important for A20 to suppress the activation of NF-κB in response to TNF-α and IL-1 signaling. (A) A20-deficient MEFs and (B) HEK293T cells stably expressing control vector, wild-type A20, or A20 ZnF7mt were stimulated with TNF-α (10 ng/ml) for the indicated times. Cell lysates were prepared and subjected to western blot analyses with the indicated antibodies. (C) A20-deficient MEFs stably expressing control vector, mouse wild-type A20, or A20 ZnF7mt were stimulated with IL-1α (10 ng/ml) for the indicated times. Cell lysates were prepared and subjected to western blot analyses with the indicated antibodies.

TNF-α in combination with the protein synthesis inhibitor cycloheximide (CHX). Cell viability was detected by the MTT assay. The viability of A20-deficient MEFs reconstituted with OTUmt or wild-type A20 was significantly greater than that of the control A20-deficient MEFs. This suggests that the deubiquitinating activity of A20 is not crucial for suppressing TNF-α-induced apoptosis (Fig. 2A). Although reconstitution with ZnF4mt partially retained the viability of A20-deficient MEFs treated with TNF-α plus CHX, reconstitution with ZnF7mt failed to improve cell viability (Fig. 2A). Because both ZnF4mt and ZnF7mt lack ubiquitin E3 ligase activity [23], these results suggest that, although the ubiquitin E3 ligase activity of A20 is partially involved in suppressing TNF-α-induced apoptosis, ZnF7 plays an essential role in the suppression of apoptosis by A20 independently of its enzymatic activity.

The activation of apoptosis signaling was also analyzed by determining levels of cleaved caspase-3 and its downstream target, PARP. The cleavage of caspase-3 and PARP was significantly suppressed by reconstituting A20-deficient MEFs with wild-type A20, OTUmt, and, to a lesser extent, ZnF4 (Fig. 2B). However, reconstitution with ZnF7mt did not suppress apoptosis signaling in A20-deficient MEFs (Fig. 2B). In HEK293T cells, even though cell viabilities were not improved (Fig. 2C), TNF-α-induced cleavage of

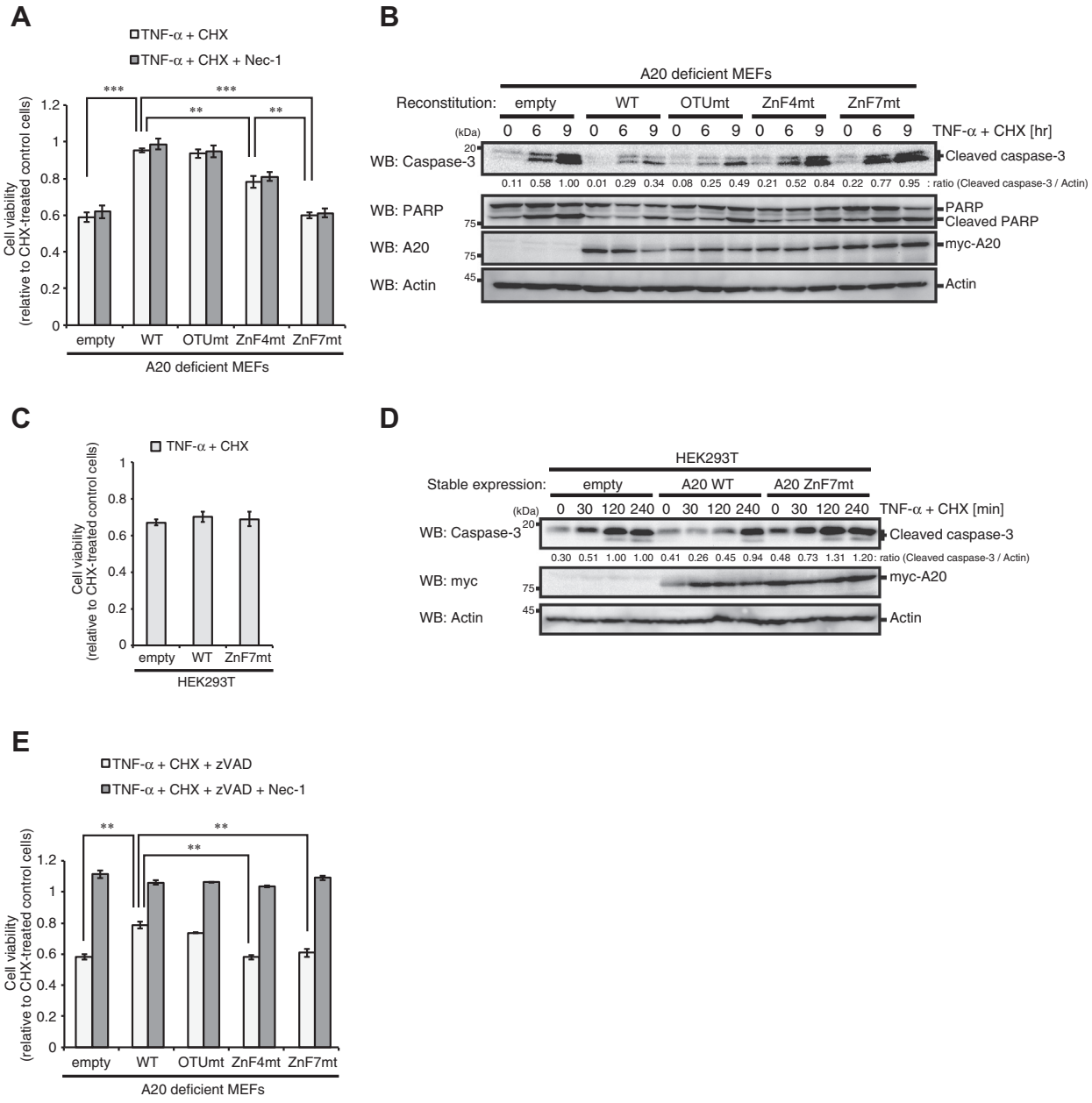

Fig. 2. ZnF7 is important for the suppression of TNF-α-induced apoptosis by A20. (A) A20-deficient MEFs stably expressing control vector, mouse wild-type A20, or A20 mutants were treated with TNF-α (10 ng/ml) plus CHX (10 μg/ml) together with or without Necrostatin-1 (Nec-1) (20 μM) for 16 h. After treatment, cell viability was determined using the MTT assay. Results are shown as ratios to the viability of control cells treated with CHX (10 μg/ml) alone. Data are expressed as means ± S.D. ($n = 3$). Asterisks indicate the significant differences ($^{***}p < 0.001$, $^{**}p < 0.01$) calculated by Student's *t*-test. (B) A20-deficient MEFs stably expressing control vector, wild-type A20, or A20 mutants were treated with TNF-α (10 ng/ml) plus CHX (10 μg/ml) for the indicated times. Cell lysates were prepared and subjected to western blot analyses with the indicated antibodies. (C) HEK293T cells stably expressing control vector, human wild-type A20, or A20 ZnF7mt were treated with TNF-α (10 ng/ml) plus CHX (10 μg/ml) for 16 h. After treatment, cell viability was determined as in (A). (D) HEK293T cells stably expressing control vector, wild-type A20, or ZnF7mt were treated with TNF-α (10 ng/ml) plus CHX (10 μg/ml) for the indicated times and then subjected to western blot analyses as in (B). (E) A20-deficient MEFs stably expressing control vector, wild-type A20, or A20 mutants were treated with TNF-α (10 ng/ml), CHX (10 μg/ml), and z-VAD-fmk (100 μM) together with or without Nec-1 (20 μM) for 16 h. After treatment, cell viability was determined as in (A).

caspase-3 was significantly reduced by overexpression of wild-type A20, but not ZnF7mt, especially 120 min after stimulation (Fig. 2D).

Caspase inhibition promotes programmed necrosis (necroptosis) instead of apoptosis upon TNF-α stimulation [27], and TNF-α-induced necroptosis depends on RIP1's kinase activity [28]. TNF-α plus CHX-induced reduction in cell viability was not blocked by the RIP1 kinase inhibitor Necrostatin-1 (Fig. 2A), suggesting that treatment with TNF-α plus CHX primarily causes apoptosis of A20-deficient MEFs. To investigate the involvement of A20 in TNF-α-induced necroptosis, we analyzed the viability of A20-deficient MEFs after treatment with TNF-α, CHX, and the caspase inhibitor z-VAD-fmk. Although reconstitution with wild-type A20 or OTUmt improved the viability of A20-deficient MEFs, reconstitution with ZnF4mt or ZnF7mt did not affect cell viability (Fig. 2E). Reduction in cell viability by treatment with TNF-α, CHX, and z-VAD-fmk was completely blocked by Necrostatin-1 (Fig. 2E). Taken together, these results suggest that A20 suppresses TNF-α-induced necroptosis in a manner that depends on its ubiquitin E3 ligase activity.

3.3. cIAP1/2 are required for the suppression of TNF-α-induced apoptosis by A20

Because cIAP1/2 are involved in apoptosis signaling [6,29,30], the involvement of cIAP1/2 in the A20-mediated suppression of TNF-α-induced apoptosis was analyzed. TNF-α can induce apoptosis in the absence of cIAP1/2. Thus, cells were treated with the cIAP1/2 antagonist MV-1, which promotes autoubiquitination and degradation of cIAP1/2 [31], prior to TNF-α stimulation. Although treatment with MV-1 alone did not affect the viability of A20-deficient MEFs, co-treatment with MV-1 and TNF-α significantly reduced their survival. Importantly, reconstitution of A20-deficient MEFs with wild-type A20 failed to block the apoptosis induced by TNF-α plus MV-1 (Fig. 3A, B). These results suggest that cIAP1/2 are required for A20 to suppress TNF-α-induced apoptosis.

3.4. ZnF7 is required for A20 to suppress the formation of complex-II

We hypothesized that A20 regulates cIAP1/2:TRAF2 in response to TNF-α signaling because A20 binds to cIAP1/2. cIAP1/2 promote polyubiquitination of RIP1 within complex-I, and depletion of cIAP1/2 causes a reduction in RIP1 polyubiquitination [29]. The formation of complex-I was determined by pull-down assays in GST-TNF-α-stimulated HEK293T cells. Polyubiquitination of RIP1 and recruitment of cIAP1 and TRAF2 to complex-I were detected in control cells (Fig. 4A). Recruitment of cIAP2 to complex-I was not detectable, most likely because of its low expression [26]. In contrast, polyubiquitination of RIP1 was significantly reduced. Moreover, recruitment of cIAP1 and TRAF2 to complex-I was almost completely suppressed in A20-overexpressing cells (Fig. 4A). As shown in previous studies [29,32], depletion of cIAP1/2 by MV-1 reduced the polyubiquitination of RIP1 similar to the overexpression of A20 (Fig. 4B). This suggests that the reduction in the polyubiquitination of RIP1 was caused by A20-mediated abrogation of the recruitment of cIAP1/2:TRAF2 to complex-I in A20-overexpressing cells. Despite the absence of cIAP1/2:TRAF2 in complex-I, the LUBAC component HOIL-1 and A20 were recruited to complex-I (Fig. 4A), which suggests that A20 binds to LUBAC-dependent polyubiquitin chains in complex-I. Consistent with this hypothesis, HOIL-1 and A20 still bound to complex-I even in cells where cIAP1/2 were depleted by MV-1 treatment (Fig. 4B and C). This cIAP1/2-independent recruitment of LUBAC to complex-I may be involved in the residual polyubiquitination of RIP1 and NF-κB activation upon TNF-α stimulation in MV-1-treated cells (Fig. 4B and D).

We next analyzed the effect of A20 on the formation of complex-II by immunoprecipitation of caspase-8 in lysates from HEK293T cells treated with TNF-α and CHX. The association of caspase-8 with un-ubiquitinated RIP1 and FADD and the cleavage of caspase-8 were increased in response to this combined treatment. In contrast, both changes were significantly repressed by the expression of wild-type A20, but not ZnF7mt (Fig. 4E). This suggests that A20 suppresses the formation of complex-II in a ZnF7-dependent manner. Because cIAP1/2 are required for the suppression of apoptosis by A20 (Fig. 3A and B), these results suggest that A20 suppresses TNF-α-induced apoptosis by controlling cIAP1/2.

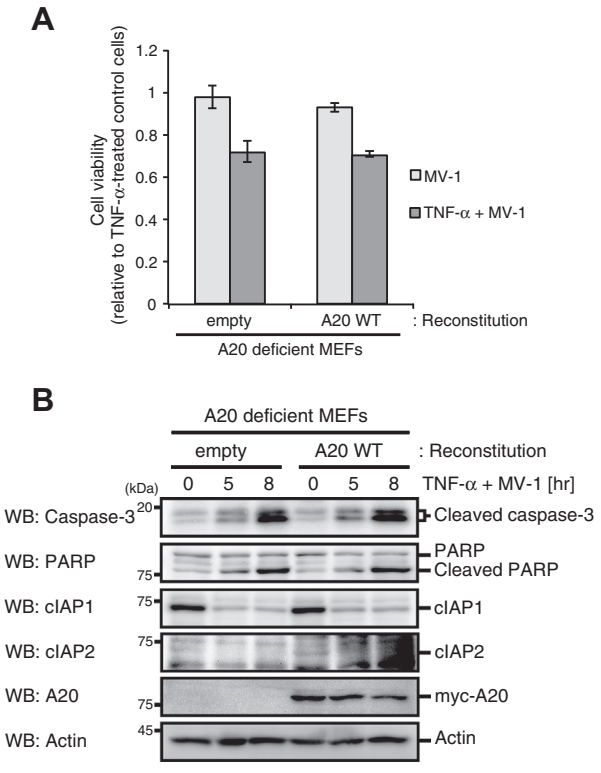

Fig. 3. cIAP1/2 are required for A20 to suppress TNF-α-induced apoptosis. (A) A20-deficient MEFs stably expressing control vector or wild-type A20 were treated with TNF-α (10 ng/ml) plus MV-1 (10 μM) or MV-1 alone for 16 h. After treatment, cell viability was determined using the MTT assay. Results are shown as ratios to the viabilities of controls cells treated with TNF-α (10 ng/ml) alone. Data are expressed as means ± S.D. ($n = 3$). (B) A20-deficient MEFs stably expressing control vector or wild-type A20 were treated with TNF-α (10 ng/ml) plus MV-1 (10 μM) for the indicated times. After incubation, cell lysates were prepared and subjected to western blot analysis with the indicated antibodies.

4. Discussion

cIAP1/2 are crucial ubiquitin E3 ligases that regulate NF-κB activation and apoptosis signaling. Under non-stimulated conditions, cIAP1/2 promote Lys48-linked polyubiquitination of NF-κB-inducing kinase (NIK), a key protein kinase involved in the activation of non-canonical NF-κB, and thereby induce its proteasomal degradation [33,34]. The lymphotoxin-β receptor, a member of the TNF receptor superfamily, promotes NIK activation [35]. Upon stimulation with lymphotoxin-β, A20 is expressed and binds directly to cIAP1/2 through ZnF7 [26]. The binding of A20 to cIAP1/2 promotes the dissociation of TRAF2 from TRAF3, breaking the molecular bridge that joins cIAP1/2 and NIK and thereby inhibiting polyubiquitination and the subsequent degradation of NIK (Supplementary Fig. S1A).

In the current study, we found that ZnF7 and cIAP1/2 are required for A20 to suppress TNF-α-induced apoptosis. Because our recent study showed that A20 binds directly to cIAP1/2 through ZnF7 and regulates their targets [26], these results suggest that A20 suppresses TNF-α-induced apoptosis by controlling cIAP1/2. Our present data show that overexpressing A20 prevents the recruitment of cIAP1/2:TRAF2 to complex-I and polyubiquitination of RIP1. Because cIAP1/2:TRAF2 associate with RIP1 through TRAF2 [7], these data suggest that the binding of A20 to cIAP1/2 suppresses the association between TRAF2 and RIP1 and the subsequent cIAP1/2-mediated polyubiquitination of RIP1. Because the presence of un-ubiquitinated RIP1 in complex-II promotes apoptosis, the suppression of RIP1 polyubiquitination by A20 is not involved in repressing TNF-α-induced apoptosis. Given that the expression of A20 is induced by TNF-α [36], we hypothesize that the binding of A20 to cIAP1/2 causes a shift in the target of cIAP1/2 from RIP1 to another protein involved in suppressing apoptosis. This target shift may inhibit both NF-κB activation and apoptosis signaling in the late phase of TNF-α signaling (Supplementary Fig. S1B). This unknown target should be determined in further studies.

A20 is frequently mutated or deleted, especially in the region encoding ZnF7, in B-cell lymphomas [18,19]. Because ZnF7 is important for suppressing NF-κB, and the constitutive NF-κB

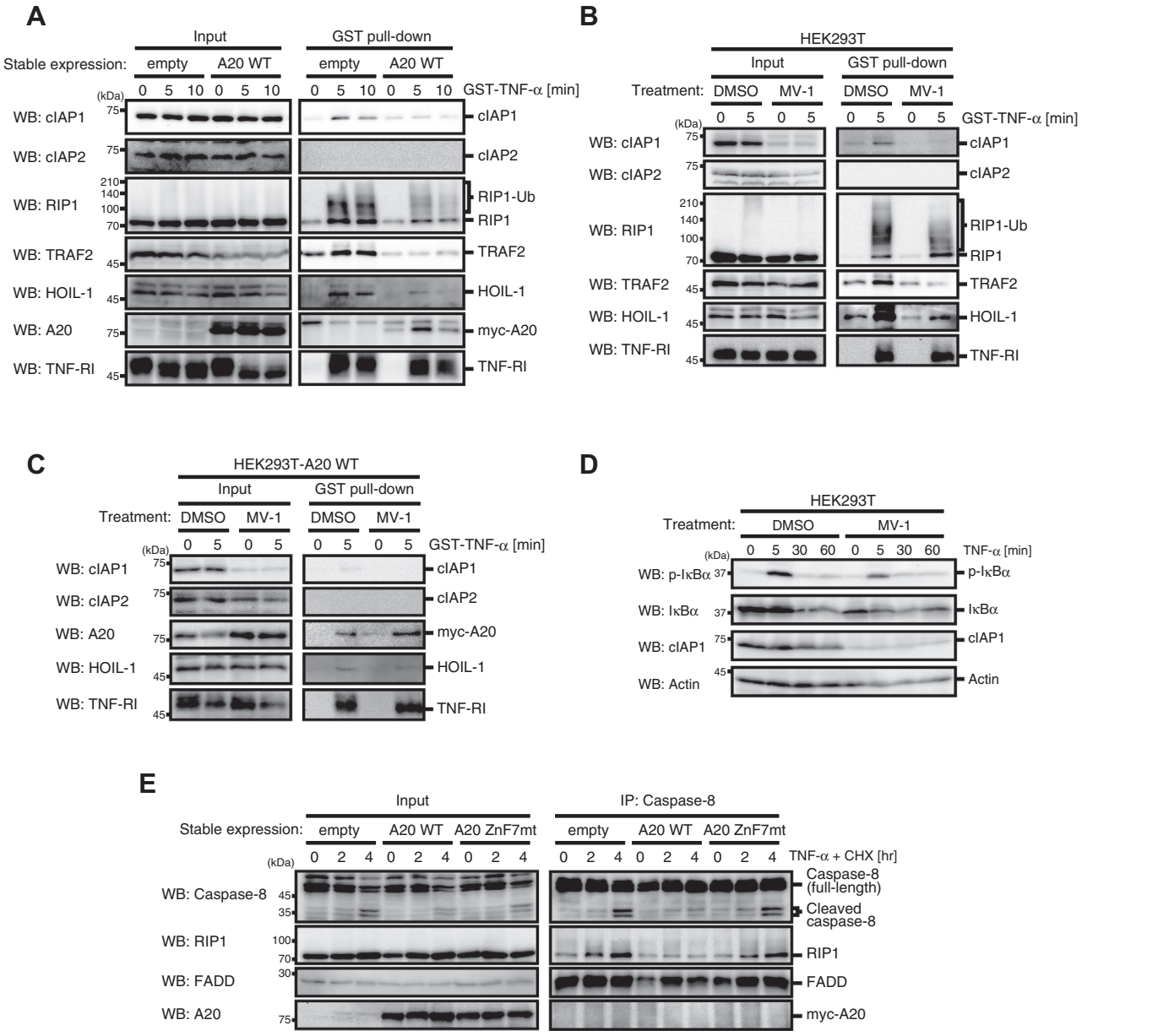

Fig. 4. ZnF7 is required for A20-mediated suppression of the formation of complex-II. (A) HEK293T cells stably expressing control vector or wild-type A20 were treated with GST-TNF-α (50 ng/ml) for the indicated times. After incubation, GST pull-down assays were performed as described in Section 2. Input and pull-down samples were subjected to western blot analyses with the indicated antibodies. (B) HEK293T cells and (C) HEK293T cells stably expressing wild-type A20 were pre-incubated with MV-1 (10 μM) or DMSO (0.1%) for 24 h and then treated with GST-TNF-α (50 ng/ml) for the indicated times. After incubation, GST pull-down assays and subsequent western blot analyses were performed as in (A). (D) HEK293T cells were pre-incubated with MV-1 (10 μM) for 24 h and then treated with TNF-α (10 ng/ml) for the indicated times. Cell lysates were prepared and subjected to western blot analyses with the indicated antibodies. (E) HEK293T cells stably expressing control vector, wild-type A20, or A20 ZnF7mt were treated with TNF-α (10 ng/ml) plus CHX (10 μg/ml) for the indicated times. Cell lysates were prepared and subjected to immunoprecipitation assays (IP) with an anti-caspase-8 antibody. Input and precipitated samples were subjected to western blot analyses with the indicated antibodies.

activation is involved in tumorigenesis [37], it is reasonable to conclude that de-regulated NF-κB activation results from mutations or deletions of *A20* and is a major cause of B-cell lymphomas. However, our present data also suggest that B-lymphocytes with mutations or deletions in *A20* are prone to undergo apoptosis upon stimulation with TNF-α. Emerging evidence revealed that apoptotic cells secrete mitogenic factors to induce compensatory proliferation of neighboring cells [38,39]. Although this compensatory proliferation was observed primarily in epithelial cells, recent studies showed that lymphocytes also have an ability to promote compensatory proliferation [40]. Because chronic mitogenic stimuli often cause tumorigenesis [38], we speculate that the susceptibility of B-lymphocytes with mutations or deletions in *A20* to TNF-α-induced apoptosis may be involved in the development of B-cell lymphomas through the induction of persistent compensatory proliferation.

In conclusion, we found that ZnF7 is important for the suppression of TNF-α-induced apoptosis by A20. Because overexpression of A20 promotes the survival of glioma stem cells and tumor progression [41], up-regulation as well as down-regulation of the activity of A20's ZnF7 may be involved in tumorigenesis by inducing resistance to apoptosis. Therefore, our present results suggest that inhibiting ZnF7 may be a novel therapeutic strategy for A20-overexpressing cancers.

Acknowledgements

We thank A. Ma for A20-deficient MEFs, Y. Demizu for MV-1, J. Inoue for the helpful inputs, and N. Fujiwara for the technical assistance. This work was supported in part by a Grant-in-aid for Scientific Research C (No. Y., 25460356) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.04.022.

References

[1] Fiers, W. (1991) Tumor necrosis factor. Characterization at the molecular, cellular and in vivo level. FEBS Lett. 285, 199–212.
[2] Sugarman, B.J., Aggarwal, B.B., Hass, P.E., Figari, I.S., Palladino Jr., M.A. and Shepard, H.M. (1985) Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells in vitro. Science 230, 943–945.
[3] Naude, P.J., den Boer, J.A., Luiten, P.G. and Eisel, U.L. (2011) Tumor necrosis factor receptor cross-talk. FEBS J. 278, 888–898.
[4] Micheau, O. and Tschopp, J. (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114, 181–190.
[5] Vucic, D., Dixit, V.M. and Wertz, I.E. (2011) Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. Nat. Rev. Mol. Cell Biol. 12, 439–452.
[6] Mahoney, D.J. et al. (2008) Both cIAP1 and cIAP2 regulate TNFα -mediated NF-κB activation. Proc. Natl. Acad. Sci. U.S.A. 105, 11778–11783.
[7] Vince, J.E. et al. (2009) TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (TNF) to efficiently activate NF-κB and to prevent TNF-induced apoptosis. J. Biol. Chem. 284, 35906–35915.
[8] Gerlach, B. et al. (2011) Linear ubiquitination prevents inflammation and regulates immune signalling. Nature 471, 591–596.
[9] Ikeda, F. et al. (2011) SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis. Nature 471, 637–641.
[10] Tokunaga, F. et al. (2011) SHARPIN is a component of the NF-κB-activating linear ubiquitin chain assembly complex. Nature 471, 633–636.
[11] Ea, C.K., Deng, L., Xia, Z.P., Pineda, G. and Chen, Z.J. (2006) Activation of IKK by TNFα requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol. Cell 22, 245–257.
[12] Walczak, H. (2011) TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer. Immunol. Rev. 244, 9–28.
[13] DiDonato, J.A., Mercurio, F. and Karin, M. (2012) NF-κB and the link between inflammation and cancer. Immunol. Rev. 246, 379–400.
[14] Varfolomeev, E.E. and Ashkenazi, A. (2004) Tumor necrosis factor: an apoptosis JuNKie? Cell 116, 491–497.
[15] Wang, L., Du, F. and Wang, X. (2008) TNF-α induces two distinct caspase-8 activation pathways. Cell 133, 693–703.
[16] Wertz, I.E. et al. (2004) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-κB signalling. Nature 430, 694–699.
[17] Lee, E.G., Boone, D.L., Chai, S., Libby, S.L., Chien, M., Lodolce, J.P. and Ma, A. (2000) Failure to regulate TNF-induced NF-κB and cell death responses in A20-deficient mice. Science 289, 2350–2354.
[18] Compagno, M. et al. (2009) Mutations of multiple genes cause deregulation of NF-κB in diffuse large B-cell lymphoma. Nature 459, 717–721.
[19] Kato, M. et al. (2009) Frequent inactivation of A20 in B-cell lymphomas. Nature 459, 712–716.
[20] Shembade, N., Ma, A. and Harhaj, E.W. (2010) Inhibition of NF-κB signaling by A20 through disruption of ubiquitin enzyme complexes. Science 327, 1135–1139.
[21] Jin, Z., Li, Y., Pitti, R., Lawrence, D., Pham, V.C., Lill, J.R. and Ashkenazi, A. (2009) Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. Cell 137, 721–735.
[22] Bellail, A.C., Olson, J.J., Yang, X., Chen, Z.J. and Hao, C. (2012) A20 ubiquitin ligase-mediated polyubiquitination of RIP1 inhibits caspase-8 cleavage and TRAIL-induced apoptosis in glioblastoma. Cancer Discov. 2, 140–155.
[23] Skaug, B., Chen, J., Du, F., He, J., Ma, A. and Chen, Z.J. (2011) Direct, noncatalytic mechanism of IKK inhibition by A20. Mol. Cell 44, 559–571.
[24] Tokunaga, F. et al. (2012) Specific recognition of linear polyubiquitin by A20 zinc finger 7 is involved in NF-κB regulation. EMBO J. 31, 3856–3870.
[25] Verhelst, K., Carpentier, I., Kreike, M., Meloni, L., Verstrepen, L., Kensche, T., Dikic, I. and Beyaert, R. (2012) A20 inhibits LUBAC-mediated NF-κB activation by binding linear polyubiquitin chains via its zinc finger 7. EMBO J. 31, 3845–3855.
[26] Yamaguchi, N., Oyama, M., Kozuka-Hata, H. and Inoue, J. (2013) Involvement of A20 in the molecular switch that activates the non-canonical NF-κB pathway. Sci. Rep. 3, 2568.
[27] Holler, N. et al. (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat. Immunol. 1, 489–495.
[28] Degterev, A. et al. (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat. Chem. Biol. 4, 313–321.
[29] Bertrand, M.J.M. et al. (2008) cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Mol. Cell 30, 689–700.
[30] Varfolomeev, E., Goncharov, T., Fedorova, A.V., Dynek, J.N., Zobel, K., Deshayes, K., Fairbrother, W.J. and Vucic, D. (2008) C-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor α (TNFα)-induced NF-κB activation. J. Biol. Chem. 283, 24295–24299.
[31] Varfolomeev, E. et al. (2007) IAP antagonists induce autoubiquitination of c-IAPs, NF-κB activation, and TNFα-dependent apoptosis. Cell 131, 669–681.
[32] Varfolomeev, E., Goncharov, T., Maecker, H., Zobel, K., Komuves, L.G., Deshayes, K. and Vucic, D. (2012) Cellular inhibitors of apoptosis are global regulators of NF-κB and MAPK activation by members of the TNF family of receptors. Sci. Signal. 5, ra22.
[33] Vallabhapurapu, S. et al. (2008) Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-κB signaling. Nat. Immunol. 9, 1364–1370.
[34] Zarnegar, B.J. et al. (2008) Noncanonical NF-κB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. Nat. Immunol. 9, 1371–1378.
[35] Dejardin, E. et al. (2002) The lymphotoxin-β receptor induces different patterns of gene expression via two NF-κB pathways. Immunity 17, 525–535.
[36] Dixit, V.M. et al. (1990) Tumor necrosis factor-α induction of novel gene products in human endothelial cells including a macrophage-specific chemotaxin. J. Biol. Chem. 265, 2973–2978.
[37] Karin, M. (2006) Nuclear factor-κB in cancer development and progression. Nature 441, 431–436.
[38] Kuraishy, A., Karin, M. and Grivennikov, S.I. (2011) Tumor promotion via injury- and death-induced inflammation. Immunity 35, 467–477.
[39] Tamori, Y. and Deng, W.M. (2014) Compensatory cellular hypertrophy: the other strategy for tissue homeostasis. Trends Cell Biol. 24, 230–237.
[40] Tchao, N.K. and Turka, L.A. (2012) Lymphodepletion and homeostatic proliferation: implications for transplantation. Am. J. Transplant. 12, 1079–1090.
[41] Hjelmeland, A.B. et al. (2010) Targeting A20 decreases glioma stem cell survival and tumor growth. PLoS Biol. 8, e1000319.