The deubiquitinase USP7 stabilizes Maf proteins to promote myeloma cell survival

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Edited by George N. DeMartino

The Maf proteins, including c-Maf, MafA, and MafB, are critical transcription factors in myelomagenesis. Previous studies demonstrated that Maf proteins are processed by the ubiquitin–proteasome pathway, but the mechanisms remain elusive. This study applied MS to identify MafB ubiquitination-associated proteins and found that the ubiquitin-specific protease USP7 was present in the MafB interactome. Moreover, USP7 also interacted with c-Maf and MafA and blocked their polyubiquitination and degradation. Consistently, knockdown of USP7 resulted in Maf protein degradation along with increased polyubiquitination levels. The action of USP7 thus promoted Maf transcriptional activity as evidenced by luciferase assays and by the up-regulation of the expression of Maf-modulated genes. Furthermore, USP7 was up-regulated in myeloma cells, and it was negatively associated with the survival of myeloma patients. USP7 promoted myeloma cell survival, and when it was inhibited by its specific inhibitor P5091, myeloma cell lines underwent apoptosis. These results therefore demonstrated that USP7 is a deubiquitinase of Maf proteins and promotes MM cell survival in association with Maf stability. Given the significance of USP7 and Maf proteins in myeloma genesis, targeting the USP7/Maf axle is a potential strategy to the precision therapy of MM.

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Multiple myeloma (MM)[5] is a malignant disorder of clonal plasma cells (1), and it is widely associated with genetic and cytogenetic aberrations. These characteristic events include various gene mutations (such as TP53, KRAS, NRAS, MYC, and BRAF) and chromosomal abnormalities (including trisomies, monosomies, deletions, and chromosomal translocations) (2). The chromosomal translocations are mainly observed between chromosome 14 that represents IgH and other specific chromosomes. The most common ones are t(4;14)(p16:q32), t(6;14)(p21:q32), t(11;14)(q13:q32), t(14;16)(q32:q23), and t(14;20)(q32:q12), which have been found in more than half of MM cell lines and MM patients (3). Among these translocations, t(8:14), t(14:20), and t(14:16) lead to the high expression of MafA, MafB, and c-Maf, respectively (3), and predict a poor clinical outcome of patients with MM. In addition to chromosomal translocations, other genetic events also up-regulate the expression of Maf proteins in MM cells (4). MafA, MafB, and c-Maf belong to the basic zipper Maf transcription factor family and up-regulate the transcription of several critical genes, including cyclin D2 (CCND2), AMP-activated protein kinase-related protein kinase (ARK5), and integrin β7 (ITGB7) in the development and progression of MM (5). These genes promote MM cell adhesion, migration, and invasion (6). Maf proteins therefore could be developed as a promising therapeutic target for MM (7, 8).

The past decade witnessed developments in cancer treatment by targeting the ubiquitin–proteasome pathway (9, 10). Protein ubiquitination is a multiple-step process by adding the small protein ubiquitin to the protein substrates under the direction of specific ubiquitin-conjugating enzymes and ubiquitin ligases. Notably, protein ubiquitination is a reversible process in which the attached ubiquitin molecules could be removed (or hydrolyzed) by a group of proteins called deubiquitinases or ubiquitin-specific proteases (11). Among the Maf
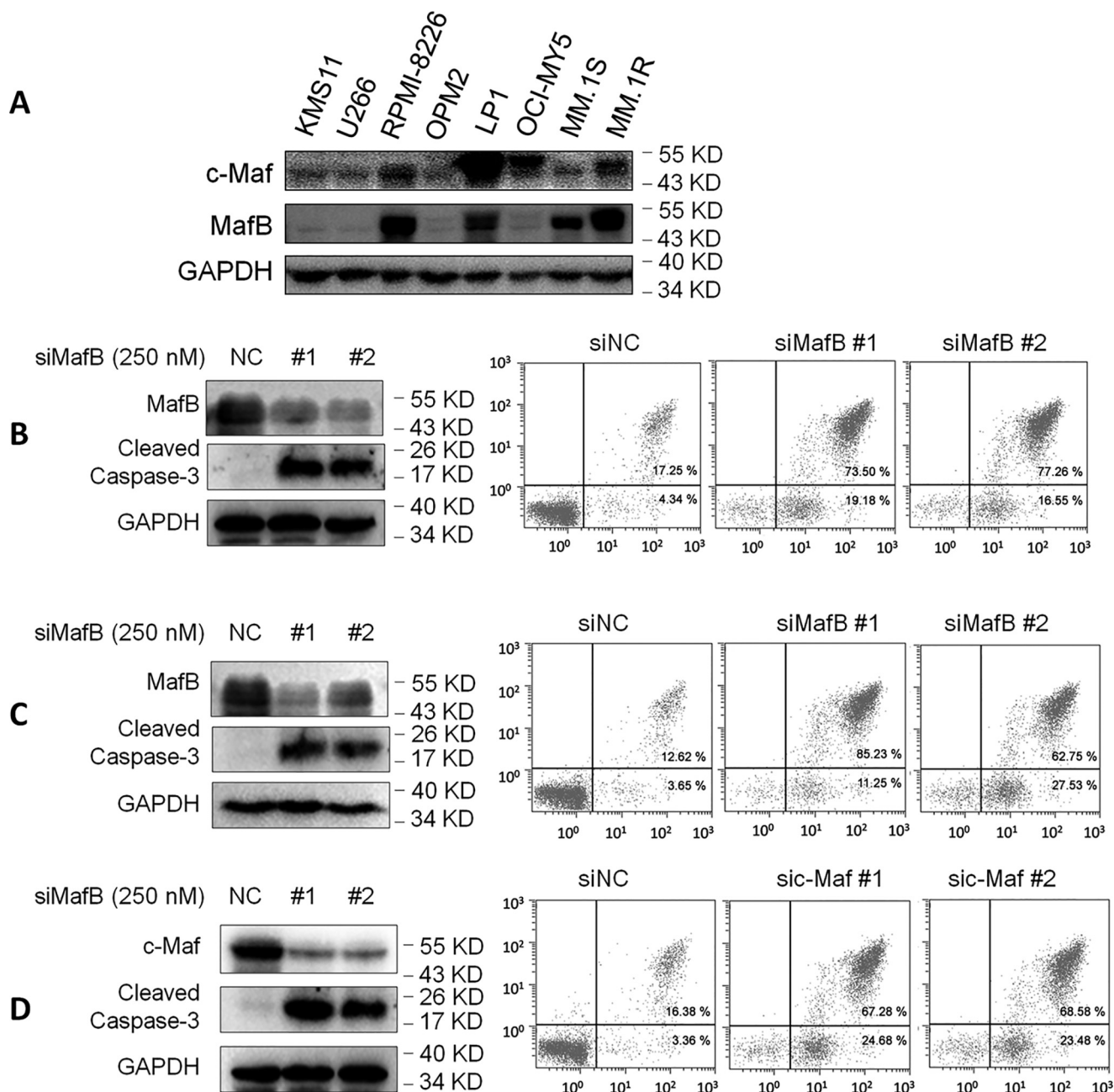
Figure 1. Inhibition of Maf proteins leads to MM cell apoptosis. *A,* Maf protein expression profiles in MM cell lines. *B* and *C,* MafB was knocked down by siRNAs (target sequences: *#1,* 5′-TGCTCAAGTTCGACGTGAA-3′; *#2,* 5′-CCAGTCTTGCAGGTATAAA-3′) from RPMI-8226 (*B*) and LP1 (*C*) for 72 h, followed by immunoblotting and annexin V/PI staining and flow cytometric analyses. *D,* c-Maf (target sequences: *#1,* 5′-GGACGCGTACAAGGAGAAA-3′; *#2,* 5′-CGCACCT-GGAAGACTACTA-3′) was knocked down from OCI-MY5, followed by immunoblotting and flow cytometric analysis. *NC,* negative control.

family proteins, c-Maf has been well-documented to be ubiquitinated by ubiquitin-conjugating enzyme UBE2O (12), ubiquitin ligase HERC4 (13), or TMEPAI/NEDD4 (14), but the ubiquitination process of MafB and MafA remains elusive. This study applied co-immunoprecipitation–coupled MS to identify MafB-associated ubiquitination enzymes and found that the ubiquitin-specific protease 7 (USP7) could interact with and stabilize all MafA, MafB, and c-Maf proteins by preventing their polyubiquitination. Moreover, inhibition of USP7 leads to Maf protein degradation and MM cell apoptosis, which forms a framework for the precision treatment of Maf-expressing MM.

Results

Down-regulation of Maf protein leads to MM cell apoptosis

The Maf genes have been reported to be highly expressed in MM cells and primary MM cells in association with various characteristic chromosomal translocations and other unknown events, but the protein expressions were largely unknown. Therefore, we first evaluated the expression profiles of Maf proteins in MM cell lines. As shown in Fig. 1*A,* c-Maf was found in most cell lines, especially in LP1, OCI-MY5, MM1.R, and RPMI-8226, and in contrast, MafB was highly expressed in LP1, MM1.S, MM1.R, and RPMI-8226 but not in OCI-MY5 and oth-

ASBMB

J. Biol. Chem. (2020) 295(7) 2084–2096 **2085**

Table 1
MafB-associated ubiquitination-associated enzymes identified by AP/MS

Classification	Gene names	Protein ID	Total MS spectral counts									Sequence coverage	Mass
			MafB_1	MafB_2	MafB_3	MafB-MG132_1	MafB-MG132_2	MafB-MG132_3	NC_1	NC_2	NC_3		
												%	*kDa*
E2	*UBE2O*	Q9C0C9	0	3	12	0	2	7	0	0	0	16.4	141.29
E3	*HUWE1*	Q7Z6Z7-2	159	64	53	75	25	68	0	0	0	30.2	480.19
E3	*ARIH1*	Q9Y4X5	0	6	11	0	3	10	0	0	0	28.9	64.117
E3	*RAD18*	Q9NS91	0	7	15	0	4	15	0	0	0	41.8	56.222
Dub	*USP7*	Q93009	6	4	25	6	4	10	0	0	0	28.9	128.3
Dub	*USP9X*	Q93008-1	0	2	7	0	2	14	0	0	0	9.4	290.46

ers. To find out whether inhibition of these Maf proteins could lead to MM cell apoptosis, RPMI-8226, LP1, and OCI-MY5 cells were transfected with Maf-specific siRNAs, followed by annexin V/PI staining and flow cytometric analysis. The results showed that MM cells underwent apoptosis as demonstrated by a marked increase in the fraction of annexin V–positive cells at both MafB (Fig. 1, *B* and *C*) and c-Maf (Fig. 1*D*) knockdowns. Consistent with this finding, cleaved caspase-3 was also markedly increased in all cell lines with Maf gene knockdown (Fig. 1, *B*–*D*). These findings further suggest the Maf proteins could be potential therapeutic targets for MM.

USP7 interacts with Maf proteins

We previously reported that ubiquitination-associated enzymes were involved in the ubiquitination of c-Maf (13). To find the associated enzymes regulating MafB ubiquitination, we performed an AP/MS assay, as described previously (13). The MS data were analyzed based on three independent paired studies (including the following three groups: control vector, MafB plasmid, and MafB plasmid + MG132 treatment). To identify potential interacting proteins with MafB, the criteria were set as follows: 1) the minimal average of unique peptides ≥2 with a *p* value <0.05 in both MafB and MafB + MG132 groups; 2) no unique peptides were found in at least two of three of the tested samples or the average number of the unique peptides in the control group <2. Based on these criteria, 264 proteins were identified by AP/MS in association with MafB (Table S1). Notably, several ubiquitination-associated enzymes include the ubiquitin-conjugating enzyme UBE2O, ubiquitin ligases ARIH1, HUWE1, and RAD18, and deubiquitinases USP7 and USP9x (Table 1). Notably, our previous study has demonstrated that UBE2O interacts with and induces MafB and c-Maf ubiquitination and degradation (12). In this study, USP7 was chosen for further studies. The unique peptides of USP7 are shown in Fig. 2*A*.

First, we verified the interaction between USP7 and Maf proteins at both endogenous and exogenous expression contexts. In the exogenous expression model, USP7 was co-transfected with MafB, MafA, or c-Maf plasmids into HEK293T cells followed by IP/IB assay, and the results showed that Maf proteins were co-immunoprecipitated with USP7 (Fig. 2, *B*–*D*), suggesting USP7 interacted with Maf proteins. To verify this interaction at the endogenous setting in MM cells, cell lysates directly from MM cell lines RPMI-8226, OCI-MY5, and LP1 cells were subjected to IP/IB analyses. The results showed that USP7 bound to endogenous MafB and c-Maf proteins in RPMI-8226/LP1 (Fig. 2*E*) and RPMI-8226/OCI-MY5 (Fig. 2*F*) cell lines,

respectively. To further characterize this interaction, a series of USP7 truncates were prepared (Fig. 2*G*), followed by co-transfection with a MafB plasmid into HEK293T cells. The cell lysates were then subjected to co-IP and IB assays. As shown in Fig. 2*H*, all truncates were successfully expressed and identified by a specific antibody; however, all truncates except the catalytic domain were detected from MafB precipitates (Fig. 2*I*), suggesting the TRAF and the UBL domains probably mediated the interaction between Maf and USP7 because the presence of either TRAF or UBL domain was sufficient for the interaction.
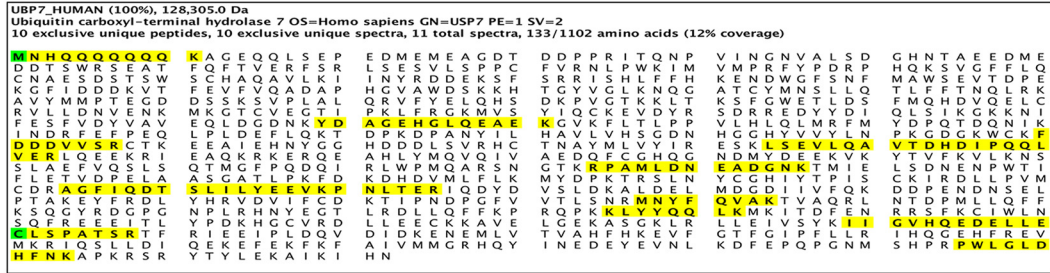
USP7 prevents polyubiquitination of Maf proteins

As a typical ubiquitin-specific protease, USP7 has been shown to catalyze the ubiquitin chains or prevent the ubiquitination from substrate proteins such as MDM2 (15), CDC25A (16), SIRT7 (17), N-Myc (18), and PHF8 (19). To find out whether USP7 modulates Maf ubiquitination, we first evaluated the ubiquitination levels of Maf proteins in HEK293T cells by co-transfecting both USP7 and Maf plasmids, followed by a co-IP/IB assay. As shown in Fig. 3, *A*–*C*, all three Maf proteins were heavily polyubiquitinated in the overexpression of Ub. However, USP7 markedly reduced the polyubiquitination levels of the Maf proteins (Fig. 3, *A*–*C*). This finding was confirmed in MM cells. Lentivirus-mediated USP7 markedly decreased the ubiquitination levels of MafB in RPMI-8226 and LP1 cells (using an anti-MafB antibody for immunoprecipitation, see Fig. 3*D*). Similarly, c-Maf ubiquitination was decreased by lentiviral USP7 in MM cells (using an anti-c-Maf antibody for immunoprecipitation, see Fig. 3*E*). To further confirm this finding, MafB ubiquitination was measured in HEK293T cells by knocking down USP7 with its specific siRNA. As shown in Fig. 3*F*, the MafB polyubiquitination level was decreased by USP7, but it was increased when USP7 was knocked down. These results thus concluded that USP7 prevents the polyubiquitination levels of Maf proteins in MM cells.
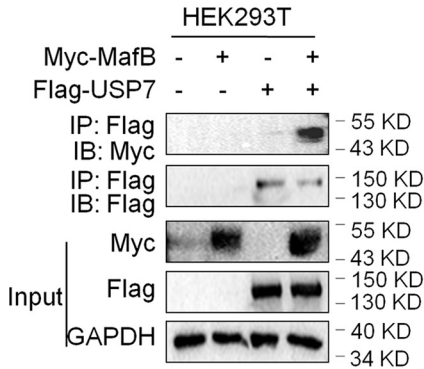
USP7 stabilizes Maf proteins

The above results demonstrated that USP7 interacts with Maf proteins and prevents their polyubiquitination. Because typical polyubiquitination might lead to protein degradation, we next wondered whether USP7 increased Maf protein stability. To this end, the USP7 plasmid was co-transfected into HEK293T cells with MafB, c-Maf, or MafA followed by IB assays. The results showed that USP7 significantly increased the protein levels of MafB (Fig. 4*A*), c-Maf (Fig. 4*B*), and MafA (Fig. 4*C*) in a concentration-dependent manner. These results were further confirmed by CHX chase assays as

A

UBP7_HUMAN (100%), 128,305.0 Da
Ubiquitin carboxyl–terminal hydrolase 7 OS=Homo sapiens GN=USP7 PE=1 SV=2
10 exclusive unique peptides, 10 exclusive unique spectra, 11 total spectra, 133/1102 amino acids (12% coverage)

B HEK293T

C HEK293T

D HEK293T

E

RPMI-8226   LP1

F

RPMI-8226   OCI-MY5

G

H

I
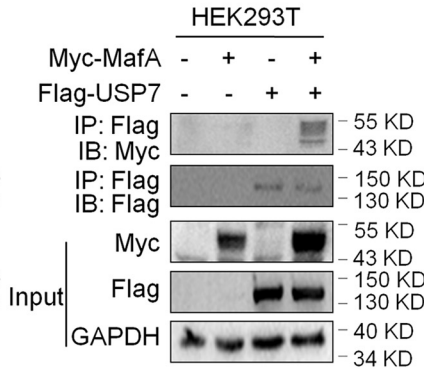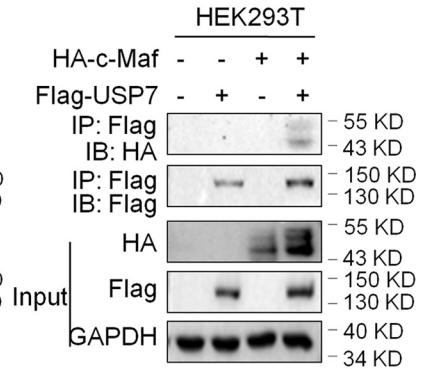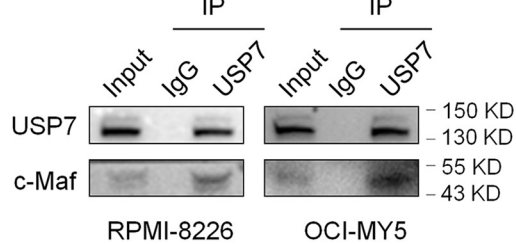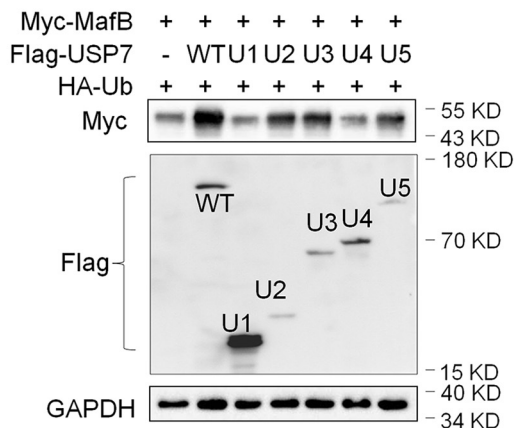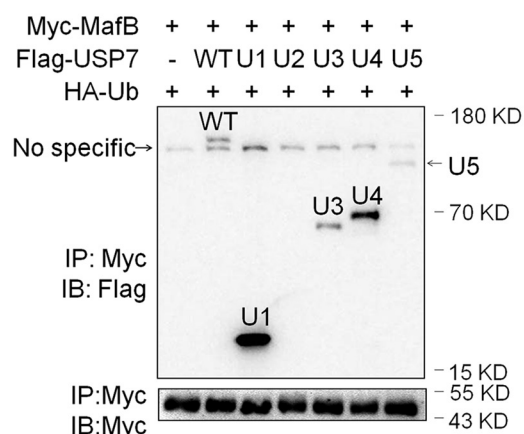
Figure 3. USP7 deubiquitinates Maf proteins. *A–C*, HEK293T cells were co-transfected with USP7, Ub, and MafB (*A*), MafA (*B*), or c-Maf (*C*) plasmids for 48 h; cell lysates were then prepared for the IP/IB assays using specific antibodies as indicated. *D*, MM cell lines RPMI-8226 and LP1 were infected with lentiviral USP7 for 96 h; cell lysates were prepared and subjected to IP with a MafB-specific antibody followed by IB with a Ub-specific antibody. *E*, RPMI-8226 cells were infected with lentiviral USP7 for 96 h; cell lysates were then prepared and subjected to IP with a anti-c-Maf–specific antibody followed by IB with anti-Ub–specific antibody. *F*, HEK293T cells were co-transfected with MafB, Ub, and USP7 plasmids, followed by siUSP7 transfection for 48 h; cell lysates were prepared for the IP/IB assays.

shown in Fig. 4, *D–F*. When *de novo* synthesis of Maf proteins was inhibited by CHX, USP7 significantly slowed down Maf degradation and prolonged their half-lives. Notably, knockdown of USP7 led to decreased Maf proteins in MM cell lines (Fig. 4, *G–I*). To find out whether Maf down-regulation by USP7 was via the ubiquitin–proteasome pathway, two siUSP7 sequences were introduced into MM cells followed by treatment of MG132, a typical proteasome inhibitor. The results showed that a marked decrease was seen in both c-Maf and MafB proteins when USP7 was knocked down, but this decrease were strikingly reversed by MG132 (Fig. 4*J*). Therefore, all the above investigations demonstrated that USP7 prevents Maf protein ubiquitination and increases their stability.

USP7 promotes the transcriptional activity of Maf proteins

Maf proteins are key transcription factors that up-regulate the gene expression by activating the promoters of specific target genes, including CCND2, ITGB7, and ARK5 via the specific recognition element (20, 21). The above studies demonstrated that USP7 stabilized the Maf protein, and therefore, we wondered whether USP7 promoted Maf transcriptional activity. To

this end, a MARE-driving firefly luciferase reporter (MARE-.Luci) was co-transfected into HEK293T cells with MafB in the presence or absence of a USP7 plasmid for 24 h before being subjected to luciferase assays (14). As shown in Fig. 5*A*, MafB up-regulated luciferase activity, and it was strikingly increased by the addition of USP7, suggesting that USP7 promoted MafB transcriptional activity. Similar findings were found in c-Maf. As shown in Fig. 5*B*, in the presence of USP7, the transcriptional activity of c-Maf was also markedly increased. Moreover, both ITGB7 and CCND2, two representative genes regulated by Maf proteins, were induced by USP7 (Fig. 5*C*). In contrast, when USP7 was knocked down by siUSP7 from the MM cell line RPMI-8226, the expression of these two genes was accordingly down-regulated (Fig. 5*D*). These results thus concluded that USP7 enhances Maf proteins transcriptional activity by preventing their degradation.

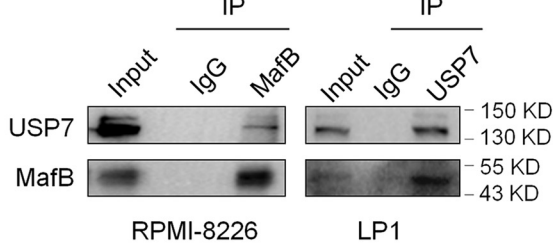### USP7 promotes MM cell survival and it is correlated with poor prognosis of patients with myeloma

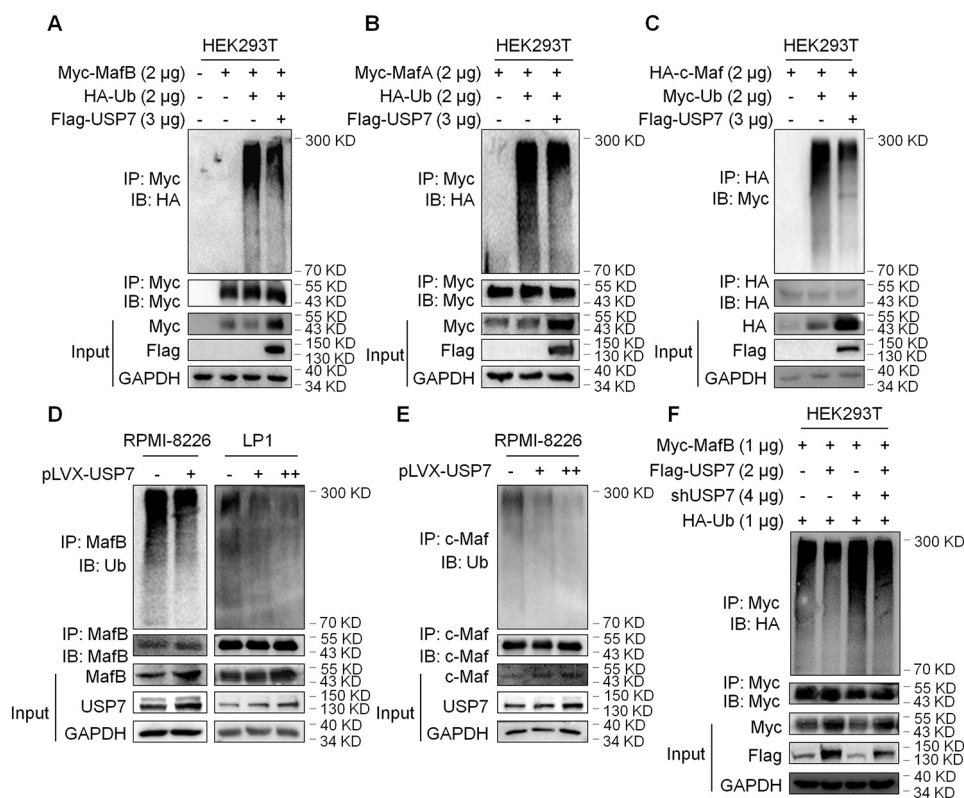The aforementioned studies have shown that USP7 stabilizes Maf proteins and promotes their oncogenic transcriptional activity. Therefore, we wondered whether USP7 was also

Figure 2. USP7 interacts with Maf proteins. *A*, USP7 was identified by AP/MS/MS, and the unique peptides are highlighted in *yellow*. *B–D*, HEK293T cells were co-transfected with FLAG-USP7 and MafB (*B*), MafA (*C*), or c-Maf (*D*) plasmids for 48 h, and cell lysates were subjected to IP with an anti-Flag antibody and subsequent IB with indicated antibodies. *E*, cell lysates from MM cell lines RPMI-8226 and LP1 were incubated with an anti-MafB or USP7 antibody overnight, followed by IB with an anti-USP7 or anti-MafB antibody. *F*, cell lysates from MM cell lines RPMI-8226 and OCI-MY5 were incubated with an anti-USP7 antibody overnight, followed by IB with an anti-USP7 or anti-c-Maf antibody. *G*, The schematic draw of USP7 domains. *H* and *I*, HEK293T cells were co-transfected with Flag-USP7 truncates, HA-Ub and Myc-MafB for 44 h, followed by MG132 (20 μM) treatment for 4 h. Cell lysates were prepared for IP with an anti-Myc antibody and IB with an anti-Flag antibody.
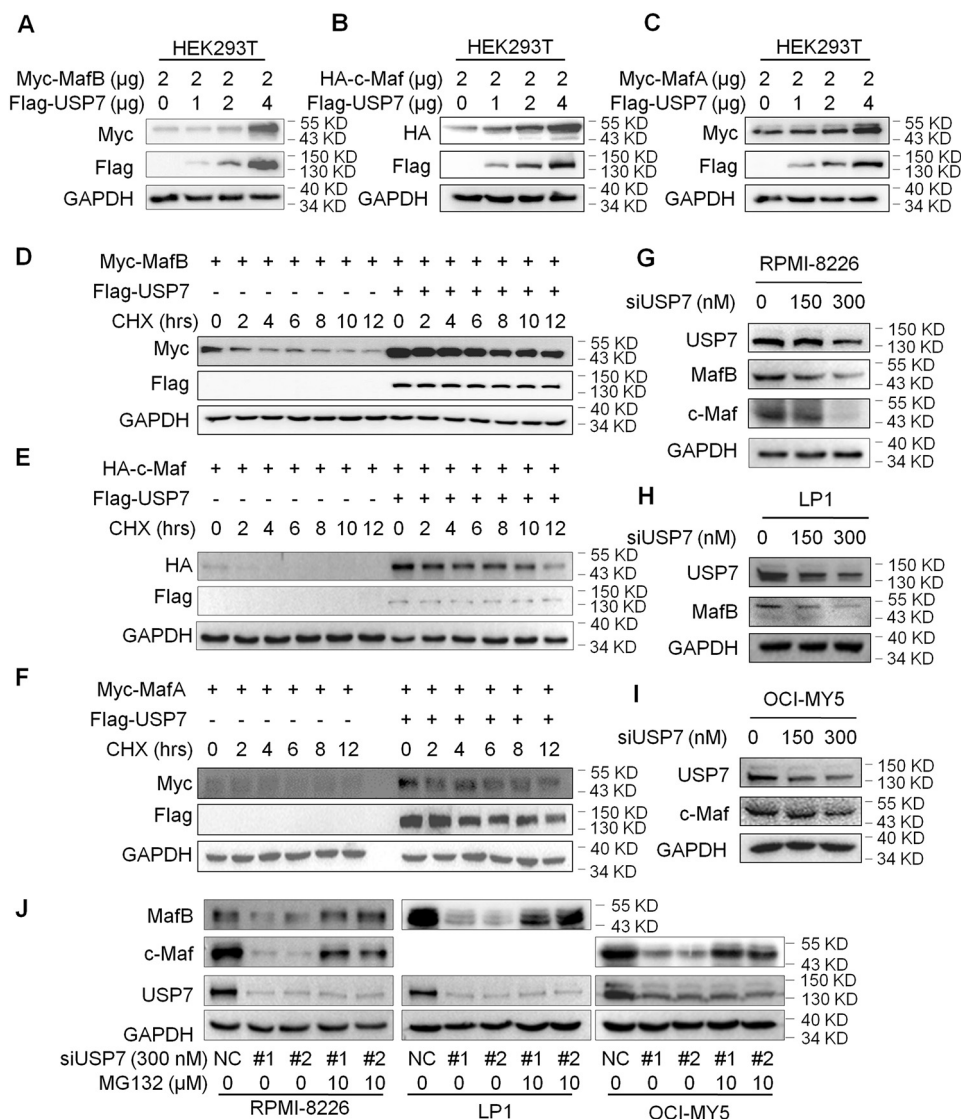
Figure 4. USP7 stabilizes Maf proteins. *A–C,* HEK293T cells were co-transfected with USP7 and MafB (*A*), c-Maf (*B*), or MafA (*C*) plasmids for 48 h, followed by cell lysate preparations and IB assay against specific antibodies as indicated. *D–F,* HEK293T cells were co-transfected with USP7 and MafB (*D*), c-Maf (*E*), or MafA (*F*) plasmids for 36 h, followed by CHX treatment for the indicated periods before collection for cell lysate preparation and IB assays. *G–I,* USP7 was knocked down by siRNAs (*#1*) from RPMI-8226 (*G*), LP1 (*H*), and OCI-MY5 (*I*) for 72 h; cell lysates were prepared for the IB assay. *J,* USP7 was knocked down by two siRNAs from RPMI-8226, LP1, and OCI-MY5 for 60 h, followed by MG132 treatment for 12 h; cell lysates were prepared for the IB assay. *NC,* negative control.

important in MM pathophysiology. To this end, the expression profile of USP7 was evaluated in plasma cells from healthy donors (HD), patients with monoclonal gammopathy of undetermined significance (MGUS), and myeloma patients by using the public cancer database (22). The result revealed that the USP7 expression levels were significantly increased in MGUS and MM cells compared with that in the HD controls (Fig. 6*A*). This observation was verified in bone marrow cells from HD and MM patients using RT-PCR. The result showed that USP7 was significantly increased in MM patients (Fig. 6*B*). These findings indicated USP7 expression is highly associated with myeloma progression. To predict the clinical relevance of USP7 in MM patients, the correlation between USP7 expression levels and the overall survival and event-free survival time was analyzed (22). As shown in Fig. 6, *C* and *D*, USP7 expression was negatively related to both events-free survival and overall survival time of MM patients. These results suggested that USP7

probably promotes MM cell survival by stabilizing Maf proteins. Subsequently, we measured MM cell survival using RPMI-8226 and OCI-MY5 as the model cells after being infected with lentiviral USP7. As shown in Fig. 6, *E* and *F*, the viability of MM cells was markedly increased following the ectopic expression of USP7. Consistent with this finding, knockdown of USP7 by its specific siRNA led to significant reduction of MM cell viability (Fig. 6, *G* and *H*). All these results thus demonstrated that USP7 promotes MM cell survival, and it predicts poor outcome of MM patients.

Inhibition of USP7 leads to MM cell apoptosis by mediating Maf degradation

Because USP7 increases MM cell viability and contributes to the poor outcome of MM patients, we wondered whether inhibition of USP7 could mediate MM cell apoptosis by degrading Maf proteins. To this end, P5091 (Fig. 7*A*), a small molecule
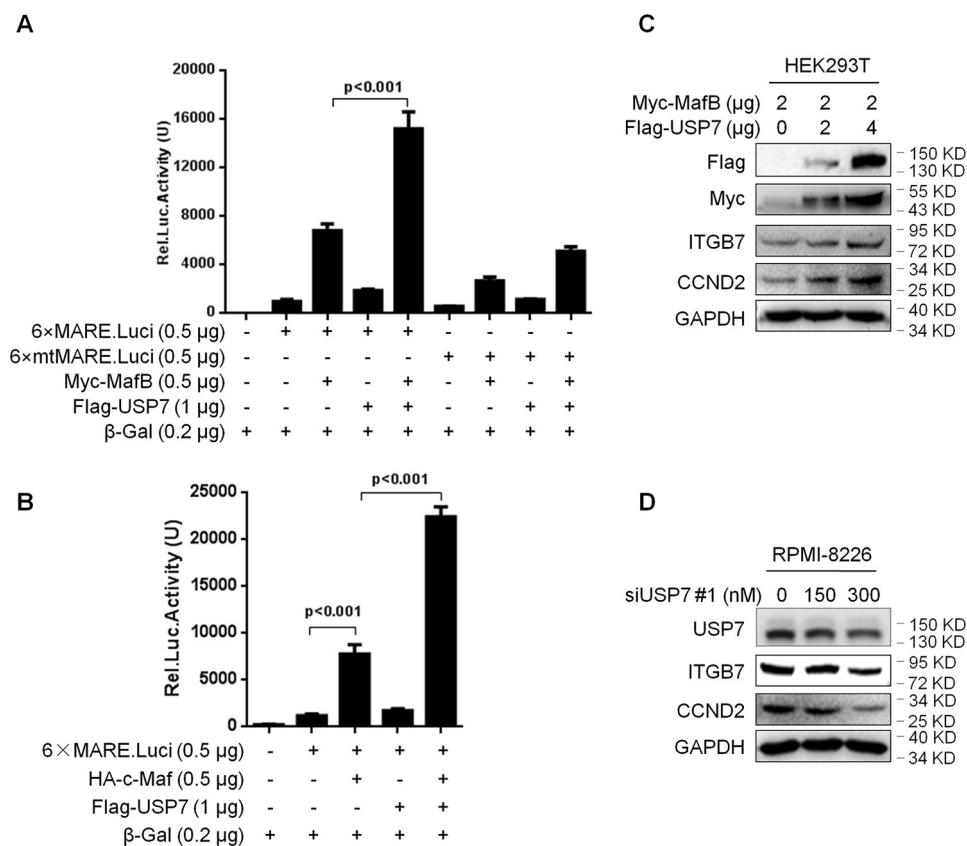
Figure 5. USP7 enhances Maf transcriptional activity and promotes the expression of targeted genes. *A,* MARE-driving luciferase plasmid was co-transfected into HEK293T cells with MafB in the presence or absence of USP7, and 24 h later, cells were collected for measurement of luciferase activity. The mutant mtMARE.Luci was used as a control. *β*-Gal was used as a transfection control. *B,* MARE-driving luciferase plasmid was co-transfected into HEK293T cells with c-Maf in the presence or absence of USP7, and 24 h later, cells were collected for measurement of luciferase activity. *C,* HEK293T cells were co-transfected with MafB and USP7 plasmids for 24 h, followed by cell lysate preparation and IB assays to measure the Maf target genes *ITGB7* and *CCND2. D,* USP7 was knocked down from RPMI-8226 cells by siUSP7 (*#1*). Forty eight hours later, cells were subjected to IB assay against specific antibodies as indicated.

inhibitor of USP7 (23), was used for the principle of concept. We first measured the effects of P5091 on Maf protein ubiquitination in MM cells. As shown in Fig. 7, *B* and *C*, P5091 significantly increased the polyubiquitination levels of both MafB and c-Maf in MM cells. Moreover, when cells were transfected with MafB, Ub, and/or USP7 plasmids followed by P5091 treatment, the IP/IB assay showed that the decreased polyubiquitination levels on MafB by USP7 were markedly increased by P5091 (data not shown). In accordance with these findings, P5091 suppressed the transcriptional activity of c-Maf and MafB that were up-regulated by USP7 (Fig. 7*D*).

Because Maf proteins are key factors in myelomagenesis, we wondered whether P5091-induced MM cell apoptosis was Maf-dependent. To this end, MafB-expressing (RPMI-8226 and LP1) and c-Maf– expressing (RPMI-8226 and OCI-MY5) cell lines as well KMS11 cells with a low expression of c-Maf (4) and lacking MafB (Fig. 1) were treated with P5091, followed by IB assays. The results showed that P5091 decreased the protein levels of MafB and c-Maf in a concentration-dependent manner in the cell lines of RPMI-8226, LP1 (Fig. 7*E*), and OCI-MY5 (Fig. 7*F*) but not in KMS11 (Fig. 7*G*). Notably, the changes on Maf proteins were associated with the cleavage of PARP, the down-regulation of prosurvival Mcl-1, and the up-regulation of pro-apoptotic Bax in RPMI-8226, LP1, and OCI-MY5 cells, but P5091 did not show a marked decrease of c-Maf and less PARP

cleavage in KMS11 (Fig. 7, *E–G*), suggesting that Maf protein degradation is associated with MM cell apoptosis induced by P5091.

Previous studies have demonstrated that USP7 can also stabilize MDM2 (24), a ubiquitin ligase of p53. P5091 was proposed to induce MM cell apoptosis by inhibiting the USP7/MDM2/p53 axle (23). However, p53 mutation is frequently seen in late stage MM, and their expression levels and mutation status are also diverse. For example, LP1 expresses WT p53, but RPMI-8226 and OCI-MY5 harbor mutated p53, and KMS11 expresses null or weak p53 (25, 26). To find out the effects of p53 in the treatment of P5091 on MM, the above MM cell lines were treated with P5091 followed by IB assays for p53. As shown in Fig. 7*E*, P5091 up-regulated the expression of p53 in both RPMI-8226 and LP1 cells in association with PARP cleavage, but p53 was not markedly affected by P5091 in OCI-MY5 (Fig. 7*F*), although OCI-MY5 cells underwent PARP cleavage. The cell apoptosis induced by P5091 was further measured in terms of annexin V staining and flow cytometric analyses. Consistent with the findings by PARP cleavage, marked annexin V–positive cells were found in both RPMI-8226 and OCI-MY5 but not in KMS11 cells. Notably, P5091 also failed to increase p53 in OCI-MY5 cells, which suggested that P5091-induced apoptosis in these cells might not be related to p53 stabilization; in contrast, Maf proteins also play a key role.
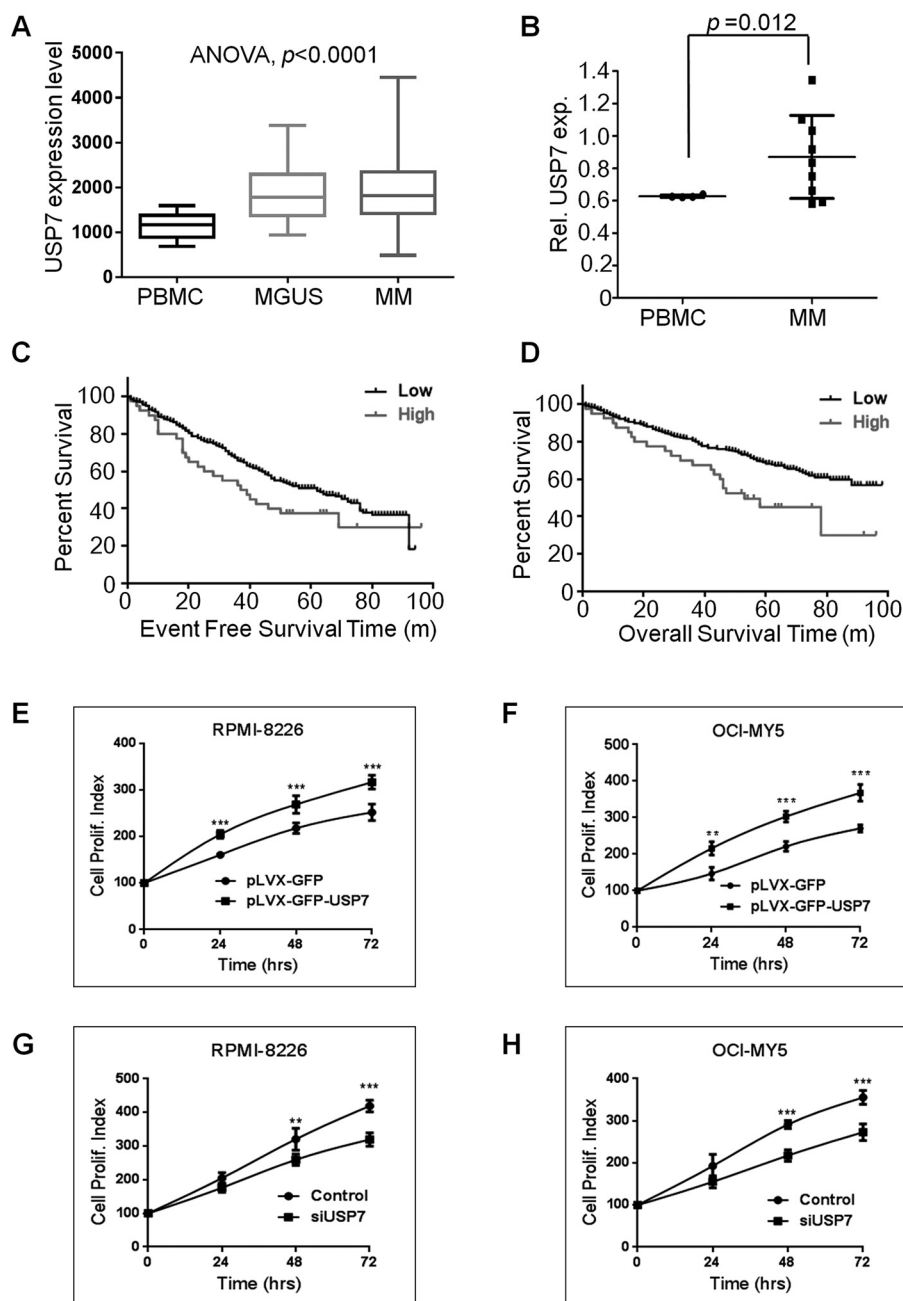
Figure 6. USP7 is overexpressed in MM cells, and it is associated with inferior outcomes in MM patients. *A,* USP7 mRNA expression was analyzed from the cDNA microarray dataset that was generated from normal bone marrow cells from healthy donors and bone marrow cells from MGUS patients or from MM patients. *B,* bone marrow cells from healthy donors or MM patients were subjected to RT-PCR using USP7-specific primers. *C,* correlation of USP7 expression level with the event-free survival of MM patients. *D,* correlation of USP7 expression level with the overall survival of MM patients. *E* and *F,* MM cell lines RPMI-8226 (*E*) and OCI-MY5 (*F*) were infected with lentiviral USP7, followed by cell viability measurement. *G* and *H,* USP7 was knocked down from MM cell lines RPMI-8226 (*G*) and OCI-MY5 (*H*) by siRNA (siUSP7#1), followed by cell proliferation measurement.

Discussion

This study identified that USP7 is a putative deubiquitinase of Maf proteins. USP7 stabilizes Maf proteins by preventing their ubiquitination and degradation. Moreover, we demonstrated that targeting USP7/Maf is a potential therapeutic strategy for the precision treatment of MM patients that express a high level of Maf.

Maf proteins belong to the basic zipper transcription factor superfamily, of which MafB, c-Maf, and MafA share highly-similar sequences. By binding to the same *cis*-acting element (Maf recognition element) in the vicinity of targeted genes, Maf proteins up-regulate the transcription of CCND2, ARK5, ITGB7, and other genes (5). Moreover, the trio of MafB, c-Maf, and MafA are highly associated with MM development and progression, and these proteins have long been proposed as a therapeutic target of MM (4, 5, 13, 20, 22, 27, 28); however, very limited progress has been made due to the complexity of the regulation of these proteins. The overexpression of Maf proteins involves multiple factors, including chromosomal translocations and the overactivation of the upstream signalings
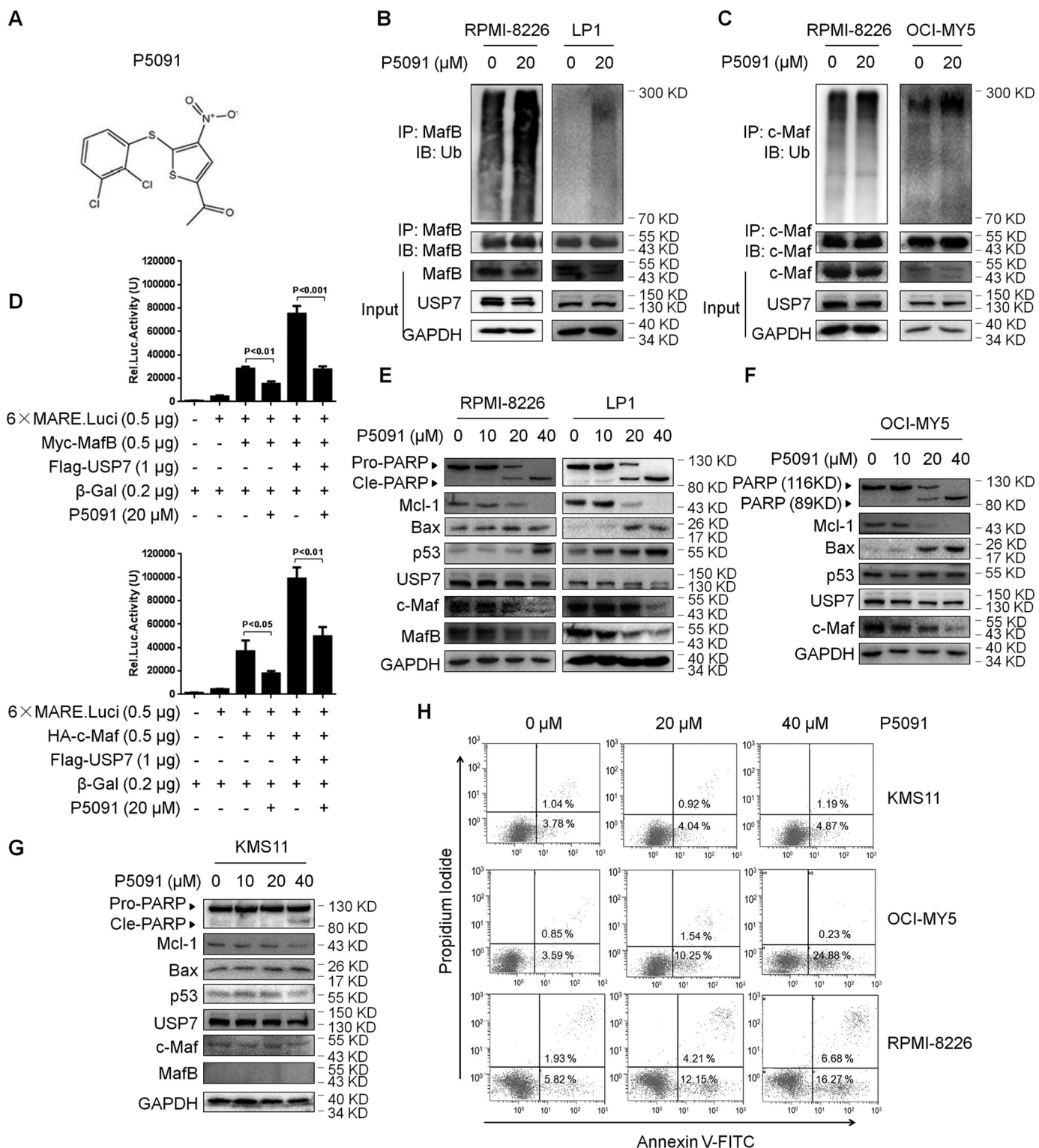
Figure 7. Inhibition of USP7 leads to MM cell apoptosis by inducing Maf protein degradation. *A,* chemical structure of the USP7-specific inhibitor P5091. *B,* RPMI-8226 and LP1 cells were treated with P5091 for 24 h, followed by IP/IB assays. *C,* RPMI-8226 and OCI-MY5 cells were treated with P5091 for 24 h, followed by IP/IB assay as indicated. *D,* MARE.Luci reporter plasmid was co-transfected into HEK293T cells with USP7 and MafB or c-Maf. Twenty four hours later, cells were treated with P5091 for another 24 h before being subjected to luciferase assay. *E–G,* RPMI-8226 and LP1 cells (*E*), OCI-MY5 (*F*), and KMS11 (*G*) cells were treated with P5091 for 24 h, followed by cell lysate preparations and IB assays against specific antibodies as indicated. *H,* KMS11, OCI-MY5, and RPMI-8226 cells were treated by P5091 for 24 h, followed by annexin V–FITC and propidium iodide staining and flow cytometric analysis.

such as mitogen-activated protein kinase and GSK-3β pathways. The stability assays demonstrated that Maf proteins could be processed via the ubiquitin–proteasome pathway.

Recently, we identified a ubiquitin-conjugating enzyme (UBE2O) (12), a ubiquitin ligase (HERC4) (13), and a deubiquitinase (USP5) (27) for the modulation of c-Maf ubiquitination

Table 2
The primers for USP7 truncates

Fragments	Primers	Sequence(5′–3′)
Full length	Forward	gatgacgacaagcttgcggccgcATGAACCACCAGCAGCAGCA
	Reverse	gccctctagactcgagcggccgcGTTATGGATTTTAATGGCCTTTTCA
U1	Forward	aaagacgatgacgacaagcttAACCACCAGCAGCAGCAGC
	Reverse	gatgagtttttgttcggatccTCACTTTGAATCCCACGCAAC
U2	Forward	aaagacgatgacgacaagcttGATTCAAAGAAGCACACAGGCTAC
	Reverse	gatgagtttttgttcggatccTCATTCCTGCCGCTCCTTC
U3	Forward	aaagacgatgacgacaagcttGAAGCCCATCTCTATATGCAAGTG
	Reverse	gatgagtttttgttcggatccTCAGTTATGGATTTTAATGGCCTTT
U4	Forward	aaagacgatgacgacaagcttAACCACCAGCAGCAGCAGC
	Reverse	gatgagtttttgttcggatccTCATTCCTGCCGCTCCTTC
U5	Forward	aaagacgatgacgacaagcttGATTCAAAGAAGCACACAGGCTAC
	Reverse	gatgagtttttgttcggatccTCAGTTATGGATTTTAATGGCCTTT

and stability. This study identified USP7 by the tandem MS assay from MafB co-immunoprecipitates. Different from USP5 that has no effect on MafA, USP7 prevents polyubiquitination and increases stability of all three Maf proteins.

USP7 is a member of cysteine protease that specifically removes the ubiquitin molecules from its substrates. USP7 can cleave both single ubiquitin molecules and polyubiquitin chains depending on the specificity of the substrates. It has demonstrated that USP7 prevents the monoubiquitination of PTEN (29) and FOXO4 (30), thereby precluding their nuclear localization and inhibiting their tumor suppressor activity. This action of USP7 on the monoubiquitination does not affect the half-lives of its client proteins. Notably, these proteins are tumor suppressors. In addition to the removal of monoubiquitination, USP7 also prevents polyubiquitination of some oncoproteins, including the ring finger protein 168 (RNF168) (31), the transcription factor Foxp3 (32), NF-κB essential modulator (33), and MDM2 (24). Therefore, USP7 exerts different mechanisms in modulating protein ubiquitination dependent on the substrate specificity and cancer types. In this study, USP7 is demonstrated to stabilize Maf proteins by preventing their polyubiquitination. In contrast, knockdown of USP7 results in Maf ubiquitination and proteasomal degradation. Moreover, inhibition of USP7 leads to MM cell apoptosis in association with Maf degradation. Therefore, our findings suggest that the USP7/Maf axle could be an anti-MM target.

Recently, a series of highly-selective inhibitors of USP7 have been identified, and these compounds display potent anti-cancer activity by targeting the substrate degradation (34). MM is a class of incurable hematological malignancy derived from plasma cells. USP7 is frequently overexpressed in MM patients and contributes to poor clinical outcome. Previous studies reported that USP7 binds to and stabilizes the ubiquitin ligase MDM2 that mediates p53 polyubiquitination and degradation, thereby suppressing p53 activity in myelomagenesis (23). Inhibition of USP7 restores p53, which could lead to MM cell apoptosis. However, p53 is probably not an ideal target in the MM treatment by inhibiting USP7 because p53 is highly mutated and deleted in MM. Drach *et al.* (35) found p53 deletions in 32.8% and 54.5% of patients with newly diagnosed and relapsed MM, respectively. Moreover, the USP7 inhibitor also leads to apoptosis of MM cells that lack p53 or harbor mutated p53. For example, RPMI-8226 undergoes apoptosis by the USP7 inhibitor, although it harbors a mutated p53 (36). Moreover, OCI-MY5, another typical MM cell line, could undergo apoptosis by

treatment of P5091, but the p53 protein level is not affected. This study suggests that Maf proteins could be a target of USP7 inhibitors in terms of MM cell apoptosis. There are at least two lines of evidence. First, USP7 stabilizes Maf proteins and promotes their oncogenic transcriptional activity, thereby contributing to the poor outcome for MM patients. Second, inhibition of P5091-induced MM cell apoptosis is associated with decreased c-Maf but not increased p53. This study suggested that it is highly probable that inhibition of USP7 selectively induces MM cell apoptosis by targeting Maf protein degradation.

In summary, this study demonstrated that USP7 is a deubiquitinase of Maf proteins. USP7 stabilizes Maf proteins and increases their transcriptional activity by preventing their polyubiquitination. Overexpression of USP7 promotes Maf transcriptional activity and MM cell proliferation. Targeting the USP7/Maf axle induces Maf-expressing MM cell apoptosis. This study thus provides a rationale for the precision therapy of Maf-expressing MM patients by inhibiting USP7.

Experimental procedures

Cell culture

Human embryonic kidney cells (HEK293 and HEK293T) were maintained in Dulbecco's modified Eagle's medium. MM cells were cultured in Iscove's modified Dulbecco's media. MM cell lines, including LP1, OCI-MY5, KMS11, MM.1S, MM.1R, and OPM2, were generously provided by Dr. Aaron Schimmer, University of Toronto. RPMI-8226 and U266 were obtained from American Type Culture Collection (ATCC, Manassas, VA). All media were supplemented with 10% fetal bovine serum, glutamine, and antibiotics.

Plasmids

MafA, MafB, and c-Maf plasmids were prepared as reported previously (13), and the USP7 plasmid was cloned from HeLa cells. Primers for USP7 and the specific domains to generate USP7 truncates were designed as shown in Table 2. The firefly luciferase reporter driven by the Maf recognition element (5′-TGCGAGTGAGGCA-3′) and its mutant version (mtMARE, 5′-**gta**GAGT**g**AG**tac**-3′) were synthesized as shown previously (28).

Chemicals and antibodies

The anti-Maf antibodies were purchased from Proteintech (Chicago, IL). Monoclonal antibodies against the HA tag, the

Myc tag, the Flag tag, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Medical and Biological Laboratories Co., Ltd. (MBL, Nagoya, Japan). An anti-USP7 antibody was purchased from Cell Signaling Technologies, Inc. (Boston, MA). MG132, CHX, and P5091 were purchased from Santa Cruz Biotechnology, Sigma, and Selleck Chemicals Inc. (Houston, TX), respectively. Horseradish peroxidase–labeled goat anti-mouse and goat anti-rabbit IgG (H+L) antibodies were purchased from Beyotime Institute of Biotechnology (Nantong, China).

Gene transfection

One day before transfection, HEK293T or HEK293 cells were seeded in 6-well plates, and when grown to 50% confluence, cells were subjected to gene delivery using polyethyleneimine (PEI), as described previously (13).

AP/MS assay

HEK293 cells were transfected with a Myc–MafB plasmid or empty vector for 44 h before being treated with dimethyl sulfoxide (DMSO) or MG132 (20 μM) for another 4 h. The cells were then collected for protein extraction using a lysis buffer containing 1% (v/v) Triton X-100, 1% sodium deoxycholate, 10% (v/v) glycerol, 50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 50 mM NaF, protease inhibitors, and 1 mM Na$_3$VO$_4$. After lysis, the clarified cell lysates (10 mg of each sample) were subjected to co-IP using anti-Myc–agarose beads (Sigma) overnight at 4 °C, as shown previously (13). The proteins bound on beads were then eluted with 150 μl of 0.15% trifluoroacetic acid (TFA) and subjected to trypsin digestion. The resulting peptides were then further treated as shown previously and subjected to LC/MS/MS assay by using a nano-ESI source (Thermo Fisher Scientific) (13). Mass spectra were acquired in a data-dependent mode with an automatic switch between a full scan and up to 10 data-dependent MS/MS scans. Target value for the full-scan MS spectra was 3,000,000 with a maximum injection time of 120 ms and a resolution of 70,000 at m/z 400. The ion target value for MS/MS was set to 1,000,000 with a maximum injection time of 120 ms and a resolution of 17,500 at m/z 400. Repeat sequencing of peptides was kept to a minimum by the dynamic exclusion of sequenced peptides for 20 s (37).

MS data process

Acquired raw files were analyzed by using MaxQuant software (version 1.5.0.30). The Andromeda probabilistic search engine was used to search peak lists against the UniProt database (2014 version, 20,226 entries). The default search parameters were used. The search included cysteine carbamidomethylation as a fixed modification, N-terminal acetylation, methionine oxidation, and Gly–Gly addition to lysine as variable modifications. The second peptide identification option in Andromeda was enabled. For statistical evaluation of the data obtained, the posterior error probability and false discovery rate were used. The false discovery rate was determined by searching a reverse database. A false discovery rate of 0.01 for proteins and peptides was permitted. Two miscleavages were allowed, and a minimum of seven amino acids per identified

peptide was required. Peptide identification was based on a search with an initial mass deviation of the precursor ion of up to 6 ppm, and the allowed fragment mass deviation was set to 20 ppm. To match identifications across different replicates and adjacent fractions, the "match between runs" option in MaxQuant was enabled within a time window of 2 min. For the determination of protein levels, at least two unmodified peptides were required for LFQ calculation. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD016020 (38, 39). Unique peptides >2 and $p < 0.05$ in both MafB and MafB + MG132 groups were selected for further studies.

CHX chase assay

After being transfected with plasmids of interest for 36 h, HEK293T cells were treated with CHX (100 μg/ml) for 0 to 12 h. Cell lysates were then prepared by 2× SDS lysis buffer, followed by SDS-PAGE and immunoblotting (IB) analyses with specific antibodies as described previously (40).

Immunoblotting

Cell lysates were prepared as described previously (13). After clarification at high speed at 4 °C, protein concentrations were determined by BCA assay (Beyotime Institute of Biotechnology). Equal amounts proteins (30 μg) were fractionated in SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes. The blots were then subjected to analysis against appropriate antibodies.

Immunoprecipitation

HEK293T cells were transfected with USP7 truncates and or MafB plasmids for 48 h. Cell lysates were then prepared in a RIPA buffer; after clarification, the clear supernatants were then immunoprecipitated with specific antibodies as described previously (13).

Luciferase assay

pGL4-MARE.Luci and pGL4-mtMARE.Luci were constructed as described previously (17). The plasmids were then co-transfected into HEK293T cells with USP7 and/or MafB plasmids. Forty eight hours later, cell lysates were subjected to luciferase analysis by using the Bright-Glo system (Promega, Madison, WI), as described previously (13). The β-gal expression was used as a transfection control. Luciferase activity was normalized to β-gal expression for each sample. All transfection experiments were performed in duplicates.

Lentiviral USP7 construction

A human USP7 cDNA was inserted into the pLVX-AcGFP lentiviral vector (Clontech). To generate lentiviral particles, HEK293T cells at 80% confluence were transfected with 10 μg of pLVX-AcGFP-USP7, 3.5 μg of VSV-G envelope glycoprotein, 2.5 μg of packaging proteins (Rev), and 6.5 μg of packaging proteins (ΔR8.74) by using PEI (Sigma) as a gene delivery carrier (13). After being washed and refreshed with media, cells were further cultured for 48 h to produce lentivirus. The lenti-

virus particle-enriched supernatants were harvested, filtered, and stored frozen at −80 °C for further use.

Knockdown with small interfering RNAs (siRNAs)

Specific siRNAs of USP7, MafB, and c-Maf were obtained from Ribobio (Guangzhou, Guangdong, China), and they were transfected into MM cells by using Lipofectamine® 2000 (Invitrogen) as the carrier (19). Forty eight or 72 h later, cells were prepared for IB assays to evaluate the knockdown efficacy.

Flow cytometry

Maf genes were knocked down from MM cells by using their specific siRNA for 72 h, or MM cells were treated with P5091 for 24 h before being collected for annexin V and PI (MultiSciences Biotech Co., Ltd., Hangzhou, Zhejiang, China) staining and flow cytometric analysis on a BD flow cytometer, as described previously (14).

Data mining

The expression level of USP7 was evaluated from the gene expression profile database (GSE2658) that is composed of healthy subjects (normal plasma cells, $n = 22$) and patients with stringently-defined MGUS/smoldering MM ($n = 24$) and symptomatic MM ($n = 351$) (22). The Kaplan-Meier plots the prognostic relevance of USP7 expression on the overall and event-free survival for MM patients ($n = 351$) (22).

Statistics

Statistical differences between the control and the experimental groups were analyzed by Student's *t* test. The expression of USP7 in MM patients was analyzed using analysis of variance. Overall and event-free survival was analyzed using the Kaplan-Meier method and compared by log-rank test as described previously (41).

Declarations

Ethics approval and consent to participate

This study was approved by the Review Board and Ethical Committee of Soochow University, and each patient provided written informed consent to donate bone marrow for this study after diagnostic and clinical procedures.

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