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Glucocorticoid modulatory element-binding protein 1 (GMEB1) interacts with the de-ubiquitinase USP40 to stabilize CFLAR$_L$ and inhibit apoptosis in human non-small cell lung cancer cells

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Abstract

Background: GMEB1 was originally identified via its interaction with GMEB2, which binds to the promoter region of the tyrosine aminotransferase (*TAT*) gene and modulates transactivation of the glucocorticoid receptor gene. In the cytosol, GMEB1 interacts with and inhibits CASP8, but the molecular mechanism is currently unknown.

Methods: Human non-small cell lung cancer cells and 293FT cells were used to investigate the function of GMEB1/USP40/CFLAR$_L$ complex by WB, GST Pull-Down Assay, Immunoprecipitation, Immunofluorescence and Flow cytometry analysis. A549 cells overexpressing green fluorescent protein and GMEB1 shRNA were used for tumor xenograft using female athymic nu/nu 4-week-old mice.

Results: We found GMEB1 interacted with CFLAR$_L$ (also known as c-FLIP$_L$) in the cytosol and promoted its stability. USP40 targeted CFLAR$_L$ for K48-linked de-ubiquitination. GMEB1 promoted the binding of USP40 to CFLAR$_L$. *USP40* knockdown did not increase CFLAR$_L$ protein level despite GMEB1 overexpression, suggesting GMEB1 promotes CFLAR$_L$ stability via USP40. Additionally, GMEB1 inhibited the activation of pro-caspase 8 and apoptosis in non-small cell lung cancer (NSCLC) cell via CFLAR$_L$ stabilization. Also, GMEB1 inhibited the formation of DISC upon TRAIL activation. CFLAR$_L$ enhanced the binding of GMEB1 and CASP8. Downregulation of GMEB1 inhibited A549 xenograft tumor growth in vivo.

Conclusions: Our findings show the de-ubiquitinase USP40 regulates the ubiquitination and degradation of CFLAR$_L$; and GMEB1 acts as a bridge protein for USP40 and CFLAR$_L$. Mechanistically, we found GMEB1 inhibits the activation of CASP8 by modulating ubiquitination and degradation of CFLAR$_L$. These findings suggest a novel strategy to induce apoptosis through CFLAR$_L$ targeting in human NSCLC cells.

Keywords: GMEB1, USP40, de-ubiquitination, CFLAR$_L$, NSCLC cells, Apoptosis

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Background

Glucocorticoid modulatory element-binding protein 1 (GMEB1) was originally identified as a nuclear protein with a molecular weight of 88 kDa [1–3]. GMEB1 interacts with GMEB2 and binds to the promoter sequence glucocorticoid modulatory element (GME) of the tyrosine aminotransferase (TAT) gene to modulate glucocorticoid receptor transactivation [4]. GMEB1 also exists in the cytosol and functions at the protein level. It interacts with the heat shock protein HSP27, but the function is not well studied. GMEB1 also binds to pro-caspases and inhibits their activation and cell apoptosis. However, it is still unknown how GMEB1 does this [5–8].

CFLAR$_L$, also known as c-FLIP$_L$, plays an important role in extrinsic ligand-induced apoptosis. In this pathway, FasL/tumor necrosis factor-alpha (TNF-α)/TRAIL binds to cell surface receptors, forms Death-Inducing Signaling Complex (DISC) and triggers the caspase-dependent apoptotic pathway. CFLAR$_L$ interacts with CASP8 via DED domains and inhibits the activation of CASP8, and thus apoptosis [9–12]. ITCH is an E3 ligase of CFLAR$_L$, which enhances apoptosis by targeting CFLAR$_L$ through ubiquitin-proteasome pathway [13, 14]. USP8 is a de-ubiquitination enzyme of CFLAR$_L$ that enhances stability and inhibits apoptosis induced by extrinsic ligands [15]. Therefore, approaches to promote the ubiquitination and degradation of CFLAR$_L$ are potential effective cancer therapies.

In the present study, we showed GMEB1 directly interacted with CFLAR$_L$ and increased its stability at the protein level. GMEB1 inhibited the activation of pro-caspase 8 via CFLAR$_L$. We found the de-ubiquitination enzyme USP40 bound to CFLAR$_L$ and GMEB, which enhanced the interaction between USP40 and CFLAR$_L$, resulting in reduced ubiquitination and degradation of CFLAR$_L$. These findings suggest GMEB1 inhibits the activation of CASP8 and apoptosis via CFLAR$_L$, which highlights potential implications for lung cancer therapy.

Methods

Reagents and antibodies

SAHA was purchased from Sigma (US). CHX, MG132 and E64D were purchased from Selleck (US). PARP (#9542), Caspase 8 (9746 L) antibodies were purchased from Cell Signaling Technology (US). Caspase 3 (NB100–56708) antibody was purchased from Imgenex (US). GMEB1 (sc-376,775), USP40 (sc-514,248) and FLIP$_L$ (sc-8346) antibodies were purchased from Santa Cruz (US). CFLAR$_L$ (ALX-804-961-0100) antibody was purchased from Enzo (US). HA (D110004) tag antibody was purchased from Sangon Biotech (China). FLAG (F7425) tag antibody was purchased from Sigma (US). His (D291–3) tag antibody was purchased from MBL (Japan). USP8 (27791–1-AP) and FADD (14906–1-AP) antibodies were purchased from proteintech (US).

Cell lines and cell culture

The human NSCLC cell lines A549, H1299, H1792. H157, H460, Calu-1 and HEK293FT cell lines were originally obtained from the American Type Culture Collection (ATCC). A549 and H1792 cell lines have been authenticated in Microread Gene Technology by STR analysis. The NSCLC cells were grown in monolayer culture in RPMI 1640 with 5% FBS (Gibco, US) at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air. HEK293FT cells were grown in DMEM with 5% FBS (Gibco, US) at 37 °C.

RNA interference and plasmid transfection

The GMEB1 siRNA targets the sequence: 5′-GCAC CAAAUUUGAUCUUCU-3′ and 5′-GCACACACAUU UGGCCUAA -3′; USP40 siRNA targets the sequence 5′-GCAGCAAAGUCGGCCAAAU-3′ and 5′-GGAU GCAGCUAACAUUGAA-3′. The siRNAs were synthesized by GenePharma and used as the manufacture's protocol.

GMEB1 and USP40 coding regions were amplified by PCR from A549 genomic DNA using following primers:

GMEB1 sense: CGGATCCGCCGCCACCATGGCTAA TGCAGAAGTGAG

GMEB1 antisense: CCTCGAGTTAATCCTCTAAGAC CACAATC

USP40 sense: CTAGCTAGCGCCGCCACCATGTCA CTTTTTTTTAAGGGTAG

USP40 antisense: CGCGGATCCTTATCTGAAGCTC CCCACG

HA tag was cloned to the N-terminal of GMEB1, His tag was cloned to the C-terminal of USP40. His-tagged, FLAG-tagged and GST-tagged CFLAR$_L$ were cloned previously by our team.

Western blot analysis

Cells were harvested and rinsed with pre-chilled PBS on ice. They were lysed in lysis buffer on ice for 30 min and then purified via centrifugation for 15 min at 4 °C. Samples of the whole-cell protein lysates (35 μg) were prepared for SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane by electro blotting. The proteins were probed with the appropriate primary and secondary antibodies. Antibody binding was detected by an HRP system according to the manufacturer's protocol [16].

Immunoprecipitation

Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM Na$_2$EDTA; 1 mM EGTA; 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate; 1 mM

Na_3VO_4; 0.5% Triton) on ice for 30 min then purified via centrifugation for 15 min at 4 °C. The supernatants were incubated with antibody at 4 °C for 1 h. Then the mixture was incubated with protein A beads (ThermoFisher) at 4 °C for 2 h. The beads were washed twice with 1 ml of lysis buffer. 20 µl 2 × SDS buffer were added for elution (100 °C, 10 min). Samples were centrifuged for western blot analysis.

GST pull-down assay
Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM Na_2EDTA; 1 mM EGTA; 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate; 1 mM Na_3VO_4; 0.5% Triton) on ice for 30 min, then purified via centrifugation for 15 min at 4 °C. The supernatants were incubated with rotation in 20 µl of Glutathione Sepharose beads (GE) at 4 °C for 2 h. Beads were washed twice with 1 ml of lysis buffer. 20 µl 2 × SDS buffer was added to beads for elution (100 °C, 10 min). Samples were centrifuged for western blot analysis.

Immunofluorescence
Cells were fixed with PHEMO buffer (0.025 M HEPES, 0.068 M PIPES, 0.003 M $MgCl_2 \cdot 6H_2O$, 0.015 M EGTA·Na_2, 10% DMSO, pH adjusted to 6.8. Additional reagents were added before use, with a final concentration as follows: 0.05% glutaraldehyde, 0.5% Triton X-100, 3.7% formaldehyde) for 10 min at room temperature before washing with PBS for 3 times. Then, cells were incubated with blocking buffer (3% BSA) for 30 min at room temperature. Afterward, cells were incubated with primary antibodies against $CFLAR_L$ (Santa Cruz, dilution at 1:500) for 1 h at room temperature. After washing with PBS for 3 times, cells were incubated with another primary antibodies GMEB1 (Santa Cruz, dilution at 1:500) or USP40 (Santa Cruz, dilution at 1:500) for 1 h. Alex Fluor 488 (Green) and Alex Fluor 568 (Red)-conjugated secondary antibodies were then applied and incubated at room temperature for 1 h. Cell nuclei were stained with DAPI. Images were captured using a confocal microscope (ZEISS, LSM700).

Flow cytometry analysis
Annexin V-FITC Apoptosis Detection Kit (Biobox Biotech, Nanjing, China) was used for cell apoptosis analysis according to the manufacture's protocol.

CASP8 activity detection
A549 cells were prepared for CASP8 activity according CASP8 Activity Apoptosis Assay Kit protocol (Sangon Biotech, Shanghai, China).

Tumor xenograft model
Fifteen female athymic nu/nu 4-week-old mice were purchased from Vital SPF Biotechnology (Beijing, China). For tumor xenograft establishments, A549 cells overexpressing green fluorescent protein or shGMEB1 RNA (1×10^6 cells/100 µL) were subcutaneously injected into the right side of the abdominal region of mice. Weight of mice and tumor size were detected every 2 days. The tumor volume was calculated as V = π × (length×width^2)/6.

Statistical analysis
GraghPad Prism version 5.00 was used for statistical analysis. All data are presented as the mean ± SD. Differences between groups were identified using Student's t-test. $P < 0.05$ was considered statistically significant.

Results
SAHA treatment reduced GMEB1 and $CFLAR_L$ protein levels in NSCLC cells
To examine the interaction between GMEB1 and $CFLAR_L$, we measured the protein levels in six NSCLC lines: A549, H1792, Calu-1, H1299, H157, and H460. Western blot assay showed GMEB1 protein levels were high in A549, H1792, Calu-1 and H1299 cells. $CFLAR_L$ protein levels were high in A549 and Calu-1 cells (Fig. 1a). $CFLAR_L$ protein levels positively correlated with GMEB1 (Additional file 1: Figure S1A). SAHA is an inhibitor of histone deacetylase and enhances TRAIL, which induces apoptosis and decreases CFLAR protein [17–19]. To detect whether SAHA also decreases GMEB1, we treated NSCLC cells with SAHA at different concentrations and time points. The results show SAHA decreased GMEB1 in a dose-dependent (Fig. 1b and Additional file 1: Figure S1D) and time-dependent manner (Fig. 1c and Additional file 1: Figure S1E). This is consistent with the effect of SAHA on $CFLAR_L$. These data indicate SAHA treatment affects GMEB1 and $CFLAR_L$ similarly in NSCLC cells.

GMEB1 enhanced the stability of $CFLAR_L$
We then characterized the biological function of the interaction between GMEB1 and $CFLAR_L$. GMEB1 was originally identified as a transcription factor, so we first determined if GMEB1 regulates $CFLAR_L$ at the transcriptional level. GMEB1 and $CFLAR_L$ relative mRNA levels were detected using q-PCR in A549 cells; GMEB1 siRNA was transfected for 24 h. Results show that *GMEB1* knock-down did not affect the relative mRNA level of $CFLAR_L$ (Fig. 2a). NSCLC cells with *GMEB1* knock-down were treated with CHX [10 µg/ml] for various time points. WB data show *GMEB1* knockdown decreased the stability of $CFLAR_L$ (Fig. 2b), while overexpression of GMEB1 increased the stability of $CFLAR_L$ (Fig. 2c). This confirms GMEB1 enhances the
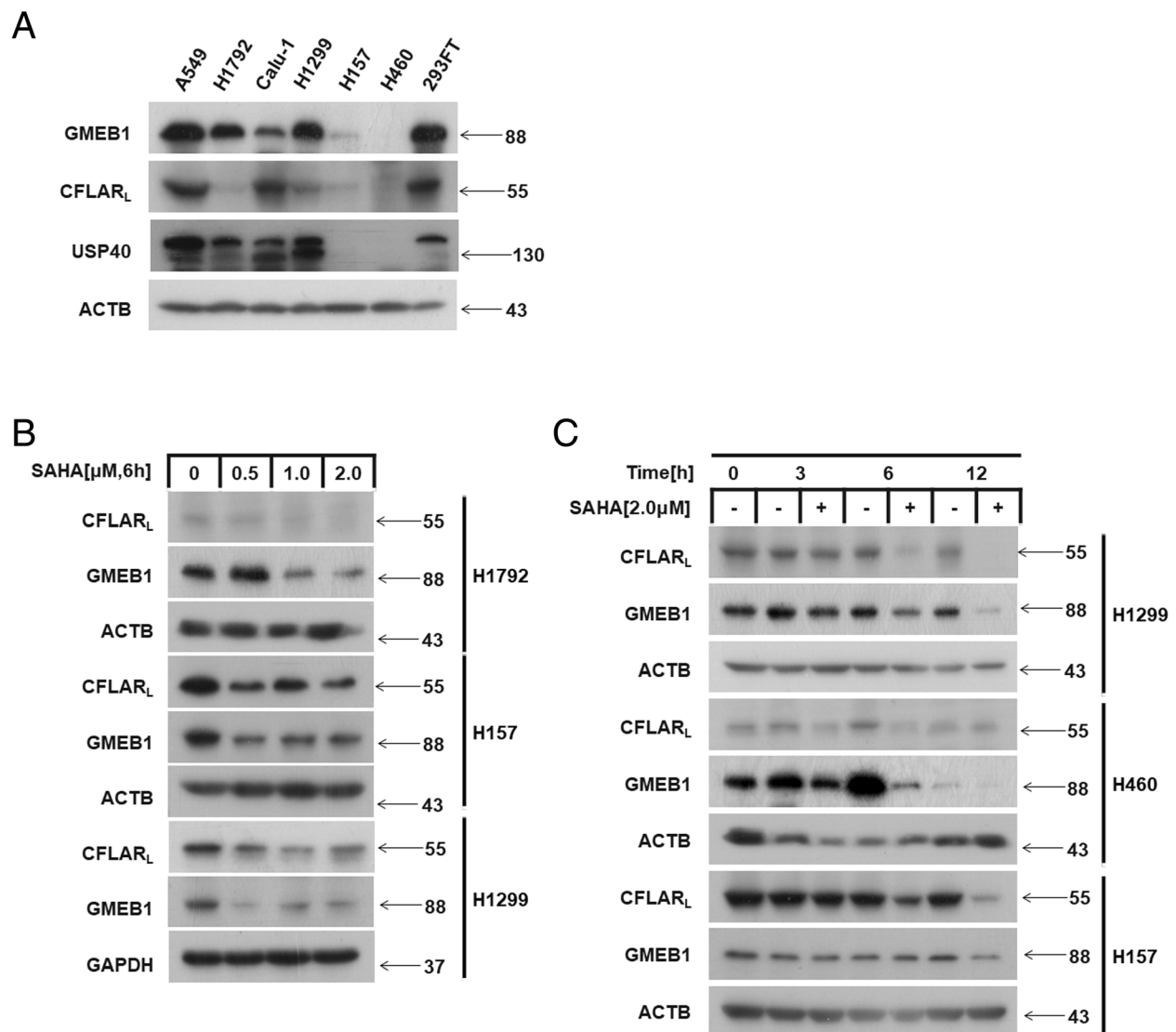
Fig. 1 SAHA treatment reduced GMEB1 and CFLAR$_L$ protein levels in NSCLC cells. **a** The protein levels of GMEB1, CFLAR$_L$ and USP40 were measured in 6 non-small cell lung cell lines and HEK293FT cell line by western blot. GMEB1 and CFLAR$_L$ protein levels decreased after SAHA treatment in dose-dependent (**b**) and time-dependent manner (**c**) in different NSCLCs

stability of CFLAR$_L$ at post-translational level. Next, we knocked down *GMEB1* by siRNA in A549, H1299 and Calu-1 cell lines and treated cells with SAHA [2.0 μM] for 6 h. Results show *GMEB1* knockdown decreased CFLAR$_L$ protein level (Fig. 2d). Overexpression of GMEB1 upregulated CFLAR$_L$ protein (Fig. 2e). To confirm the effect of GMEB1 on CFLAR$_L$, we knocked down *GMEB1* using GMEB1 shRNA in A549 cell lines and overexpressed GMEB1 using plasmid. We found that GMEB1 overexpression rescued the reduced CFLAR$_L$ protein level caused by GMEB1 knockdown (Fig. 2f). These data indicate GMEB1 plays a role in maintaining the protein level of CFLAR$_L$.

Most protein degradation occurs in either the ubiquitination-proteasome or ubiquitination-lysosome pathway. As such, de-ubiquitinases inhibit the effective degradation of proteins. Therefore, we focused how GMEB1 affects the ubiquitination of CFLAR$_L$. First, we knocked down *GMEB1* in H1299 cells and treated them with DMSO, MG132 [20 μM] and E64D [15 μM] for 6 h. MG132 inhibits the degradation of proteins by blocking proteasomes, and E64D inhibits the degradation of proteins via lysosomes. Western blot analysis shows MG132 treatment rescued the reduced CFLAR$_L$ protein level caused by *GMEB1* knockdown. This indicates CFLAR$_L$ is degraded through the proteasome pathway when GMEB1 protein levels are decreased (Fig. 2g). In addition, we designed a co-IP assay to determine whether GMEB1 affects the ubiquitination
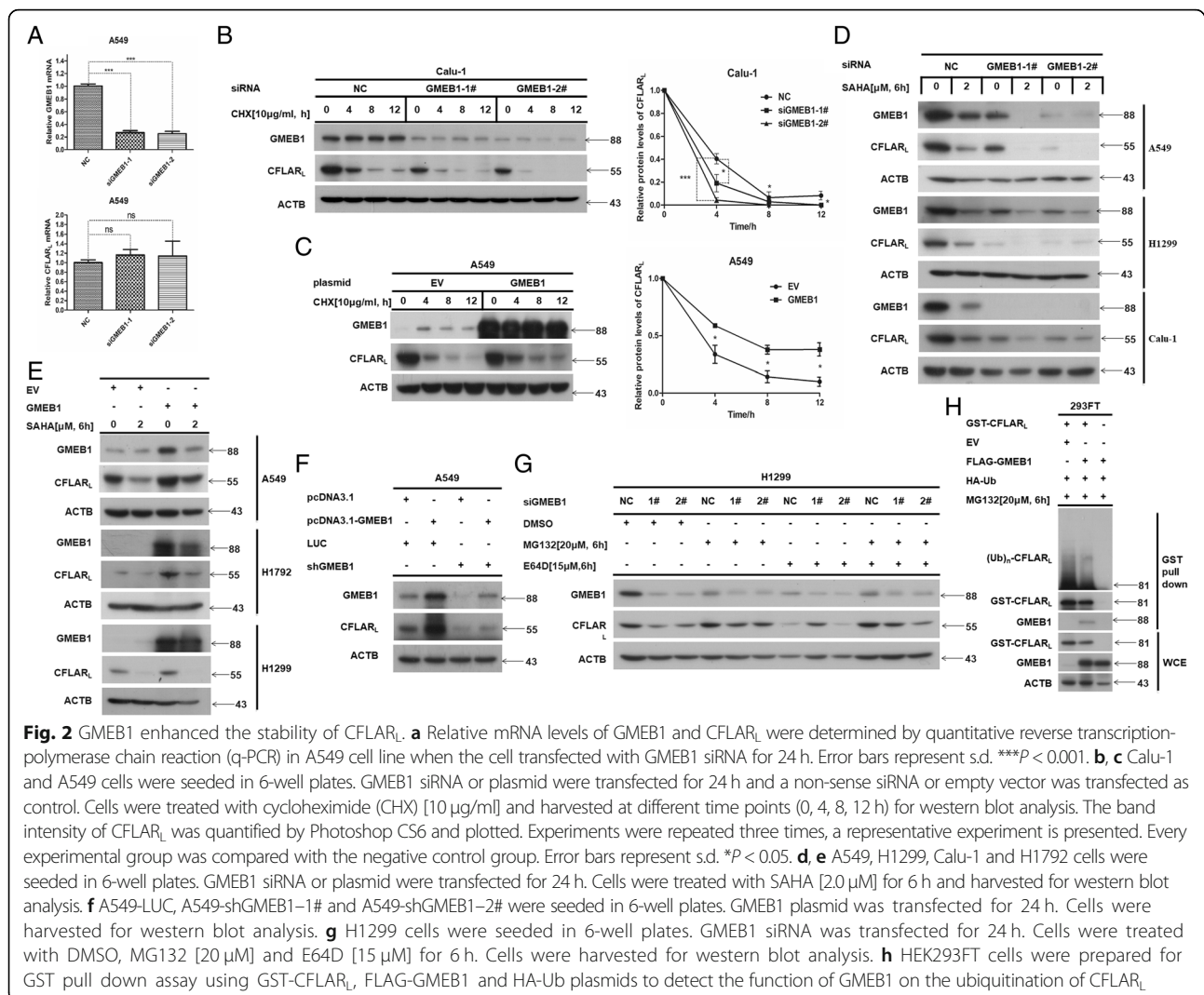
Fig. 2 GMEB1 enhanced the stability of CFLAR$_L$. **a** Relative mRNA levels of GMEB1 and CFLAR$_L$ were determined by quantitative reverse transcription-polymerase chain reaction (q-PCR) in A549 cell line when the cell transfected with GMEB1 siRNA for 24 h. Error bars represent s.d. ***$P < 0.001$. **b, c** Calu-1 and A549 cells were seeded in 6-well plates. GMEB1 siRNA or plasmid were transfected for 24 h and a non-sense siRNA or empty vector was transfected as control. Cells were treated with cycloheximide (CHX) [10 μg/ml] and harvested at different time points (0, 4, 8, 12 h) for western blot analysis. The band intensity of CFLAR$_L$ was quantified by Photoshop CS6 and plotted. Experiments were repeated three times, a representative experiment is presented. Every experimental group was compared with the negative control group. Error bars represent s.d. *$P < 0.05$. **d, e** A549, H1299, Calu-1 and H1792 cells were seeded in 6-well plates. GMEB1 siRNA or plasmid were transfected for 24 h. Cells were treated with SAHA [2.0 μM] for 6 h and harvested for western blot analysis. **f** A549-LUC, A549-shGMEB1–1# and A549-shGMEB1–2# were seeded in 6-well plates. GMEB1 plasmid was transfected for 24 h. Cells were harvested for western blot analysis. **g** H1299 cells were seeded in 6-well plates. GMEB1 siRNA was transfected for 24 h. Cells were treated with DMSO, MG132 [20 μM] and E64D [15 μM] for 6 h. Cells were harvested for western blot analysis. **h** HEK293FT cells were prepared for GST pull down assay using GST-CFLAR$_L$, FLAG-GMEB1 and HA-Ub plasmids to detect the function of GMEB1 on the ubiquitination of CFLAR$_L$

of CFLAR$_L$. Data show overexpression of GMEB1 decreased the ubiquitination of CFLAR$_L$ (Fig. 2h). Thus, we propose GMEB1 enhances the stability of CFALR$_L$ by modulating its ubiquitination level.

GMEB1 physically interacted with CFLAR$_L$ in NSCLC cells

GMEB1 interacts with CASP8 and inhibits its activation. *CFLAR$_L$* gene has high homology with *CASP8* gene, and the proteins display similar structures that may confer interaction with each other through DED domains. Thus, we determined if GMEB1 and CFLAR$_L$ bind one another via a co-immunoprecipitation (co-IP) assay in HEK293FT cells. The data show that HA-tagged GMEB1 interacted with FLAG-tagged CFLAR$_L$ (Fig. 3a and b). After GST-tagged CFLAR$_L$ was pulled down with Glutathione Sepharose beads, GMEB1 was detected using WB assay, indicating GMEB1 physically interacted with CFLAR$_L$ (Fig. 3c). An additional IP assay using A549 and H1299 cells (Fig. 3d) shows that endogenous CFLAR$_L$ interacted

with endogenous GMEB1. To further evaluate the interaction between GMEB1 and CFLAR$_L$, immunofluorescence staining experiments were conducted in Calu-1 cells. Results show GMEB1 localized in the cytosol. GMEB1 and CFLAR$_L$ were co-localized in the cytosol (Fig. 3e). We determined which domains of CFLAR$_L$ are required for this binding. Our data indicated that DED domains of CFLAR$_L$ were not necessary for interaction with GMEB1. However, P20 and P12 fragments of CFLAR$_L$ interacted with GMEB1 (Additional file 1: Figure S2A, B and C). Additional results show the N-terminal of GMEB1 was essential for interaction with CFLAR$_L$ (Additional file 1: Figure S2D and E). And, the fragment 325–573 of GMEB1, which doesn't interact with CFLAR$_L$, didn't increase the protein level of CFLAR$_L$ in A549 cell lines.

USP40 interacted with GMEB1 and CFLAR$_L$

We next aimed to identify the de-ubiquitination enzyme that targets CFLAR$_L$. We examined protein level of
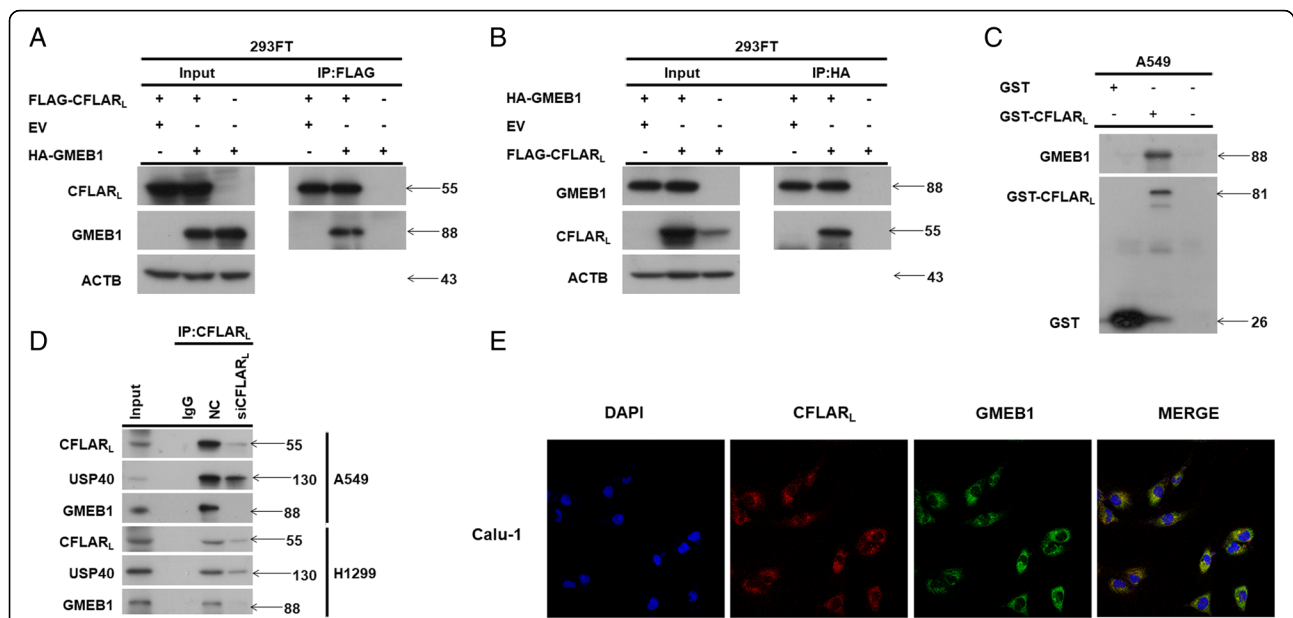
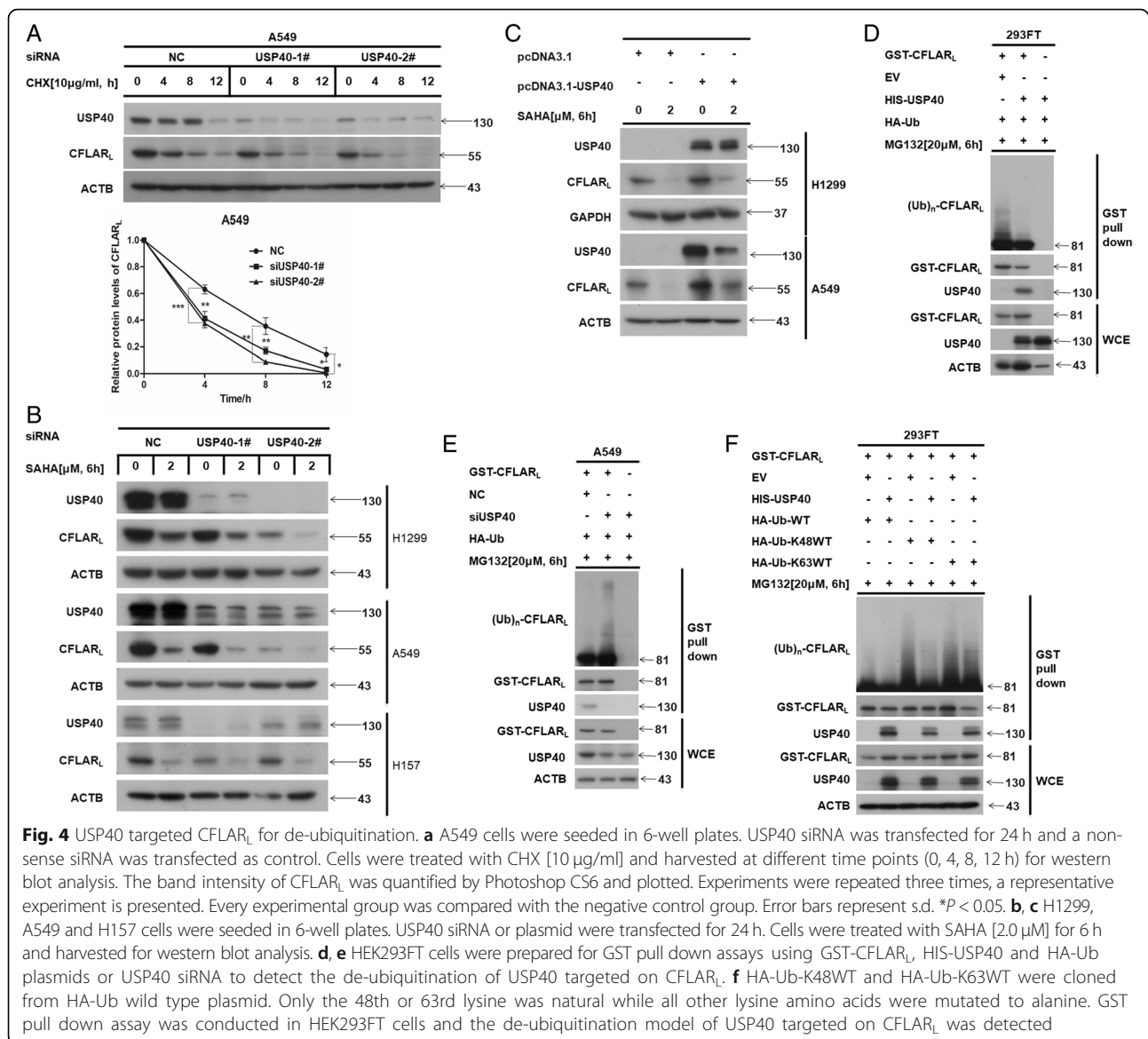**Fig. 3** GMEB1 physically interacted with CFLAR_L in NSCLC cells. **a**, **b** Co-IP assays were conducted in HEK293FT cells using FLAG-CFLAR_L and HA-GMEB1 plasmids. **c** GST-pull down assay was conducted in A549 cells using GST-CFLAR_L plasmids. **d** IP assays was conducted in A549 and H1299 cells using anti-FLIP_L (Santa Cruz, US) antibody. **e** Calu-1 cells were fixed and subjected to indirect immunofluorescence staining with anti-FLIP_L (Santa Cruz, US) and GMEB1 (Santa Cruz, US). The red signal (CFLAR_L) was obtained with anti-rabbit IgG Alexa 568-conjugated secondary Ab, and the green signals (GMEB1) were obtained with anti-mouse IgG Alexa 488-conjugated secondary Ab. Nuclei were stained with DAPI

several de-ubiquitinases (USP4, USP40, USP7, USP8 and USP22) in six NSCLC cell lines and found USP40 was positively correlated with CFLAR_L (Fig. 1a and Additional file 1: Figure S1B) and GMEB1 (Fig. 1a and Additional file 1: Figure S1C). We proposed that USP40 modulated the ubiquitination of CFLAR_L via its de-ubiquitinase activity. We then examined the interaction between GMEB1 and USP40 using Co-IP assay and found that tagged GMEB1 directly interacted with tagged USP40, which was confirmed by a reverse experiment (Additional file 1: Figure S3A and B). Given that de-ubiquitination enzymes interact with substrates, we then detected if USP40 directly interacted with CFLAR_L using Co-IP assay in 293FT cells and found that tagged USP40 did in fact interact with tagged CFLAR_L. This was also confirmed by a reverse experiment (Additional file 1: Figure S3C and D). IP assay using anti-FLIP_L showed that endogenous CFLAR_L interacted with endogenous USP40 in A549 and H1299 cells (Fig. 2d). Immunofluorescence staining results indicate that USP40 also co-localized with CFLAR_L in cytosol (Additional file 1: Figure S3E). We next aimed to identify the domains of CFLAR_L that were required for binding with USP40. Our data show that DED domains of CFLAR_L did not interact with USP40. P20 and P12 fragments of CFLAR_L interacted with USP40 (Additional file 1: Figures S2A, S3F and S3G).

USP40 targets CFLAR_L for de-ubiquitination

Previous work showed ITCH targets CFLAR_L as an E3 ligase, and USP8 is one of the de-ubiquitination enzymes

of CFLAR_L. Here, we found USP40 was positively correlated with and interacted with CFLAR_L in NSCLC cells. Therefore, USP40 may target CFLAR_L for de-ubiquitination. To confirm, we first evaluated the function of USP40 on CFLAR_L in NSCLC cell lines treated with CHX. Results show that knocking down *USP40* decreased the stability of CFLAR_L (Fig. 4a). Furthermore, we knocked down *USP40* in H1299, A549 and H157 cell lines using siRNA and treated cells with SAHA [2.0 μM] for 6 h. Data show protein level of CFLAR_L decreased after knockdown of *USP40* (Fig. 4b). Conversely, overexpression of USP40 in H1299 and A549 cell lines increased the protein level of CFLAR_L (Fig. 4c). These findings demonstrate USP40 promotes the stability of CFLAR_L at the protein level. We used GST pull-down assays to determine the role of USP40 during CFALR_L ubiquitination. Results show that overexpression of USP40 decreased the ubiquitination of CFLAR_L (Fig. 4d), and knockdown *USP40* increased the ubiquitination of CFLAR_L (Fig. 4e). Additional results show that USP40 targeted CFLAR_L for K48-linked de-ubiquitination (Fig. 4f). To confirm our proposal, we cloned mutant USP40 (C62A) (Additional file 1: Figure S4A) without enzyme activity [20]. Data show mutant USP40 (C62A) did not increase the protein level of CFLAR_L in A549 cell lines (Additional file 1: Figure S4B), but it still interacted with CFLAR_L (Additional file 1: Figure S4C). GST pull-down assay results show mutant USP40 (C62A) did not de-ubiquitinate CFLAR_L (Additional file 1: Figure S4D).

Fig. 4 USP40 targeted CFLAR$_L$ for de-ubiquitination. **a** A549 cells were seeded in 6-well plates. USP40 siRNA was transfected for 24 h and a non-sense siRNA was transfected as control. Cells were treated with CHX [10 μg/ml] and harvested at different time points (0, 4, 8, 12 h) for western blot analysis. The band intensity of CFLAR$_L$ was quantified by Photoshop CS6 and plotted. Experiments were repeated three times, a representative experiment is presented. Every experimental group was compared with the negative control group. Error bars represent s.d. *$P < 0.05$. **b**, **c** H1299, A549 and H157 cells were seeded in 6-well plates. USP40 siRNA or plasmid were transfected for 24 h. Cells were treated with SAHA [2.0 μM] for 6 h and harvested for western blot analysis. **d**, **e** HEK293FT cells were prepared for GST pull down assays using GST-CFLAR$_L$, HIS-USP40 and HA-Ub plasmids or USP40 siRNA to detect the de-ubiquitination of USP40 targeted on CFLAR$_L$. **f** HA-Ub-K48WT and HA-Ub-K63WT were cloned from HA-Ub wild type plasmid. Only the 48th or 63rd lysine was natural while all other lysine amino acids were mutated to alanine. GST pull down assay was conducted in HEK293FT cells and the de-ubiquitination model of USP40 targeted on CFLAR$_L$ was detected

Taken together, these data suggest that USP40 is a de-ubiquitinase of CFLAR$_L$.

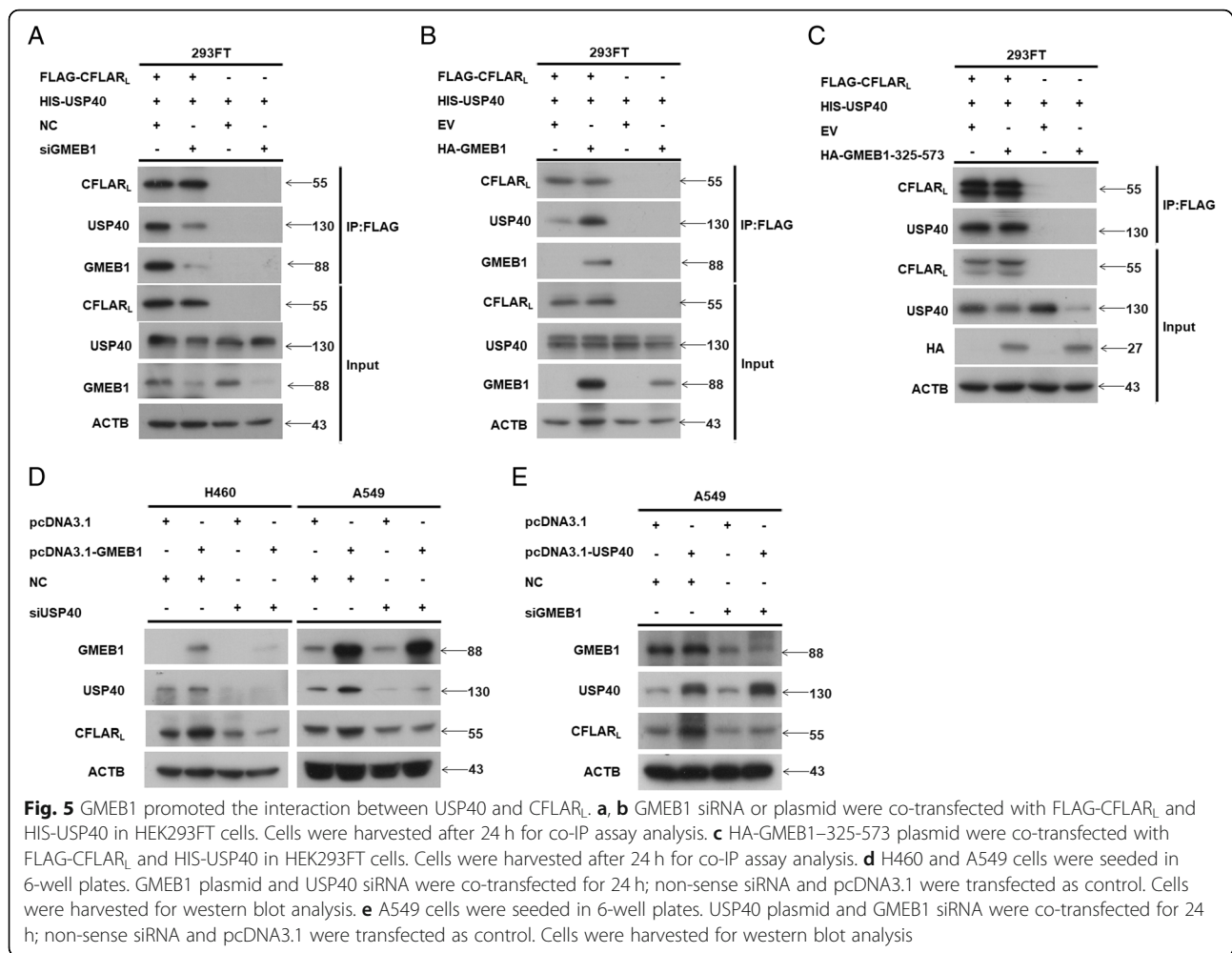### GMEB1 promoted the interaction between USP40 and CFLAR$_L$

Our findings show that GMEB1, USP40 and CFLAR$_L$ interact to form a complex. We looked into the function of GMEB1 in this complex. Data from co-IP assay in 293FT cells show that knock down of *GMEB1* weakened the interaction between CFLAR$_L$ and USP40; overexpression of GMEB1 enhanced the interaction (Fig. 5a and b, respectively). In addition, we found that overexpression of HA-GMEB1 (325–573), which did not interact with CFLAR$_L$, had no significant impact on the interaction between CFLAR$_L$ and USP40 (Fig. 5c). Our data indicate that GMEB1 acts as an adaptor protein in

the complex, and GMEB1 is essential for the interaction between CFLAR$_L$ and GMEB1.

Further, we questioned whether GMEB1 affects CFLAR$_L$ via USP40. We knocked down *USP40* and overexpressed GMEB1 in H460 and A549 cell lines and found no impact on CFLAR$_L$ (Fig. 5d). Using a reverse experiment in A549 cell lines, we showed that USP40 overexpression slightly increased CFLAR$_L$ protein when *GMEB1* was knocked down (Fig. 5e). These data indicate GMEB1 confers stability to CFLAR$_L$ via USP40.

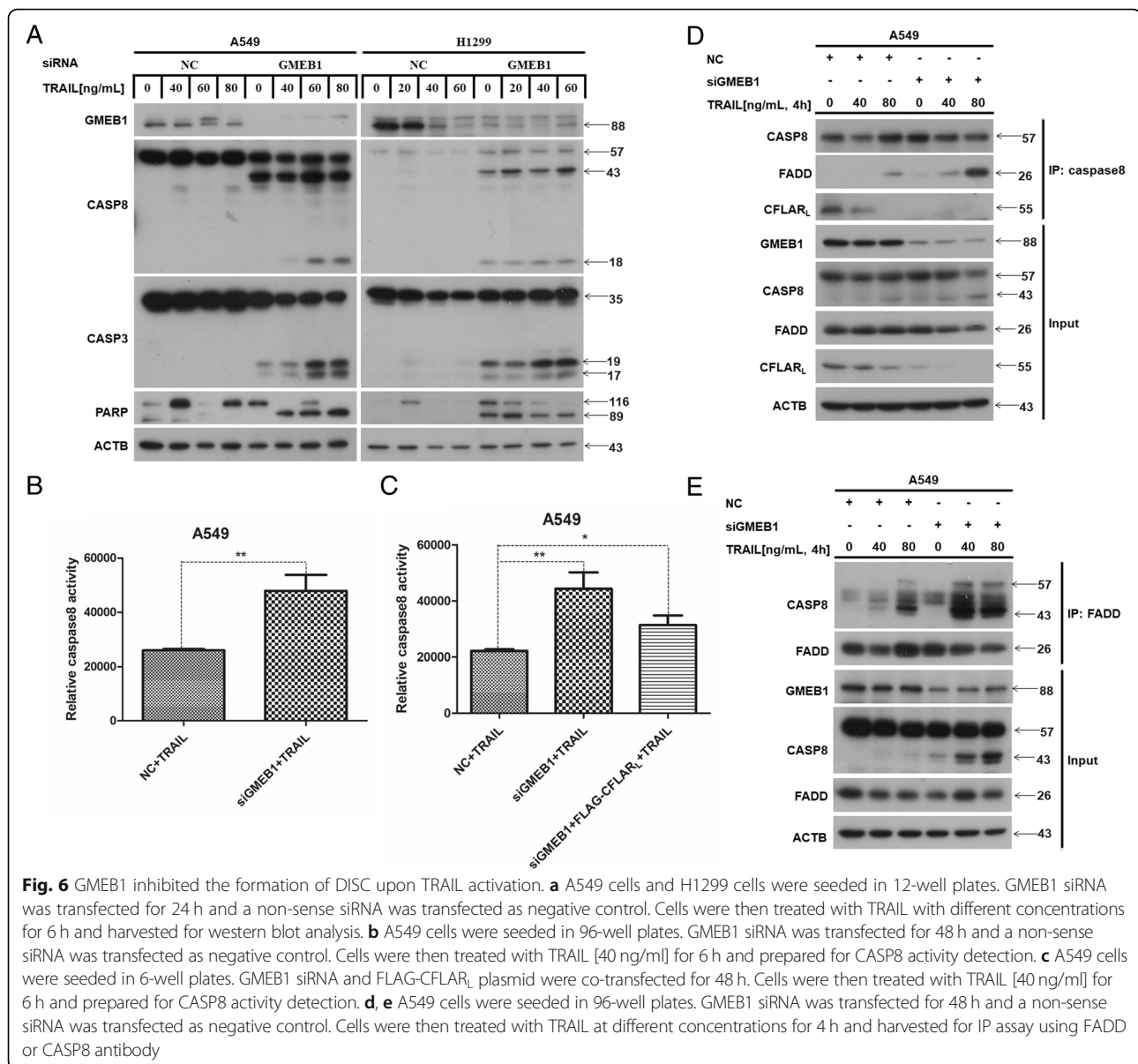### GMEB1 inhibited apoptosis via CFLAR$_L$ and DISC formation upon TRAIL exposure

GMEB1 inhibits the activation of pro-caspases, but the molecular mechanism is still unclear. Our study demonstrates that GMEB1 interacts with CFLAR$_L$ and promotes

Fig. 5 GMEB1 promoted the interaction between USP40 and CFLAR$_L$. **a**, **b** GMEB1 siRNA or plasmid were co-transfected with FLAG-CFLAR$_L$ and HIS-USP40 in HEK293FT cells. Cells were harvested after 24 h for co-IP assay analysis. **c** HA-GMEB1–325-573 plasmid were co-transfected with FLAG-CFLAR$_L$ and HIS-USP40 in HEK293FT cells. Cells were harvested after 24 h for co-IP assay analysis. **d** H460 and A549 cells were seeded in 6-well plates. GMEB1 plasmid and USP40 siRNA were co-transfected for 24 h; non-sense siRNA and pcDNA3.1 were transfected as control. Cells were harvested for western blot analysis. **e** A549 cells were seeded in 6-well plates. USP40 plasmid and GMEB1 siRNA were co-transfected for 24 h; non-sense siRNA and pcDNA3.1 were transfected as control. Cells were harvested for western blot analysis

CFLAR$_L$ stability via the de-ubiquitination enzyme USP40. We asked if GMEB1 inhibits apoptosis via CFLAR$_L$. GMEB1 siRNA was transfected in A549 and H1299 cell lines for 48 h, and then treated with TRAIL for 6 or 24 h. Western blot results show that *GMEB1* knockdown increased the level of cleaved CASP8, CASP3 and PARP (Fig. 6a). Flow Cytometry analysis shows that *GMEB1* knockdown enhanced apoptosis of A549 induced by TRAIL (Additional file 1: Figure S5B and C). Further, we transfected plasmid FLAG-CFLAR$_L$ while knocking down *GMEB1* in A549 cells. Western blot results show that overexpression of CFLAR$_L$ decreased protein levels of cleaved CASP8, CASP3 and PARP that were induced by *GMEB1* knockdown and TRAIL treatment (Additional file 1: Figure S5A). Flow Cytometry analysis show that CFLAR$_L$ overexpression partially attenuated apoptosis induced by *GMEB1* knockdown, indicating GMEB1 inhibited apoptosis through regulating CFLAR$_L$ (Additional file 1: Figure S5D and E). *GMEB1* knockdown increased the activation of CASP8 upon TRAIL treatment in A549 cells (Fig. 6b). And another similar experiment showed that

CFLAR$_L$ overexpression partially attenuated the activation of CASP8 upon knocking down *GMEB1* and TRAIL treatment (Fig. 6c). We then evaluated the formation of DISC induced by TRAIL treatment. Data show *GMEB1* knockdown promoted the interaction of FADD and CASP8 (Fig. 6d and e).

CFLAR$_L$ is critical for the interaction of GMEB1 and CASP8
GMEB1 interacts with pro-caspase 8 and inhibits its activation, but the mechanism is not clear. Our experiments indicated that GMEB1 also interacted with CFLAR$_L$, which interacted with pro-caspase 8 and inhibited its activity. We questioned which protein plays the dominant role in the interaction with GMEB1, CASP8 or CFLAR$_L$. Co-IP assays were conducted to evaluate the interaction between GMEB1 and CFLAR$_L$ while *CASP8* was knocked down using siRNA. Results show no significant change (Fig. 7a). We conducted another co-IP assay to evaluate the interaction between GMEB1 and *CASP8* (using a plasmid HIS-CASP8M that 374 and 384 Aspartic acids mutated to Alanine acids) while

Fig. 6 GMEB1 inhibited the formation of DISC upon TRAIL activation. **a** A549 cells and H1299 cells were seeded in 12-well plates. GMEB1 siRNA was transfected for 24 h and a non-sense siRNA was transfected as negative control. Cells were then treated with TRAIL with different concentrations for 6 h and harvested for western blot analysis. **b** A549 cells were seeded in 96-well plates. GMEB1 siRNA was transfected for 48 h and a non-sense siRNA was transfected as negative control. Cells were then treated with TRAIL [40 ng/ml] for 6 h and prepared for CASP8 activity detection. **c** A549 cells were seeded in 6-well plates. GMEB1 siRNA and FLAG-CFLAR$_L$ plasmid were co-transfected for 48 h. Cells were then treated with TRAIL [40 ng/ml] for 6 h and prepared for CASP8 activity detection. **d, e** A549 cells were seeded in 96-well plates. GMEB1 siRNA was transfected for 48 h and a non-sense siRNA was transfected as negative control. Cells were then treated with TRAIL at different concentrations for 4 h and harvested for IP assay using FADD or CASP8 antibody

CFLAR$_L$ was knocked down using siRNA. Results show CFLAR$_L$ knockdown decreased the interaction between GMEB1 and CASP8 (Fig. 7b). These results confirm CFLAR$_L$ directly interacts with GMEB1, and CFLAR$_L$ plays a critical role in the interaction of CASP8 and GMEB1.
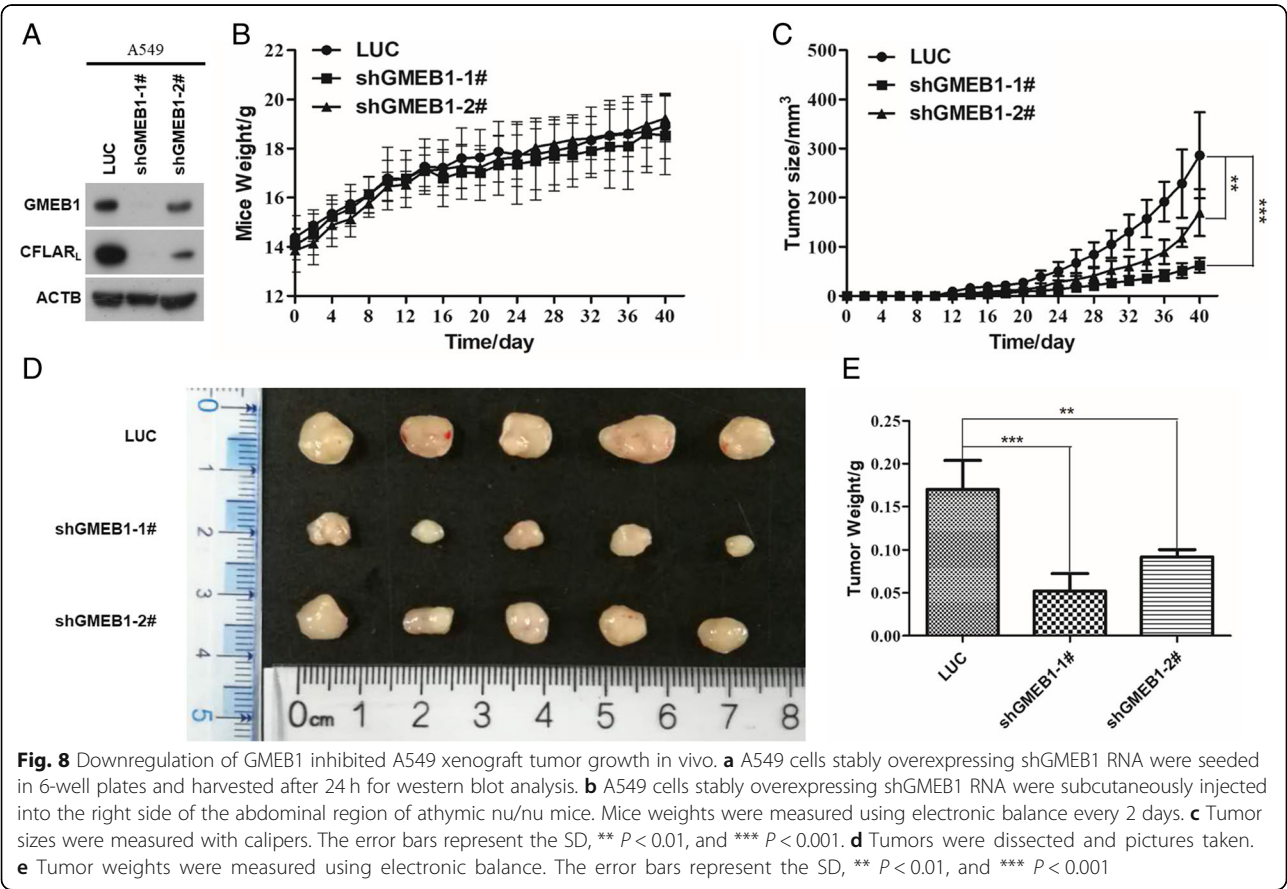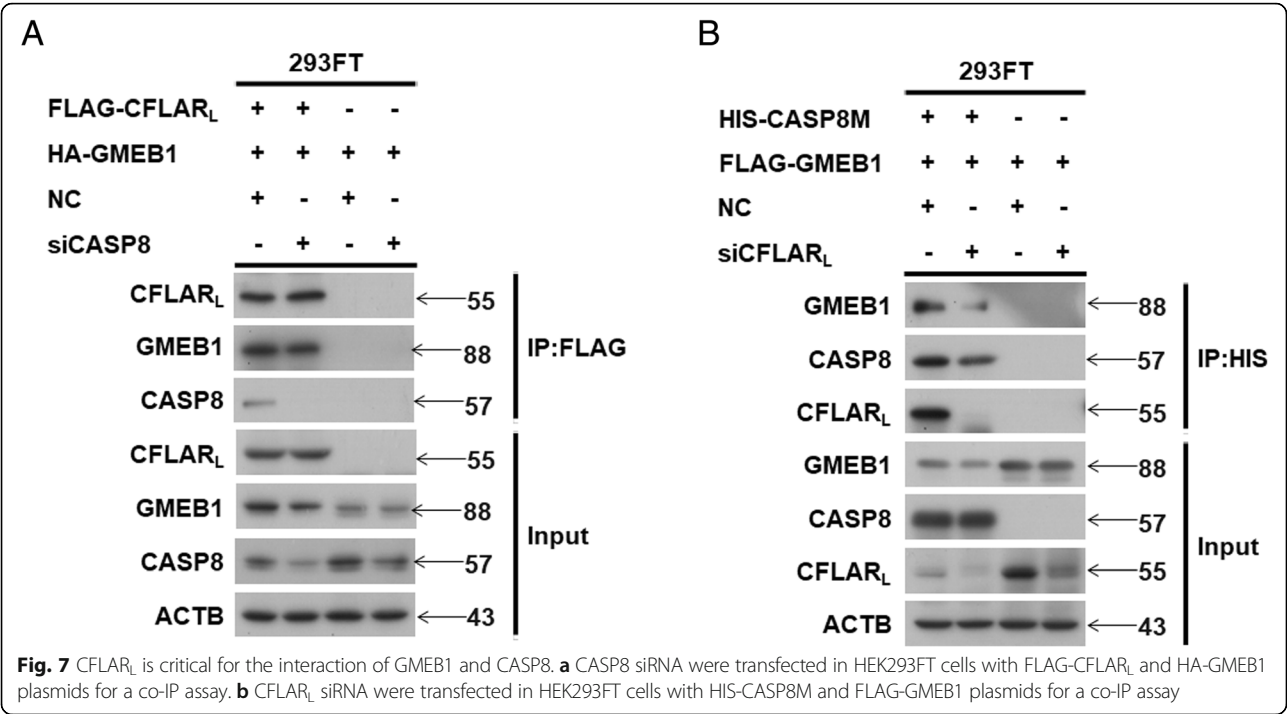
Downregulation of GMEB1 inhibited A549 xenograft tumor growth in vivo

To evaluate whether the tumor growth of A549 is regulated by GMEB1 in vivo, A549-LUC, A549-shGMEB1–1# and A549-shGMEB1–2# were subcutaneously injected into the right side of the abdominal region of athymic nu/nu mice. GMEB1 and CFLAR$_L$ protein levels were detected in A549 cell lines (Fig. 8a). Mice weights

were measured using an electronic balance, results show no significant difference among the three groups (Fig. 8b). Tumor sizes were measured with calipers (Fig. 8c). Results show tumor growth was inhibited after GMEB1 knockdown compared with the control group (Fig. 8d). And, tumor weights also support this finding (Fig. 8e). These results suggest GMEB1 plays a key role in cellular mechanisms related to apoptosis and cancer progress.

Discussion

GMEB1 is found throughout many cell types with multiple functions that are now being uncovered. GMEB1 and GMEB2 bind to the promoter sequence of the *TAT* gene and modulate GR transactivation. In the cytosol, GMEB1 interacts with HSP27, a protein chaperone with

Fig. 7 CFLAR$_L$ is critical for the interaction of GMEB1 and CASP8. **a** CASP8 siRNA were transfected in HEK293FT cells with FLAG-CFLAR$_L$ and HA-GMEB1 plasmids for a co-IP assay. **b** CFLAR$_L$ siRNA were transfected in HEK293FT cells with HIS-CASP8M and FLAG-GMEB1 plasmids for a co-IP assay

Fig. 8 Downregulation of GMEB1 inhibited A549 xenograft tumor growth in vivo. **a** A549 cells stably overexpressing shGMEB1 RNA were seeded in 6-well plates and harvested after 24 h for western blot analysis. **b** A549 cells stably overexpressing shGMEB1 RNA were subcutaneously injected into the right side of the abdominal region of athymic nu/nu mice. Mice weights were measured using electronic balance every 2 days. **c** Tumor sizes were measured with calipers. The error bars represent the SD, ** $P < 0.01$, and *** $P < 0.001$. **d** Tumors were dissected and pictures taken. **e** Tumor weights were measured using electronic balance. The error bars represent the SD, ** $P < 0.01$, and *** $P < 0.001$

many critical functions in cancer invasion, metastasis, proliferation and apoptosis [21–29]. HSP27 is related to CFLAR$_L$ and the activation of caspases [30, 31]. However, the relationship between GMEB1 and HSP27 has not been well studied. Recently, GMEB1 was reported to inhibit cell apoptosis by binding to pro-caspases and inhibiting their activation in cytosol [6]. GMEB1 interacts with pro-caspases via DED domains, but the molecular mechanism is unknown.

CFLAR$_L$ is an apoptotic inhibitor protein that interacts with pro-caspase 8 and inhibits its activation; DED domains play important roles in this interaction. The interaction between CFLAR$_L$ and pro-caspase 8 inhibits the formation of DISC [15]. As an inhibitor of histone deacetylase, SAHA is effective in treating skin T cell lymphoma clinically. It enhances the acetylation of Ku70 and disrupts the CFLAR/Ku70 complex and then triggers CFLAR poly-ubiquitination and degradation by the proteasome [32, 33]. SAHA treatment in NSCLC cells shows that SAHA decreased CFLAR$_L$ and GMEB1 protein levels in a dose-dependent and a time-dependent manner. The results indicate GMEB1 and CFLAR$_L$ are positively correlated, which was confirmed by analyzing protein levels in six NSCLC cell lines and a HEK293FT cell line. In addition, GMEB1 protein reduction by SAHA suggests a relationship similar to SAHA and CFLAR$_L$.

Given that GMEB1 acts in the nucleus as a transcription factor, we first conducted q-PCR experiments to check the function of GMEB1. We found GMEB1 affected the transcription of CFLAR$_L$. We then turned our attention to the cytosol. Knock-down and overexpression experiments showed that GMEB1 promoted the stability and directly regulated the protein level of CFLAR$_L$. Our in vitro studies indicated that GMEB1 interacts with CFLAR$_L$ outside the nucleus. DED domains of CFLAR$_L$ were not necessary for this process; P20 and P12 domains of CFLAR$_L$ accounted for the interaction with GMEB1. Our results also show that N-terminal of GMEB1 interacted with CFLAR$_L$, and GMEB1 (325–573) fragments, which did not interact with CFLAR$_L$, couldn't increase the protein level of CFLAR$_L$. These findings suggest that the function of GMEB1 is dependent on the interaction with CFLAR$_L$. Our experiments also indicate GMEB1 affects the degradation of CFLAR$_L$ through proteasome pathway. GMEB1 doesn't have ubiquitin-related enzymatic activity that directly modulates the ubiquitination of CFLAR$_L$. This suggests that another enzyme is needed in this process. Therefore, USP40 was emphasized because its expression was positively correlated with GMEB1 and CFLAR$_L$ in NSCLC cells.

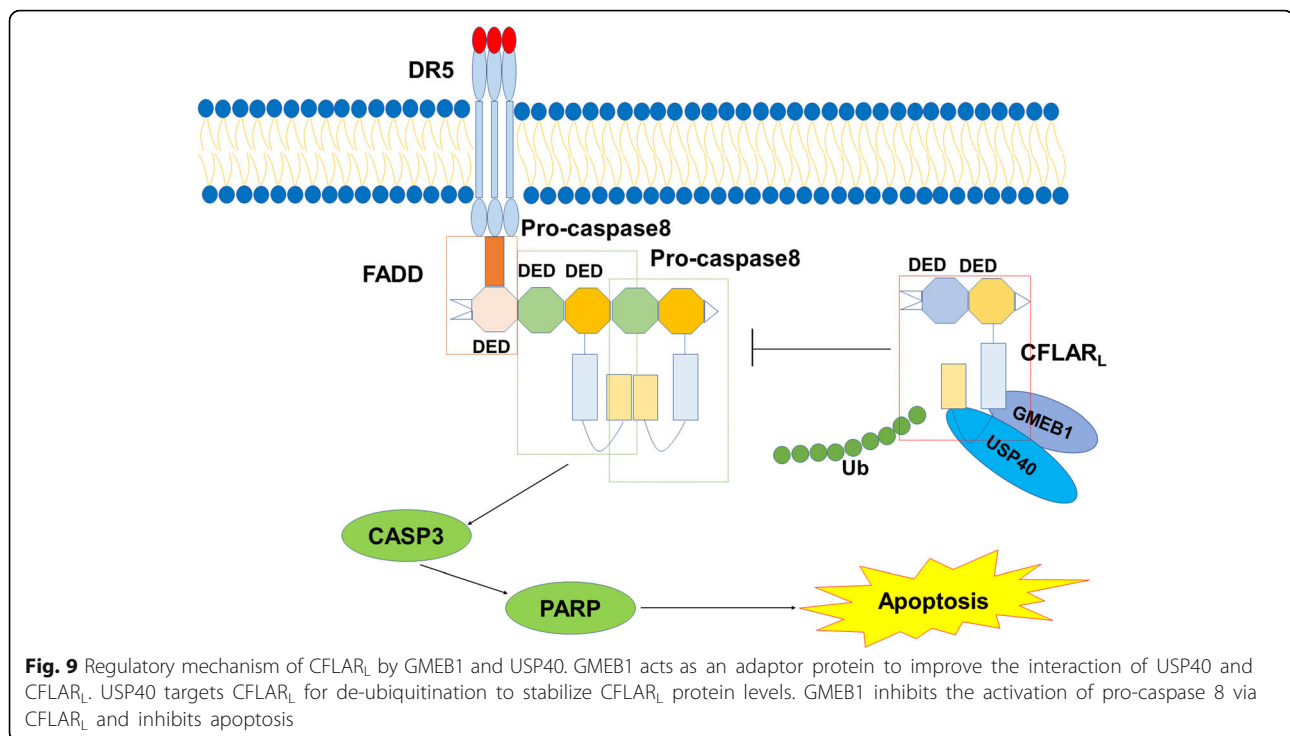CFLAR$_L$ is an effective target for cancer therapy [9–12]. ITCH, which has important roles in cell immune regulation [34], was identified as an E3 ligase targeting CFLAR$_L$. It induces apoptosis by degrading CFLAR$_L$ and activating pro-caspase 8. De-ubiquitination is an important protein modification that reverses the ubiquitination of proteins via E1/E2/E3 ligase. De-ubiquitination inhibits the degradation of proteins and drives the fate of substrate proteins [35]. USP8 regulates the morphology of the endosome by ubiquitinating proteins and is also involved in cargo sorting and membrane trafficking at the early endosome stage [36, 37]. USP8 is a de-ubiquitination enzyme of CFLAR$_L$ that promotes the stability of CFLAR$_L$ and inhibits the activation of pro-caspase 8. Changes in CFLAR$_L$ protein levels also affect the formation of DISC and apoptosis induced by extrinsic ligands.

To find the de-ubiquitinase that regulates CFLAR$_L$ by GMEB1, we focused on USP8 that was identified as a de-ubiquitinase of CFLAR$_L$. But our experiments showed that GMEB1 did not interact with USP8 (Additional file 1: Figure S4E). Western blot data from six NSCLC lines suggested USP40 protein levels are positively correlated with GMEB1 and CFLAR$_L$. This underscores the importance of USP40.

Several reports show that USP40 is correlated with late-onset Parkinson's disease and USP24 [38, 39]. In addition, USP40 affects glomerular permeability in zebrafish [40]. Our results suggest USP40 interacts with both GMEB1 and CFLAR$_L$. GMEB1 promoted the binding of USP40 with CFLAR$_L$, conferring a role as an adaptor protein. Consequently, USP40 augmented the stability of CFLAR$_L$ via its de-ubiquitinase activity. USP40 targeted CFLAR$_L$ for K48-linked de-ubiquintion. *USP40* knockdown did not increase the protein level of CFLAR$_L$ even though GMEB1 protein level was overexpressed. And, overexpression of USP40 increased the protein level of CFLAR$_L$ though *GMEB1* was knocked down. Our data indicate that USP40 is the key protein affecting GMEB1 on CFLAR$_L$.

In addition, we also found that *GMEB1* knockdown promoted the activation of pro-caspase 8 and apoptosis induced by TRAIL. CFLAR$_L$ attenuated apoptosis that was induced by GMEB1 knockdown, which highlights the function of GMEB1 in inhibiting apoptosis via CFLAR$_L$. GMEB1 inhibited the formation of DISC upon TRAIL activation.

Previous studies show GMEB1 interacts with the DED domain of CASP8 and inhibits its activation. Our results show GMEB1 did not interact with DED domain of CFLAR$_L$ which has a similar structure with CASP8. However, GMEB1 interacted with CFLAR$_L$ via the P20 and P12 domains. Co-IP results showed that *CASP8* knockdown didn't affect the interaction between GMEB1 and CFLAR$_L$, while *CFLAR$_L$* knockdown decreased the interaction between GMEB1 and CASP8. These findings suggest that CFLAR$_L$ is crucial for the interaction

Fig. 9 Regulatory mechanism of CFLAR$_L$ by GMEB1 and USP40. GMEB1 acts as an adaptor protein to improve the interaction of USP40 and CFLAR$_L$. USP40 targets CFLAR$_L$ for de-ubiquitination to stabilize CFLAR$_L$ protein levels. GMEB1 inhibits the activation of pro-caspase 8 via CFLAR$_L$ and inhibits apoptosis

between GMEB1 and CASP8. Thus, GMEB1 interacts with CFLAR$_L$, and CFLAR$_L$ interacts with CASP8 via DED domains. GMEB1 inhibits the activation of CASP8 via the function of CFLAR$_L$.

In vivo data showed *GMEB1* knockdown inhibited the A549 xenograft tumor growth, which also confirmed our results.

Conclusions

We described the interaction among GMEB1, USP40 and CFLAR$_L$ (Fig. 9). We found that GMEB1 promoted the stability of CFLAR$_L$ by de-ubiquitinase USP40. USP40 targeted CFLAR$_L$ for K48-linked de-ubiquitination. GMEB1 inhibited the activation of CASP8 and apoptosis in NSCLC via CFLAR$_L$. CFLAR$_L$ promoted the interaction between GMEB1 and CASP8. *GMEB1* knockdown inhibited tumor growth in vivo. These findings provide more in-depth knowledge that serves as potential therapies for cancer.

Additional file

Additional file 1: Figure S1. CFLAR$_L$ protein level positively correlated with GMEB1 and USP40 protein levels in NSCLC cell lines. **Figure S2.** CFLAR$_L$ directly interact with GMEB1. **Figure S3.** USP40 interacted with GMEB1 and CFLAR$_L$. **Figure S4.** USP40 targeted CFLAR$_L$ for de-ubiquitination. **Figure S5.** GMEB1 inhibited apoptosis via CFLAR$_L$. (DOCX 1283 kb)

Abbreviations

CFLAR: Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; DISC: Death-inducing signaling complex; HSP27: Heat Shock Protein Family B (Small) Member 1; ITCH: Itchy E3 Ubiquitin Protein Ligase; NSCLC: Non-small cell lung cancer; PARP: Poly ADP-ribose polymerase

Availability of data and materials
The datasets used/analyzed to support the conclusions of this article are available from the corresponding author upon reasonable request.

Authors' contributions
Xiangguo Liu, Ling Su and Yidan Lin conceived the study. Xiangguo Liu, Wentao An and Ling Su designed the experiments. Wentao An performed most of the experiments. Wentao An, Shun Yao and Xiaoyang Sun performed the animal experiments. Zhaoyuan Hou provided GMEB1 shRNA plasmids. Wentao An and Xiangguo Liu interpreted the data and wrote the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate
All the animal experiments were carried out in accordance with the approval of the Animal Research Committee of Shandong University.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing of interests.

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