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# HSP70 interacts with TRAF2 and differentially regulates TNFα signalling in human colon cancer cells

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## Abstract

Members of tumour necrosis factor (TNF) family usually trigger both survival and apoptotic signals in various cell types. Heat shock proteins (HSPs) are conserved proteins implicated in protection of cells from stress stimuli. However, the mechanisms of HSPs in TNFα-induced signalling pathway have not been fully elucidated. We report here that HSP70 over-expression in human colon cancer cells can inhibit TNFα-induced NFκB activation but promote TNFα-induced activation of c-Jun N-terminal kinase (JNK) through interaction with TNF receptor (TNFR)-associated factor 2 (TRAF2). We provide evidence that HSP70 over-expression can sequester TRAF2 in detergent-soluble fractions possibly through interacting with TRAF2, leading to reduced recruitment of receptor-interacting protein (RIP1) and IκBα kinase (IKK) signalosome to the TNFR1–TRADD complex and inhibited NFκB activation after TNFα stimuli. In addition, we found that HSP70–TRAF2 interaction can promote TNFα-induced JNK activation. Therefore, our study suggests that HSP70 may differentially regulate TNFα-induced activation of NFκB and JNK through interaction with TRAF2, contributing to the pro-apoptotic roles of HSP70 in TNFα-induced apoptosis of human colon cancer cells.

**Keywords:** TNFα • TRAF2 • NFκB • JNK • HSP70 • lipid raft • apoptosis • ubiquitination

## Introduction

Members of the tumour necrosis factor (TNF) family and their respective receptors play important roles in diverse cellular events, such as septic shock, induction of other cytokines, cell proliferation, differentiation and apoptosis [1, 2]. A sub-family of TNF ligands, including TNFα, TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL), are potent inducers of apoptosis [1, 2]. However, it has been recognized that TNF usually initiates apoptotic and antiapoptotic signals simultaneously [1, 2]. The molecular mechanisms involved in the control of survival or death in TNF signalling are under intensive investigations.

TNFα exerts its function through two distinct receptors, TNFR1 (CD120a) and TNFR2 (CD120b). TNFR1 appears to be the key mediator of TNF signalling [3]. Upon binding to its ligand, TNFR1 recruits the adaptor protein TNFR1-associated death domain protein (TRADD) directly to its cytoplasmic death domain (DD), which later serves as an assembly platform for the recruitment of other molecules, such as TNFR-associated factor 2 (TRAF2) and receptor-interacting protein (RIP1) for the activation of NF-κB, or Fas-associated protein with death domain (FADD) for the activation of caspase 8 and induction of apoptosis [4–6]. TNFR1 activation results in stimulation of cell death pathway or activation of a protective pathway mediated by NFκB, or both [4–8]. Previous studies have elucidated the mechanisms for the activation of NFκB by TNFR1, which suggest that TRAF2 and RIP1 are major players in activating NFκB by recruiting the IκBα kinase (IKK) signalosome, a complex formed by three components: IKKα (IKK1), IKKβ (IKK2) and IKKγ (also called NEMO) [7–14]. In addition to the IKK-NFκB pathway, TNRF1 also signals the activation of mitogen-activated protein kinase (MAPK) pathway (including ERK1/2 [extracellular signal-regulated kinase], JNK1/2 [c-Jun N-terminal kinase]

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and p38) and AP1 transcription factor, which may be regulated by MAPK kinase kinase (MKKKs), such as ASK1 (apoptosis signal-stimulating kinase 1) and transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) [7, 15–19]. It has been confirmed that TRADD and FADD are essential for the induction of apoptosis by TNFα [20, 21]. However, accumulating evidence have verified that the activation of NFκB usually protects cells from apoptosis through regulating the expression of many intermediators involved in the regulation of apoptosis, such as Bcl-Xl, survivin, members of the inhibitor of apoptosis (IAP) family (c-IAP1, c-IAP2, XIAP) and FLICE-like inhibitory protein (FLIP) etc. [22]. The activation of both NFκB and MAPK by TNFR1 is initiated by the TNFR1–TRADD–TRAF2–RIP complex [1, 2, 4–14]. Although NFκB activation favours antiapoptotic signals, the activation of JNK has been regarded as an apoptotic signal (although arguments exist) [1, 2, 23–27]. Up to now, a major confusion in TNFα signalling is how the cells discriminate the activation of NFκB and/or JNK. It has been suggested that TRAF2 may be the bifurcate in TNFα signalling since TRAF2-deficient cells were unable to activate JNK while leaving NFκB activation unaffected [11, 28]. However, little is known about the related regulating mechanisms responsible for the separation of JNK activation from NFκB activation.

Heat shock proteins (HSPs) constitute a highly conserved and functionally interactive network of chaperone proteins. Their collective ability to disaggregate, re-fold and re-nature mis-folded proteins offsets the otherwise fatal consequences of damaging stimuli [29–31]. HSP70, including the inducible (HSP70i) and the cognate (HSC70) forms, is expressed by almost all the cells. Various studies have suggested that HSP70 may protect cells from cell death under diverse circumstances, such as UV radiation, heat stress, chemotherapy, hypoxia, etc. [29–31]. Upon stress stimuli, HSP70 may exert anti-apoptotic roles by inhibition of TNFR1-induced activation of ASK1 and JNK, prevention of Bid cleavage and Bax translocation, stability of mitochondria, blockage of the release of cytochrome c, prevention of apoptosome formation and caspase 3 activation and impairment of the nuclear import of apoptosis-inducing factor (AIF) [29–31]. However, there are also evidence supporting the apoptotic roles of HSP70 in receptor-mediated and stress-induced cell death [32–35]. It has been shown previously that HSP70 can delay the degradation and inhibit the phosphorylation of IκBα, and impair the assembly of IKK complex by interacting with IKKγ/NEMO [36–42]. The discrepancy of these studies has been largely attributed to the differences in cell types and in apoptotic stimuli. Further investigations of HSP70 in TNFα-induced signalling and apoptosis may facilitate the detailed understanding of the related molecular mechanisms and the more efficient treatment of human cancer by using TNF-related drugs.

Lipid rafts are specialized membrane microdomains, rich in cholesterol and sphingolipids, which, by sequestering specific sets of proteins, are thought to act as organizing centers for processes such as membrane trafficking and signal transduction [43, 44]. TNFR1 has been shown to be constitutively or inducibly associated with lipid rafts in a number of cell types [45–47]. Recently, the assembly of TNFR1–TRADD–TRAF2–RIP1 in the lipid raft has been suggested to be essential for the ubiquitination of RIP1, the recruitment of IKK complex and TAK1, the activation of NFκB and the regulation of apoptosis [48–50]. In this study, we investigated the roles of HSP70 (HSP70i) in excluding TRAF2 from lipid raft *via* interaction and the effects of HSP70–TRAF2 interaction on TNFα-induced signalling pathways in human colon cancer cells.

# Material and methods

## Cells, antibodies and reagents

The human colon cancer cells HT29 and LoVo, and HEK293 cells were obtained from ATCC (Manassas, VA) and cultured under standard conditions. The antibodies against ASK1, Bcl-Xl, caspase 3, caspase 8, caveolin-1, cIAP1, FADD, HA tag, Myc tag, RIP1, TNFR1, TRADD and TRAF2, the antibodies against IκBα, IKKα, IKKβ, JNK1/2, NEMO, p38, p65 sub-unit of NFκB (RelA) and TAK1, and the antibodies against phosphorylated ASK1 (Thr845), MKK4 (Thr261), p65/RelA (Ser536) and TAK1 (Thr184/187) were from Cell Signaling Technology (Beverly, MA). Purified recombinant IκBα, MBP and HRP-conjugated anti-phospho-MBP (Thr98) were purchased from Upstate Biotechnology (Lake Placid, NY). The agaroses for immunoprecipitations of Flag, Myc and HA, and the antibodies against Caveolin-1, HSP70i, HSC70, Rab5 and transferrin receptor (TfR) were from Abcam (Cambridge, MA). Recombinant MKK4 was obtained from Merck (Darmstadt, Germany). Recombinant human TNFα was obtained from R&D Systems (Minneapolis, MN). The quantitative ELISA kits for phosphorylated IκBα (Ser32), JNK1/2 (Thr180/Tyr182) and p38 (Thr180/Tyr182) were from Calbiochem (San Diego, CA).

## Plasmids, vector construction and transfection

For construction of HA-tagged ubiquitin (GenBank No. NM_018955, encoding 76-residue protein), Myc- or HA-tagged TRAF2 (GenBank No. NM_021138), Falg-tagged HSP70i (GenBank No. NM_005345) and the mutated vectors, pcDNA3.1 vector (Invitrogen, San Diego, CA, USA) was used. Corresponding cDNAs were amplified by PCR from the HEK293 cDNAs. All the expression vectors used in this study were confirmed by sequencing and then prepared using Endofree Plasmid Maxi kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. For the transfection of expression vectors in mammalian cells, the jetPEI reagents were used (Polyplus-transfection Company, Illkirch, France) according to the manufacturer's instructions.

## Apoptosis assay

After treatments with TNFα, cells were labelled with phycoerythrin (PE)-conjugated annexin V or the annexin V/propidium iodide (PI) labelling kit provided by Molecular Probes (Eugene, OR) following manufacturer's instructions. To accurately investigate the effects of HSP70i over-expression in TNFα-induced apoptosis, the cells were co-transfected with

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pcDNA3.1-GFP vector and HSP70i-Flag or pcDNA3.1-Flag mock vectors. After 48 hrs, cells were labelled with PE–annexin V. Otherwise, the cells were labelled with annexin V/PI 48 hrs after transfection. Samples were examined by fluorescence-activated cell sorter (FACS) analysis, and the results were analyzed using CellQuest software (Becton Dickinson, San Jose, CA). For the examination of caspase 3 activation, whole cell lysates were subjected to ELISA assays of cleaved caspase 3 by using Sandwich ELISA Kit (Cell Signaling Technology) as instructed.

## RNA quantification

Quantitative real-time RT-PCR analysis was performed by LightCycler (Roche) and SYBR RT-PCR kit (Takara, Dalian, China). Data were normalized by the level of β-actin. Primer sequences were available upon request.

## Western blot assay

Western blot assay was performed as described by us previously [51]. Bands were revealed using Supersignal West Femto Maximum Sensitivity substrate (Pierce, Rockford, IL), following the manufacturer's instructions. The signal intensity of the indicated band was quantified by densitometric analysis using a Gene Gnome densitometer (Syngene Bio Imaging Systems, Frederick, MD).

## p65/RelA nuclear translocation assay

To examine the nuclear translocation of NF-κB, the extracted nuclear proteins, using NE-PER nuclear reagents (Pierce), were Western blotted to detect the presence of p65/RelA sub-unit in the nucleus.

## Assay of luciferase reporter gene expression

Cells were transfected with the pNFκB-Luc luciferase reporter plasmid, pTA-Luc control luciferase plasmid (Panomics, Fremont, CA) or indicated vectors and treated as indicated. Total amounts of plasmid DNA were equalized *via* empty control vector. Luciferase activities were measured with Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Data are normalized for transfection efficiency by dividing Firefly luciferase activity with that of TA-Luc.

## Sucrose density gradient centrifugation

Lipid rafts were isolated by sucrose density gradient centrifugation essentially as described [48, 50]. Cells were harvested in ice-cold PBS and lysed in 1 ml of MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl) containing 1% Triton X-100, 10 μg/ml benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 mM $NaVO_4$, 10 mM NaF and 1 mM PMSF, incubated on ice for 30 min., and vortexed extensively. One millilitre of an 80% sucrose solution in MES-buffered saline was mixed with the lysate, and this was overlaid with 2 ml of a 30% sucrose solution in MES-buffered saline, followed by 1 ml of a 5% sucrose solution in MES-buffered saline. The samples were centrifuged in a Beckman SW55Ti rotor at 200,000 × *g* overnight at 4°C as described. Then 0.5 ml fractions were taken from the top of the gradient to which 250 μl of 2 × SDS gel-loading buffer was added. Finally, 30 μl of each fraction was subjected to SDS-PAGE and Western blotting.

## Immunoprecipitation

Cells were rinsed twice with ice-cold PBS, and solubilized in lysis buffer containing 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min. on ice. Lysates were centrifuged (15,000 × *g*) at 4°C for 15 min. Proteins were immunoprecipitated for 2 hrs with indicated antibodies respectively. The pre-cleared Protein A/G PLUS-agarose beads (Pierce) were incubated with immunocomplexes for another 2 hrs and washed four times with the lysis buffer. The samples were separated by SDS-PAGE, transferred to a PVDF membrane and detected by Western blot analysis.

## *In vitro* kinase assay

For the analysis of kinases activity, cells were lysed in lysis buffer containing 20 mM Tris pH 7.5, 300 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 2 mM *p*-nitrophenyl phosphate, 1 mM sodium orthovanadate, 10% glycerol, 1% Triton X-100 and 1 mM PMSF. Then the indicated kinases were immunoprecipitated with specific Abs and protein A/G beads (Pierce). After washing extensively with the lysis buffer for three times, the beads were suspended in the kinase assay buffer containing 25 mM Tris, pH 7.5, 5 mM β-glycerol phosphate, 2 mM DTT, 0.1 mM sodium orthovanadate and 10 mM $MgCl_2$. Then 100 μM ATP and 2 μg substrates were added and incubated for 30 min. at 30°C. The reaction was terminated by adding SDS sample buffer. The phosphorylated MBP were detected by Western blot using phospho-MBP Ab.

## RNA interference

For transient transfection, 21-nucleotide sequences of siRNA duplexes targeting 5′-GUUCCCCUUAACUUGUGACUU-3′ (for TRAF2), 5′-GGUGGAGAUCAUCGCCAACUU-3′ (for HSP70i), 5′-UGAACCCCACCAACACAGUUU-3′ (for HSC70) and the corresponding duplexes containing 3-nucleotide mutations (as scrambled control) were synthesized. SiRNA duplexes were transfected using Genesilencer Transfection Reagent (Genlantis, San Diego, CA) according to the standard protocol.

## Statistical analysis

All the experiments were repeated at least three times. Results are given as mean ± S.E.M. or mean ± S.D. Comparisons between two groups were done using Student's t-test analysis. Multiple comparisons were done with a one-way ANOVA followed by Fisher's least significant difference analysis or Mann–Whitney's U-test as indicated. Statistical significance was determined as $P < 0.05$.


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# Results

## HSP70i over-expression inhibits TNFα-induced NFκB activation, whereas it promotes the activation of JNK

To determine the effects of HSP70 in human colon cancer, we over-expressed Flag-tagged HSP70i in HT29 cells and LoVo cells (Fig. 1A), and then treated the cells with 20 ng/ml TNFα. We found that HSP70i over-expression inhibited TNFα-induced phosphorylation of IκBα (Fig. 1B) and the nuclear translocation of p65/RelA sub-unit of NFκB (Fig. 1C). Correspondingly, we found that HSP70i over-expression inhibited TNFα-induced IKKβ activation (Fig. 1D), NFκB gene reporter expression (Fig. 1E) and NFκB-regulated Bcl-xl and c-IAP1 gene expression (Fig. 1F–H). Moreover, we found that HSP70i over-expression promoted TNFα-induced JNK activation (Fig. 1I) and p38 activation (Fig. 1J). These data indicated that HSP70i over-expression could distinguish the effects of TNFα on NFκB and JNK activation in human colon cancer cells.

## HSP70i over-expression sensitizes human colon cancer cells to TNFα-induced apoptosis

To assess the effects of HSP70 on TNFα-induced apoptosis, we co-transfected HSP70i-Flag and pcDNA3.1-GFP vectors in HT29 cells and treated the cells with TNFα for 24 hrs. We found that the transfection efficiency was about 30.4 ± 3.2% (GFP-positive cells). After TNFα treatments, we found that cells over-expressing HSP70i showed elevated percentages of annexin $V^+$ cells in $GFP^+$ populations (Fig. 2A and B). Meanwhile, we also treated HSP70i-transfected cells (HSP70i-Flag) with 20 ng/ml TNFα for 24 hrs. We found that mock vector-transfected cells (Mock) were resistant to TNFα treatments (20 ng/ml), whereas HSP70i over-expression could increase the percentage of apoptotic cells after TNFα treatments (Fig. 2C). Similar effects were observed in LoVo cells (data not shown). To further evaluate the apoptosis, we examined the activity of caspase 3 by ELISA assays of the cleaved caspase 3 fragments. We found that caspase 3 activity was increased significantly in HSP70i-over-expressed cells (Fig. 2D). However, we found that HSP70i over-expression did not affect TNFα-induced activation of caspase 8 (data not shown), indicating that HSP70 may not affect FADD-induced caspase 8 activation. Moreover, we found that the JNK inhibitor SP600125 could inhibit the HSP70i over-expression-induced increase in apoptosis and caspase 3 activation (Fig. 2B and D), indicating that HSP70 sensitizes human colon cancer cells to TNFα-induced apoptosis by activating JNK and caspase 3.

## HSP70 is recruited into lipid rafts together with TNFR1, TRADD, FADD, TRAF2 and RIP1 upon TNFα ligation

HSP70 is a chaperone widely distributed within diverse compartments of cells [32–34]. It has been shown previously that HSP70 can be sequestered in lipid rafts [52]. Therefore, we examined whether HSP70 could be recruited into lipid rafts after TNFα treatments. We found that HSP70 (HSP70i and HSC70), as well as TNFR1, TRADD, FADD, TRAF2 and RIP1, was absent in lipid rafts before TNFα treatments (data not shown). However, these molecules were present in lipid rafts (fractions 2–4) within 5 min. of TNFα treatments (Fig. 3A).

## HSP70 is rapidly released from lipid raft together with TRAF2

Then we examined the dynamic alterations of TNFα signalling intermediates in lipid rafts (combination of fractions 2–4) after TNFα treatments. We found that 15 min. after TNFα stimuli, IKK complex and TAK1 were also recruited into lipid rafts, and the levels of TNFR1, TRADD, HSP70, TRAF2, FADD and RIP1 within lipid rafts were increased (Fig. 3B). However, 30 min. after TNFα treatments, the levels of HSP70 and TRAF2 were decreased. One hour after TNFα treatments, the levels of HSP70 and TRAF2 (especially HSP70i) were diminished in lipid rafts. These data (Fig. 3B) indicated that HSP70 is released from lipid rafts after its recruitment into lipid rafts, and HSP70 may be associated with TRAF2 after TNFα stimuli.

## Over-expression of HSP70i sequesters TRAF2 in detergent-soluble fractions and disrupts the assembly of IKK complex in lipid raft

The above results have suggested that endogenous HSP70 may be involved in the lipid raft *versus* non-raft distribution of TRAF2 (Fig. 3B). To examine the effects of over-expressed HSP70 in the distribution of TRAF2, we treated HSP70i- or mock-transfected HT29 cells with TNFα. We found that the TRAF2 in lipid rafts was greatly reduced in HSP70i-over-expressed cells, and the levels of RIP1, IKK complex and TAK1, but not FADD and TRADD, were also decreased (Fig. 3C). On the other hand, the levels of HSP70i and TRAF2 in soluble fractions of HSP70i-over-expressed cells were significantly increased (Fig. 3C). Therefore, HSP70i may have sequestered TRAF2 in detergent-soluble fractions, and interfered with the complex formation of RIP1 with TRAF2 and the recruitment of IKK and TAK1 in lipid rafts.

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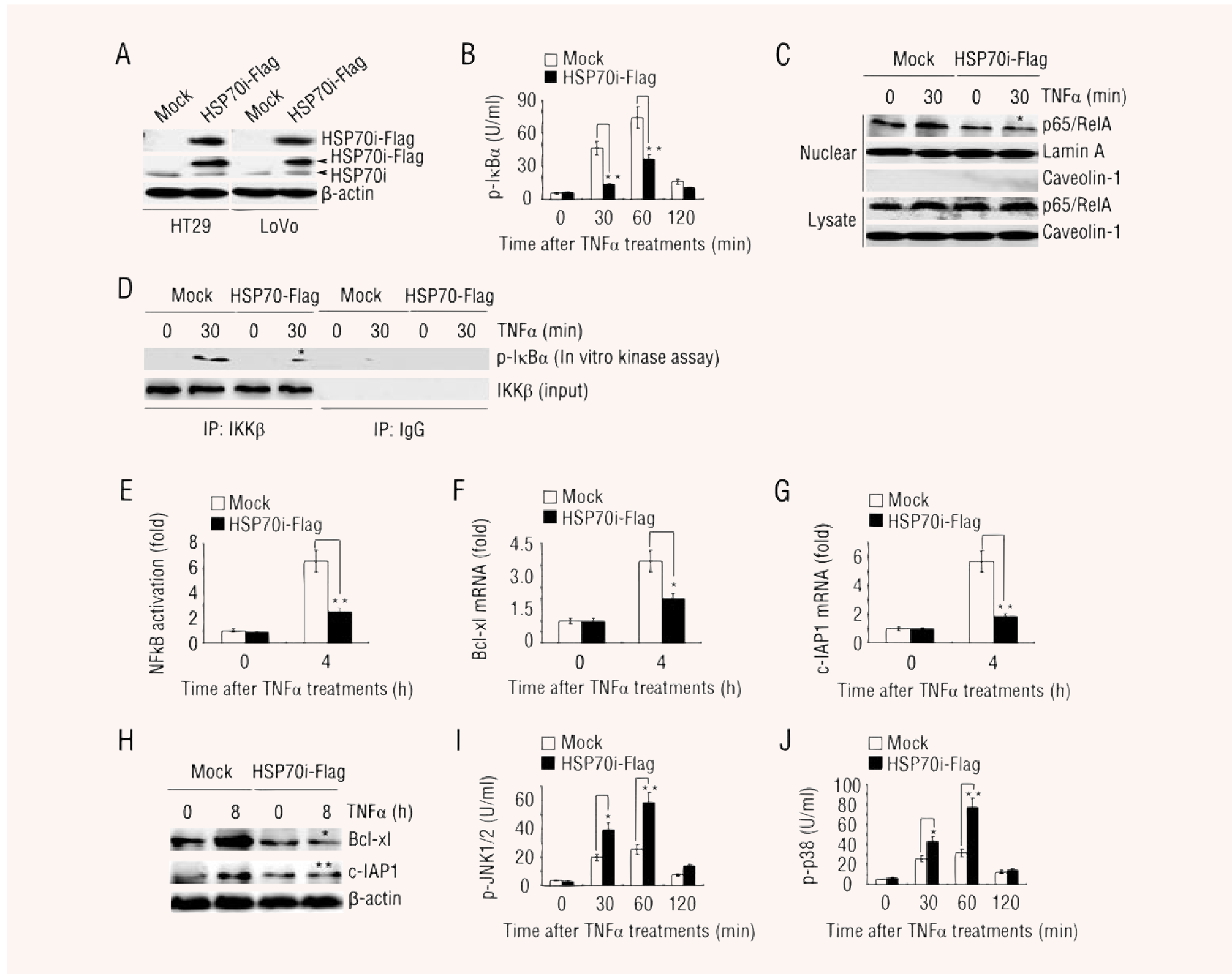


**Fig. 1** HSP70i over-expression inhibits TNFα-induced NFκB activation and promotes TNFα-induced JNK/p38 activation. (**A**) Western blot assays of HT29 and LoVo cells stably transfected with HSP70i-Flag vector or pcDNA3.1-Flag vector (Mock) using anti-Flag Ab or anti-HSP70i Ab. (**B**) ELISA assays of phosphorylated IκBα (p-IκBα) in 100 μg lysates derived from mock- or HSP70i-Flag-transfected cells that were treated with 20 ng/ml TNFα as indicated. Results were presented as mean ± S.D. of triplicate samples. **, $P < 0.01$ (ANOVA). (**C**) Western blot assays of p65/RelA in nuclear fractions and cell lysates in mock- or HSP70-Flag-transfected cells treated with 20 ng/ml TNFα as indicated. Lamin A and Caveolin-1 were examined as loading control and as indicators for the extraction efficiency. *, the relative signal intensity of nuclear p65/RelA to Lamin A in HSP70i-transfected cells 30 min. after TNFα treatment (20 ng/ml) was about 26.3% of that in mock-transfected cells. (**D**) IKKβ kinase activity assay. IKKβ was immunoprecipitated (IP) with IKKβ Ab or control IgG plus protein A/G agarose. The in vitro kinase assay was performed by using recombinant IκBα as substrate. *, the relative signal intensity of p-IκBα to input IKKβ in HSP70i-transfected cells 30 min. after TNFα treatment (20 ng/ml) was about 18.3% of that in mock-transfected cells. (**E**) NFκB gene reporter assay. Mock- or HSP70-Flag-transfected cells were transiently transfected with pNFκB-Luc and pTA-Luc vectors for 48 hrs. Then cells were treated with 20 ng/ml TNFα for 4 hrs. The luciferase activity was determined by dual-luciferase assays of the lysates. Data are expressed as fold increase relative to untreated mock cells, and presented as mean ± S.D. of triplicate samples. **, $P < 0.01$ (Student's t-test). (**F, G**) Quantitative RT-PCR assays of Bcl-xl (**F**) and c-IAP1 (**G**) mRNA expression in mock- or HSP70-transfected cells treated with 20 ng/ml TNFα as indicated. Results were expressed as fold increase in the mRNA levels as compared to mock-transfected cells without TNFα treatments, and presented as mean ± S.D. of triplicate samples. *, $P < 0.05$; **, $P < 0.01$ (Student's t-test). (**H**) Western blot assays of Bcl-xl and c-IAP1 protein expression in cell lysates. *, the relative signal intensity of Bcl-xl to β-actin in HSP70i-transfected cells 8 hrs after TNFα treatment (20 ng/ml) was about 13.2% of that in mock-transfected cells; **, the relative signal intensity of c-IAP1 to β-actin in HSP70i-transfected cells 8h after TNFα treatment (20 ng/ml) was about 28.5% of that in mock-transfected cells. (**I, J**) ELISA assays of phosphorylated JNK1/2 (p-JNK1/2, **I**) and p38 (p-p38, **J**) in 100 μg lysates derived from mock- or HSP70-Flag-transfected cells that were treated with 20 ng/ml TNFα as indicated. Results were presented as mean ± S.D. of triplicate samples. *, $P < 0.05$; **, $P < 0.01$ (ANOVA).


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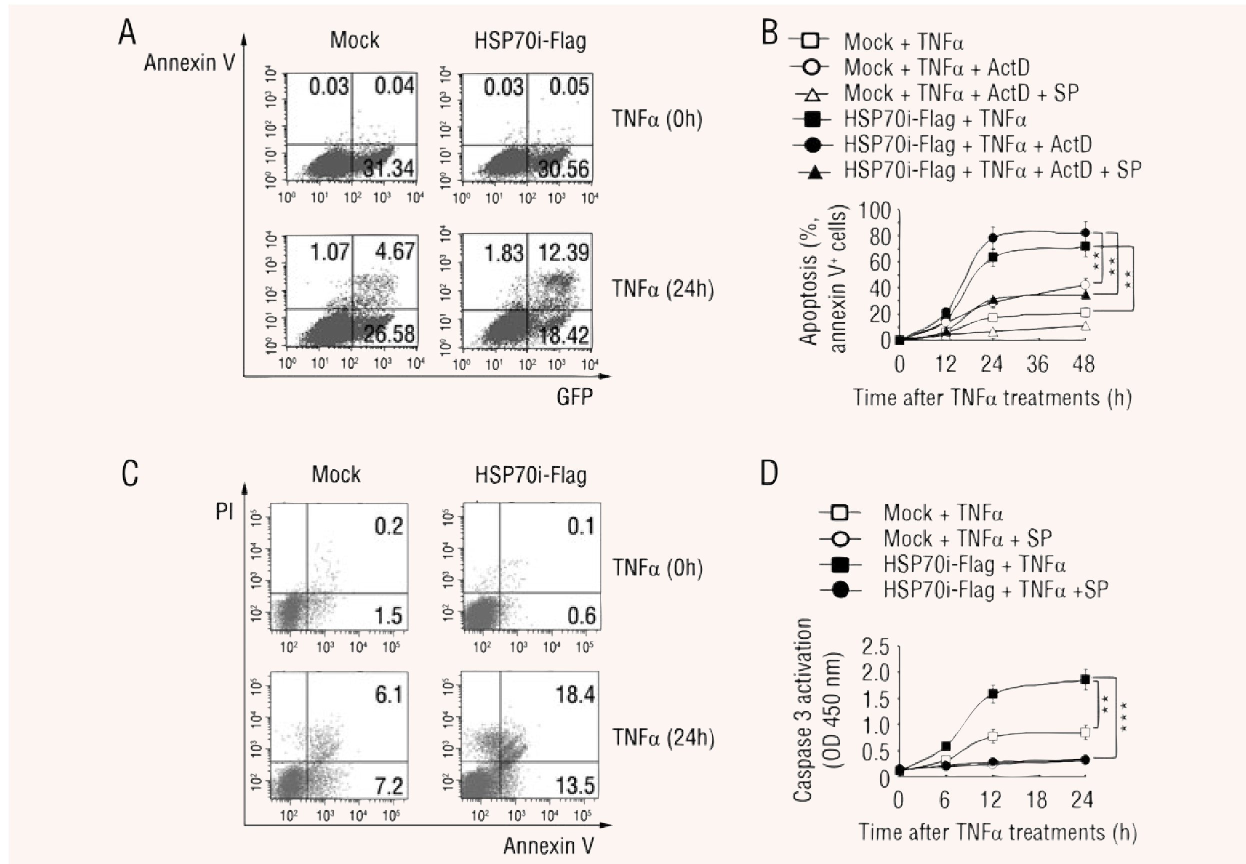


**Fig. 2** HSP70i over-expression promotes apoptosis of human colon cancer cells in response to TNFα. (**A, B**) Apoptosis assay. HT29 cells were co-transfected with pcDNA3.1-GFP vector and HSP70i-Flag or pcDNA3.1-Flag (Mock) vectors, treated with 20 ng/ml TNFα in the presence or absence of 1 μg/ml actinomycin D (ActD) and/or 5 μM SP60025 (SP, JNK inhibitor) for 24 hrs or as indicated, stained with PE-annexin V (**A**) or annexin V/PI (**B**) and analyzed by FACS. The apoptotic cells (the annexin V–positive cells) were indicated as the percentages of whole populations (**A**). Results in (**B**) were presented as percentage of annexin $V^+$ cells to that of $GFP^+$ populations and expressed as mean ± S.D. of triplicate samples. **, $P < 0.01$ (Mann–Whitney's U-test). (**C**) HT29 cells were transfected with HSP70i-Flag or pcDNA3.1-Flag (Mock) vectors, treated as described in (**A** and **B**), stained with PE–annexin V and analyzed by FACS. The apoptotic cells (the annexin V–positive cells) were indicated as the percentages of whole populations. (**D**) ELISA assay of cleaved caspase 3 in 100 μg lysates derived from mock- or HSP70-Flag-transfected cells that were treated with 20 ng/ml TNFα in the presence or absence of 5 μM SP60025 (SP, JNK inhibitor) as indicated. Results were presented as mean OD at 450 nm ± S.D. of triplicate samples. **, $P < 0.01$; ***, $P < 0.001$ (Mann–Whitney's U-test).

## Over-expression of HSP70i promotes TRAF2 ubiquitination

Previously, it has been suggested that RIP1 but not TRAF2 was ubiquitinated in lipid rafts [48]. TRAF2 ubiquitination plays important roles in the recruitment of IKK complex and the activation of TRAF2-associated kinases [53–56]. Therefore, we next examined the effects of HSP70i over-expression on the ubiquitination of TRAF2. In mock-transfected HT29 cells, TNFα could induce the ubiquitination of TRAF2 and RIP1 (Fig. 4A and B). However, in HSP70i-transfected cells, we found that the ubiquitination of TRAF2 was increased, whereas the RIP1 ubiquitination was somehow decreased (Fig. 4A and B). Similar effects were observed in LoVo cells (data not shown).

It has been suggested that K48-linked ubiquitination of target proteins by E3 ligase is susceptible for degradation, whereas K63-linked ubiquitination is essential for activation [53]. Therefore, we examined whether HSP70i-mediated increase in ubiquitinated TRAF2 was beneficial to the activation of TRAF2. In HEK293 cells, we co-transfected Flag-tagged HSP70i,

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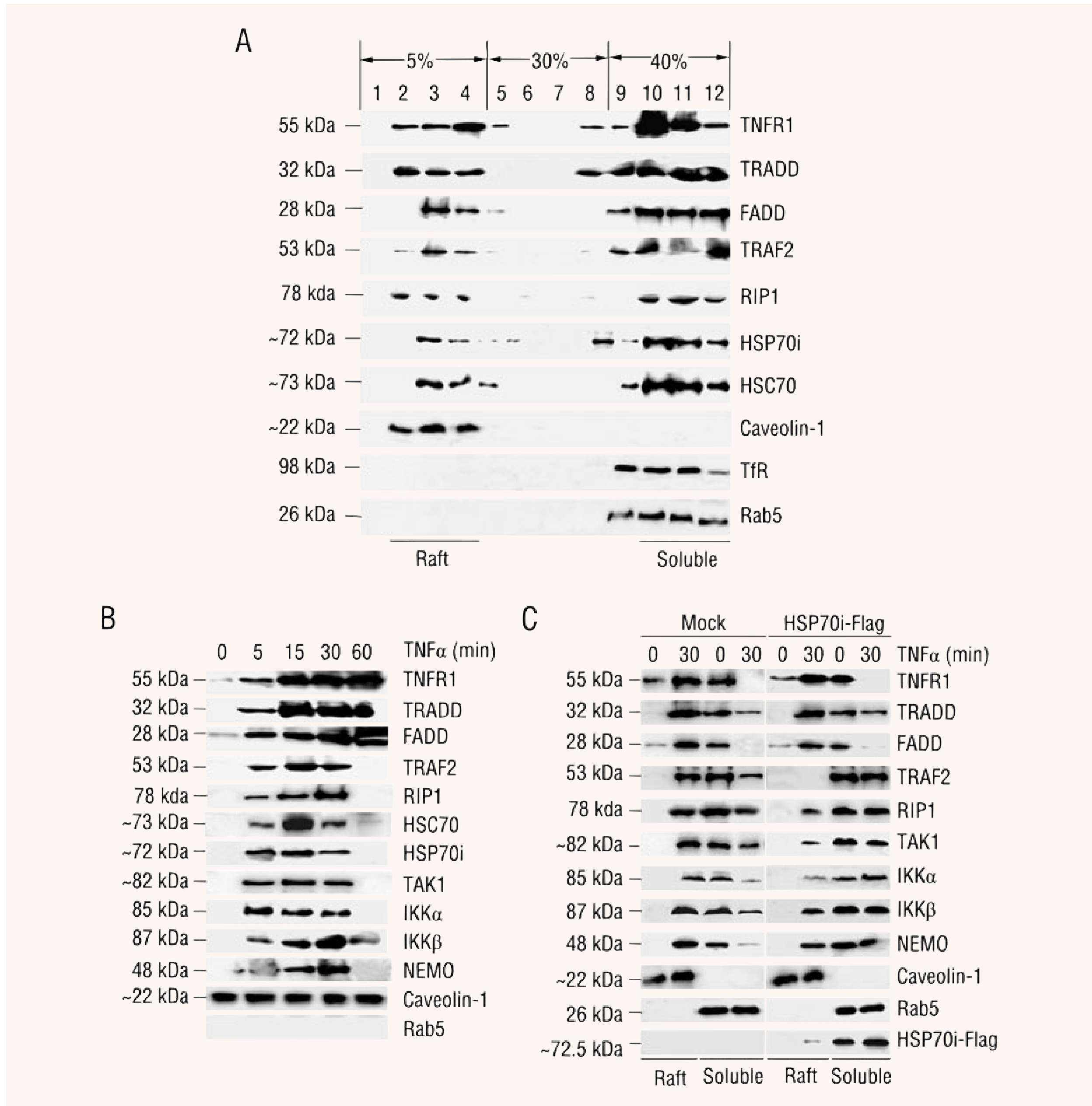


**Fig. 3** HSP70i over-expression promotes the distribution of TRAF2 in detergent-soluble fractions and decreases the recruitment of RIP1, IKK complex and TAK1 in lipid rafts. (**A**) HSP70 (inducible [HSP70i] and cognate [HSC70] HSP70) as well as TNFR1, TRAF2, RIP1, TAK1 and IKK complex were recruited into lipid rafts of wild-type HT29 cells 5 min. after TNFα (20 ng/ml) treatments. Lipid rafts were isolated by sucrose density gradient centrifugation. The indicated components in each fraction (1–12) were examined by Western blots (equal volume of fractions was loaded). The raft-localized (Raft) caveolin-1 and the non–raft-localized (Soluble) Rab5 and transferrin receptor (TfR) were examined to display the efficiency of lipid raft isolation. (**B**) Western blot assay of the dynamic alterations of the indicated components within lipid rafts (combination of fractions 2–4) after TNFα (20 ng/ml) treatments of HT29 cells. (**C**) Mock (pcDNA3.1-Flag)- or HSP70i-Flag-transfected HT29 cells were treated with 20 ng/ml TNFα for 30 min., and then the lipid rafts (fractions 2–4 combination) and soluble fractions (fractions 10–12 combination) were isolated. The indicated components in equal volumes were detected by Western blots.


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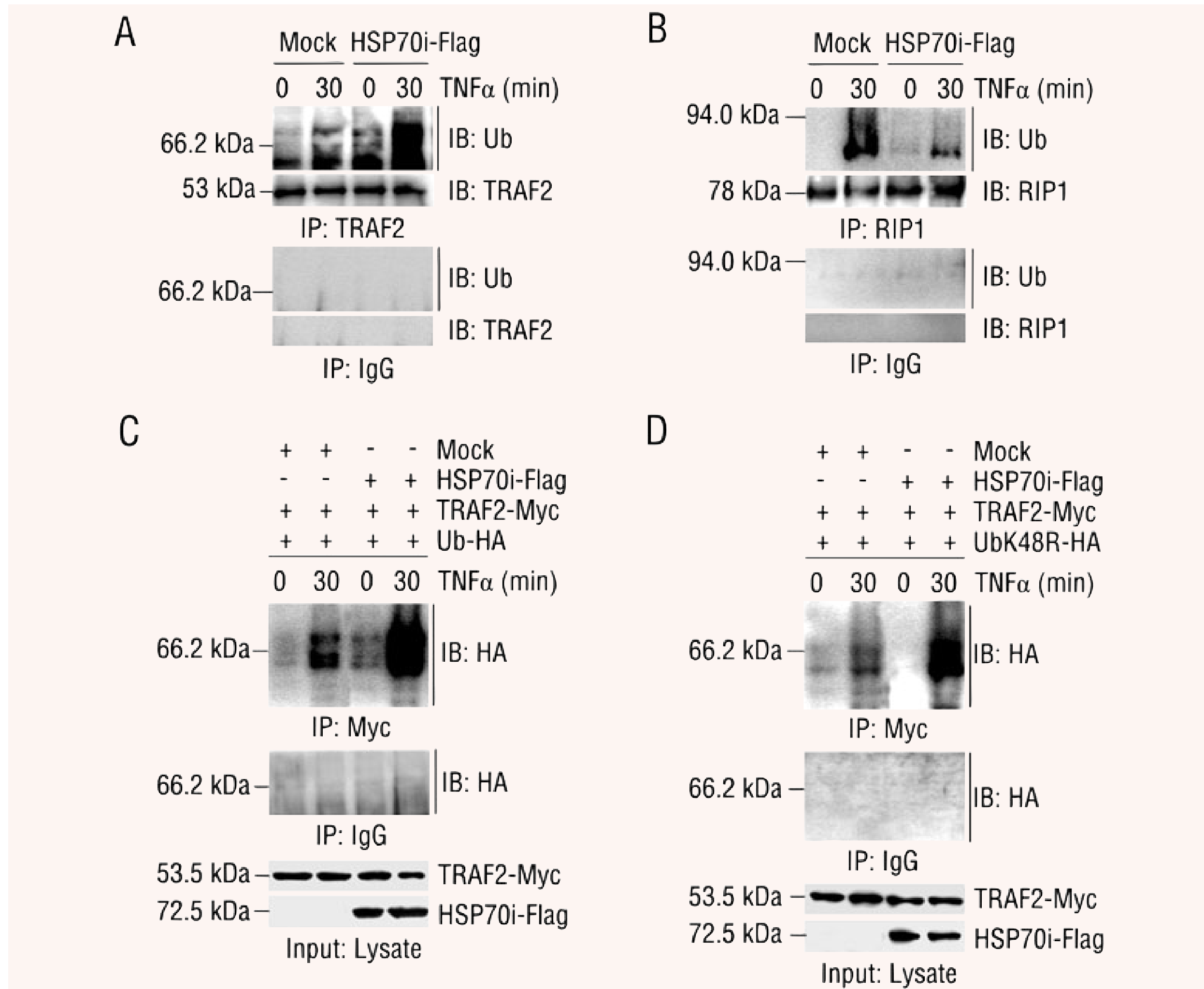


**Fig. 4** HSP70i over-expression promotes TRAF2 polyubiquitination but inhibits RIP1 polyubiquitination in response to TNFα. (**A, B**) Mock (pcDNA3.1-Flag)- or HSP70i-Flag-transfected HT29 cells were treated with 20 ng/ml TNFα for 30 min. Then TRAF2 and RIP1 in cell lysates (100 μg) were immunoprecipitated (IP) with TRAF2 Ab (**A**) or RIP1 Ab (**B**), respectively. Polyubiquitination was examined by immunoblotting (IB) with ubiquitin Ab and HRP-linked pre-adsorbed anti-mouse IgG. Control IgG was also used in the IP processes. (**C, D**) HSP70i possibly promotes K63-linked polyubiquitination of TRAF2. HEK293 cells were transiently transfected with vectors encoding HSP70i-Flag, TRAF2-Myc and HA-tagged ubiquitin (Ub-HA, **C**) or HA-tagged mutated ubiquitin (Lys48 mutated into Arg, UbK48R-HA, **D**) for 48 hrs. Then TRAF2 in cell lysates (100 μg) were immunoprecipitated (IP) with Myc agarose, and the ubiquitination was examined by immunoblotting (IB) with HA Ab and HRP-linked pre-adsorbed anti-rabbit IgG. Control IgG agaroses was also used in the IP processes.

Myc-tagged TRAF2 and HA-tagged ubiquitin vectors, and treated the cells with TNFα. We found that in HEK293 cells, HSP70i over-expression could also promote the polyubiquitination of TRAF2 (Fig. 4C). Moreover, we found that TNFα mainly induces the K63-linked ubiquitination of TRAF2 in HSP70i-Flag- and TRAF2-Myc-co-transfected HEK293 cells since K48R (Lys48 mutated into Arg) mutate of ubiquitin could not affect the polyubiquitination of TRAF2 (Fig. 4D).

## HSP70i interacts with the TRAF domain of TRAF2

TRAF2 can interact with diverse proteins, such as RIP1, TRADD, TAK1, ASK1 and IKKγ *via* different domains [1, 2]. The above results (Figs 3 and 4) indicated that HSP70 may interact with TRAF2. In wild-type HT29 cells, we found that HSP70 (including HSP70i and HSC70) could be co-immunoprecipitated with TRAF2

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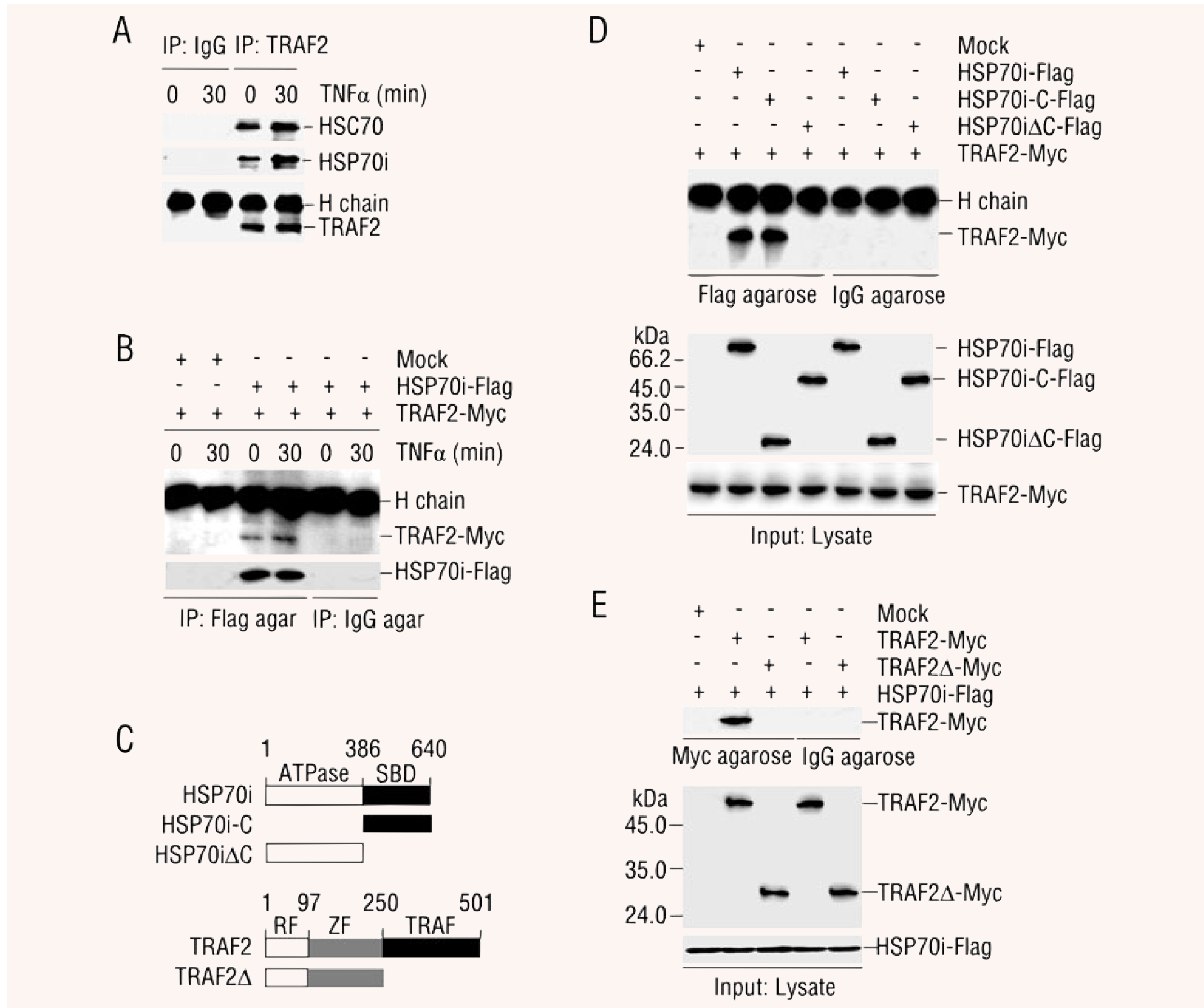


**Fig. 5** HSP70i interacts with TRAF2. (**A**) Endogenous interaction of TRAF2 with HSP70. HT29 cells were treated with or without 20 ng/ml TNFα for 30 min. Then TRAF2 in cell lysates (100 μg) were immunoprecipitated (IP) with TRAF2 Ab or IgG. HSP70 (HSP70i and HSC70) in the immunocomplex was examined by Western blot. (**B**) Exogenously over-expressed HSP70i interacts with TRAF2. HEK293 cells were transiently transfected with indicated vectors for 48 hrs. Then HSP70i-Flag in cell lysates (100 μg) were immunoprecipitated (IP) with Flag agarose (Flag agar) or IgG agarose (IgG agar). Then the TRAF2-Myc contained in the immunocomplex was examined by Western blot using anti-Myc Ab. (**C**) Illustration of the structure of HSP70i and TRAF2 and the mutates used in the interaction assays. SBD, substrate-binding domain; RF, ring finger domain; ZF, zinc finger domain; HSP70i-C, C-terminus of HSP70i; HSP70iΔC, deletion of the C-terminus of HSP70i; TRAF2Δ, deletion of TRAF domain in TRAF2. (**D**) C-terminus of HSP70i interacts with TRAF2. HEK293 cells were transiently transfected with indicated vectors for 48 hrs. Then HSP70i and the mutates in cell lysates (100 μg) were immunoprecipitated (IP) with Flag agarose or IgG agarose. Then the TRAF2-Myc contained in the immunocomplex was examined by Western blot using anti-Myc Ab. The expression of the transfected vectors in cell lysates was also examined by Western blot. (**E**) TRAF domain of TRAF2 interacts with HSP70i. HEK293 cells were transiently transfected with indicated vectors for 48 hrs. Then TRAF2-myc and the mutates in cell lysates (100 μg) were immunoprecipitated (IP) with Myc agarose or IgG agarose. Then the HSP70i-Flag contained in the immunocomplex was examined by Western blot using anti-Flag Ab. The expression of the transfected vectors in cell lysates was also examined by Western blot.

after TNFα treatments (Fig. 5A). In HT29 cells co-transfected with TRAF2-Myc and HSP70i-Flag, we found that HSP70i-Flag could also be co-immunoprecipitated with TRAF2-Myc after TNFα treatments (Fig. 5B).

Next we determined the segments in HSP70i involved in interaction with TRAF2. HSP70i contains an N-terminal ATPase domain (ATPase) and a C-terminal substrate-binding domain (SBD) (Fig. 5C). In HEK293 cells co-transfected with TRAF2-Myc and


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Flag-tagged HSP70i mutate vectors, we found that TRAF2-Myc could be co-immunoprecipitated with HSP70i-C-Flag but not HSP70iΔC (deletion of C-terminal SBD) (Fig. 5D). TRAF2 contains an N-terminal ring finger domain (RF), an intermediate zinc finger domain (ZF) and a C-terminal TRAF domain (Fig. 5C). In HEK293 cells co-transfected with HSP70i-Flag and TRAF2 mutates, we found that HSP70i-Flag could not be co-immunoprecipitated with TRAF2 without TRAF domain (TRAF2Δ) (Fig. 5E). Therefore, the C-terminal domain of HSP70i was responsible for its interaction with the TRAF domain of TRAF2.

## Over-expression of HSP70i promotes the oligomerization of TRAF2 and the activation of ASK1 and TAK1 induced by TNFα

HSP70i over-expression inhibited TNFα-induced NFκB activation (Fig. 1A–E), which may be consistent with the findings that HSP70i over-expression could disrupt the recruitment of IKK complex to lipid rafts (Fig. 3C) and partially inhibit the RIP1 polyubiquitination (Fig. 4B) because the recruitment of IKK complex into lipid rafts and the RIP1 ubiquitination may be essential for TNFα-induced NFκB activation [1, 2, 6–10, 13, 14, 48]. However, HSP70i over-expression–mediated JNK activation in TNFα signalling still lacked explanations despite the observation of increased TRAF2 ubiquitination by HSP70i over-expression (Fig. 4A).

To investigate the mechanisms involved in HSP70-mediated JNK activation, we first examined the activating status of ASK1 and TAK1, two major MKKKs involved in activation of JNK/p38 in TNFα signalling [15–19], after TNFα treatments. We found that the levels of phosphorylated ASK1 and TAK1 were increased in HSP70i-over-expressed cells (Fig. 6A), and the kinase activity of ASK1 and TAK1 was also increased (Fig. 6B).

Oligomerization of TRAF2 through the TRAF domain is crucial for the activation of both NFκB and JNK [57, 58]. Therefore, we examined the oligomerization of TRAF2 in detergent-soluble fractions after TNFα treatments in HT29 cells co-transfected with Myc- and HA-tagged TRAF2 vectors. In mock-transfected cells, the oligomerization of TRAF2 in detergent-soluble fractions was rare, whereas the oligomerization of TRAF2 in lipid rafts was strong in response to TNFα treatments (Fig. 6C). However, the oligomerization of TRAF2 in the detergent-soluble fractions of HSP70i-over-expressing cells was greatly promoted (Fig. 6C), indicating that HSP70 over-expression may facilitate the oligomerization of TRAF2 in detergent-soluble fractions.

Association of ASK1 and TAK1 with TRAF2 is involved in the activation of JNK [15–19]. We then examined the TRAF2-associated ASK1 and TAK1 and their kinase activity. We found that HSP70i over-expression increased the levels of TRAF2-associated ASK1 and TAK1 and the kinase activity of TRAF2-immunoprecipitated complex for recombinant MKK4 (Fig. 6D), indicating that HSP70–TRAF2 interaction may favour the recruitment of ASK1/TAK1 and the activation of ASK1/TAK1.

To elucidate the roles of HSP70-mediated exclusion of TRAF2 from lipid raft, we examined the localization of HSP70-associated TRAF2 and TRAF2-associated phosphorylated ASK1 and TAK1. We found that the HSP70-associated TRAF2 was mainly distributed in detergent-soluble fractions, and the TRAF2-associated p-ASK1 and p-TAK1 was also in the soluble fractions (Fig. 6E), indicating that HSP70 over-expression may favour the activation of ASK1 and TAK1 in the soluble fractions.

## Silence of HSP70 does not affect the lipid raft recruitment of TRAF2 but prolongs the presence of TRAF2 in lipid raft

The above results (Fig. 3C) have suggested a potential role of HSP70 in the regulation of TRAF2 distribution in lipid raft. Therefore, we silenced HSP70 expression by siRNA duplexes specific for HSP70 (HSP70i and HSC70) (Fig. 7A). We found that the recruitment of TRAF2 into lipid rafts 5 min. after TNFα stimuli was not affected by HSP70 silence, indicating that HSP70 may not be essential for the inclusion of TRAF2 in lipid rafts (Fig. 7B). However, we found that the TRAF2 presence in lipid rafts was prolonged by HSP70 silence (Fig. 7B), indicating that HSP70 may be required for sequestration of TRAF2 in detergent-soluble fractions. Moreover, we found that the recruitment of RIP1, TAK1 and IKK complex in lipid rafts was elevated in HSP70-silenced cells (Fig. 7B), which may be due to the prolonged presence of TRAF2 in lipid rafts.

## Silence of HSP70 inhibits TRAF2 ubiquitination

Up to now, there has been no confirmation about the cellular compartments of TRAF2 ubiquitination. To examine the effects of HSP70 on TRAF2 ubiquitination, we measured the ubiquitination status of TRAF2 in HSP70 (both HSP70i and HSC70)-silenced cells after TNFα treatments. We found that the TRAF2 polyubiquitination was decreased (Fig. 7C), whereas RIP1 ubiquitination was increased (Fig. 7D) in HSP70-silenced cells, which may be explained by the observation that HSP70 silence prolonged the presence of RIP1 in lipid raft (Fig. 7B). Considering that lipid rafts have been shown to be essential for RIP1 ubiquitination and activation [48–50], it may be inferred that lipid rafts may be the major sites of RIP1 ubiquitination but not of TRAF2 ubiquitination.

## Silence of HSP70 promotes IKK-NFκB activation but inhibits JNK activation

In HSP70-silenced cells, we found that TNFα-induced activation of IκBα and IKKβ was promoted (Fig. 7E and F), and the TNFα-induced activation of NFκB gene reporter expression was also increased (Fig. 7G), which may be due to the elevated RIP1 ubiquitination and increased IKK complex in lipid raft (Fig. 7B and D). On the contrary, we found that TNFα-induced

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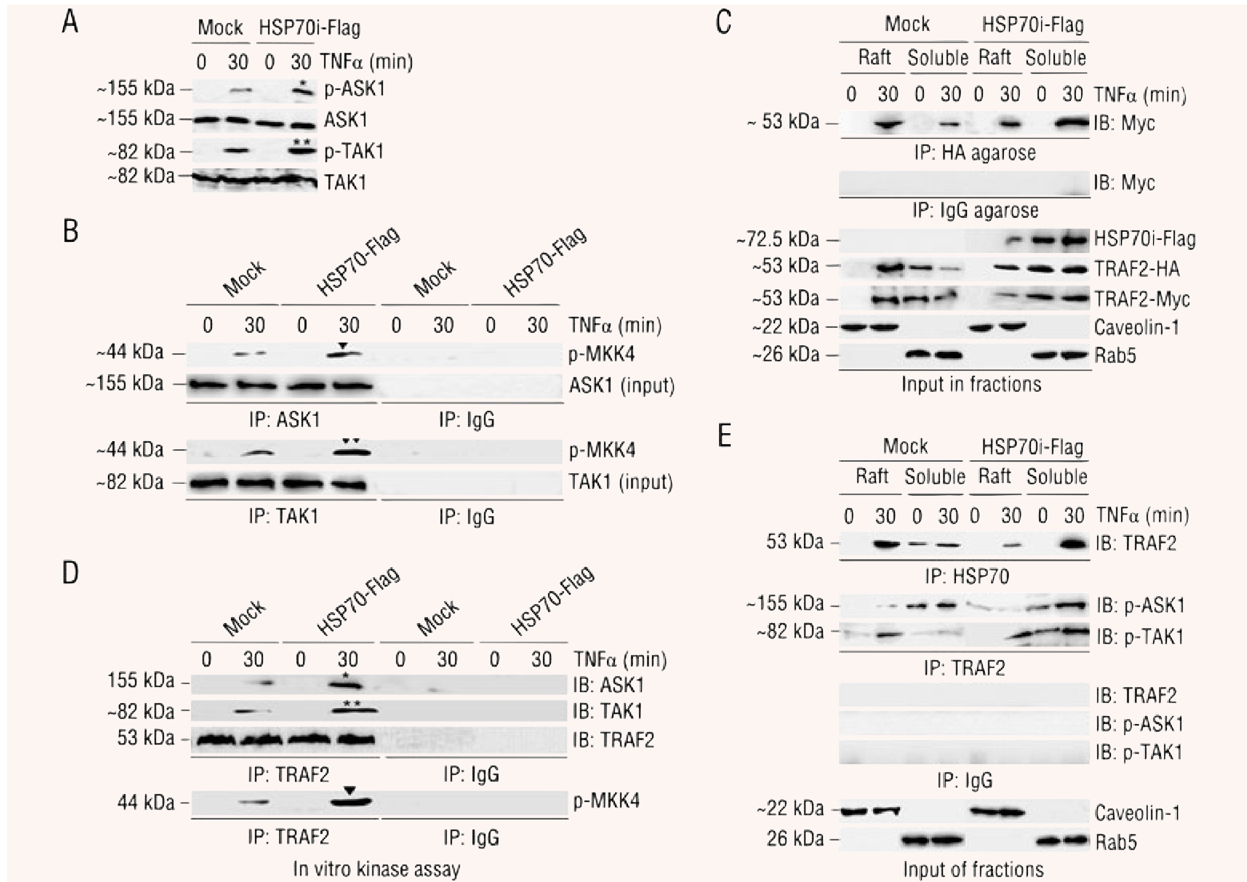


**Fig. 6** HSP70i over-expression promotes the activation of ASK1/TAK1, the oligomerization of TRAF2, the association of ASK1/TAK1 with TRAF2 in soluble fractions in response to TNFα. (**A, B**) HSP70i over-expression promotes TNFα-induced phosphorylation and activation of ASK1 and TAK1. Mock (pcDNA3.1-Flag)- or HSP70i-Flag-transfected HT29 cells were treated with 20 ng/ml TNFα for 30 min. Then the phosphorylated ASK1 (p-ASK1) and TAK1 (p-TAK1) contained in cell lysates (**A**) were examined by Western blot. In (**B**), ASK1 and TAK1 contained in cell lysates (100 μg) were immunoprecipitated (IP) with ASK1 and TAK1 Ab as indicated or IgG (as control). The kinase activity of ASK1 and TAK1 was measured by *in vitro* kinase assays in the presence of recombinant MKK4 as substrate. *, the relative signal intensity of p-ASK1 to total ASK1 in HSP70i-transfected cells 30 min. after TNFα treatment (20 ng/ml) was about 2.64 folds to that in mock-transfected cells; **, the relative signal intensity of p-TAK1 to total TAK1 in HSP70i-transfected cells 30 min. after TNFα treatment (20 ng/ml) was about 2.18 folds to that in mock-transfected cells; ▼, the relative signal intensity of p-MKK4 to input ASK1 in HSP70i-transfected cells 30 min. after TNFα treatment (20 ng/ml) was about 2.88 folds to that in mock-transfected cells; ▼▼, the relative signal intensity of p-MKK4 to input TAK1 in HSP70i-transfected cells 30 min. after TNFα treatment (20 ng/ml) was about 2.72 folds to that in mock-transfected cells. (**C**) HSP70i promotes the oligomerization of TRAF2 in soluble fractions. Mock (pcDNA3.1-Flag)- or HSP70i-Flag-transfected HT29 cells were transiently transfected with Myc- and HA-Tagged TRAF2. Forty-eight hours later, cells were treated with 20 ng/ml TNFα, and the raft fractions (combination of fractions 2–4) and soluble fractions (combination fractions 10–12) were immunoprecipitated (IP) with HA agarose for TRAF2-HA. The IgG agarose was used as control. The associated TRAF2-Myc was immunoblotted by using Myc Ab. The expression of transfected vectors and markers of raft (Caveolin-1)/soluble (Rab5) fractions were examined by Western blot. (**D**) HSP70i promotes the association of TRAF2 with ASK1 and TAK1. Cells were treated as in (**A**). Then TRAF2 in cell lysates (100 μg) was immunoprecipitated with TRAF2 Ab or IgG. The associated ASK1 and TAK1 contained in the immunocomplex were examined by immunoblotting (IB). Otherwise, the immunocomplex was subjected to in vitro kinase assay in the presence of recombinant MKK4. *, the relative signal intensity of TRAF2-associated ASK1 to input TRAF2 in HSP70i-transfected cells 30 min. after TNFα treatment (20 ng/ml) was about 3.24 folds to that in mock-transfected cells; **, the relative signal intensity of TRAF2-associated TAK1 to input TRAF2 in HSP70i-transfected cells 30 min. after TNFα treatment (20 ng/ml) was about 4.03 folds to that in mock-transfected cells; ▼, the relative signal intensity of p-MKK4 to input TRAF2 in HSP70i-transfected cells 30 min. after TNFα treatment (20 ng/ml) was about 5.22 folds to that in mock-transfected cells. (**E**) HSP70 promotes the association of HSP70 with TRAF2 and the association of TRAF2 with ASK1/TAK1 in soluble fractions. Mock- or HSP70i-Flag-transfected HT29 cells were treated with 20 ng/ml TNFα for 30 min. Then the raft fractions (combination of fractions 2–4) and soluble fractions (combination fractions 10–12) were immunoprecipitated (IP) with HSP70 Ab (for HSP70i and HSC70) and TRAF2 Ab as indicated, or IgG (as control). The associated TRAF2 and ASK1/TAK1 were immunoblotted (IB) as indicated. Markers of raft (Caveolin-1)/soluble (Rab5) fractions were examined by Western blot as loading control.


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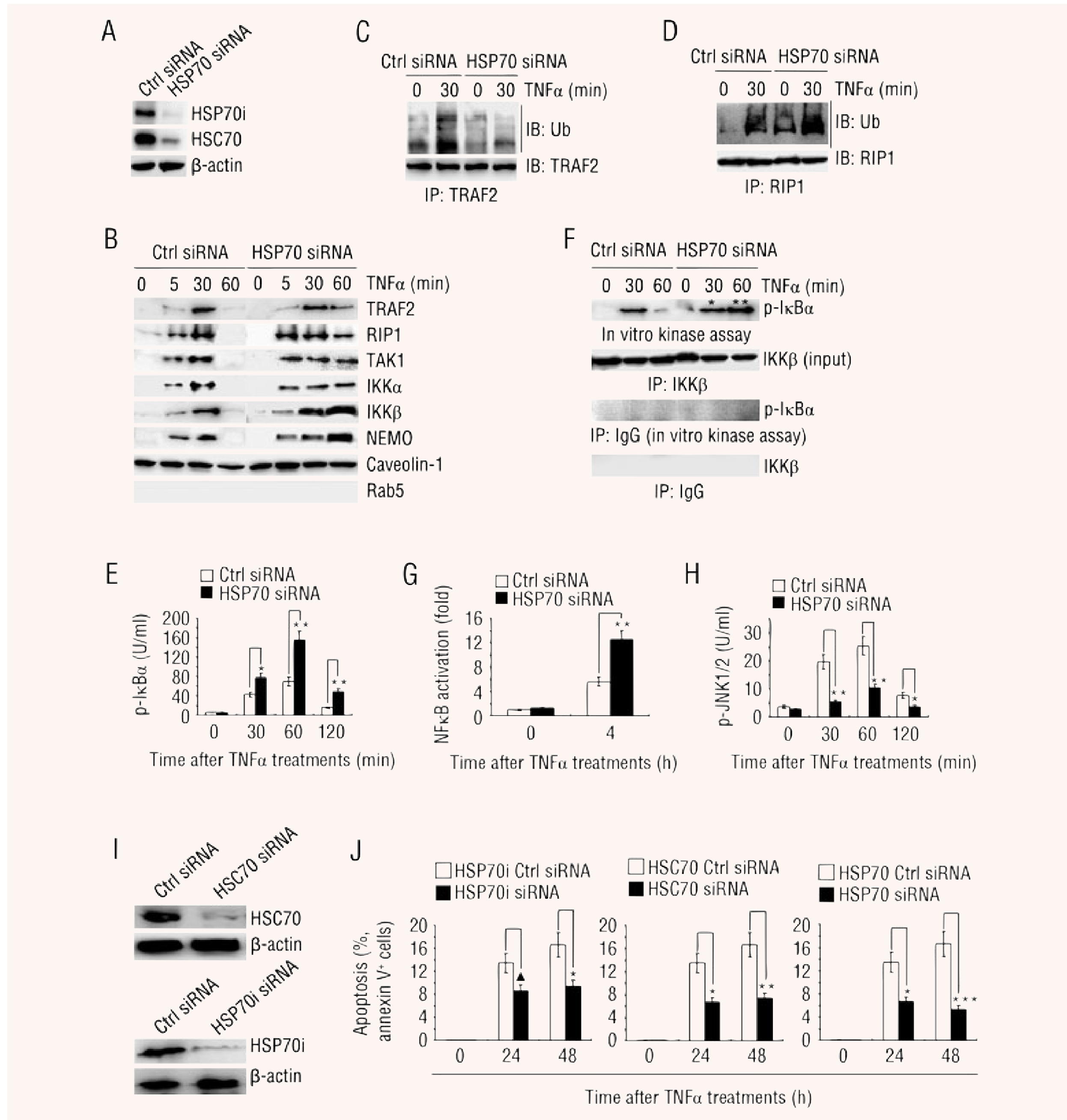


**Fig. 7** HSP70 silence inhibits TRAF2 ubiquitination, prolongs the presence of TRAF2-RIP1-TAK1-IKK in lipid rafts, promotes RIP1 ubiquitination and NFκB activation and inhibits JNK activation. (**A**) HT29 cells were transiently silenced with siRNA duplexes specific for HSP70 (HSP70 siRNA, for HSP70i and HSC70) or scrambled control siRNA duplexes (Ctrl siRNA) for 48 hrs. Then HSP70i and HSC70 contained in cell lysates were examined by Western blot. (**B**) HT29 cells silenced with indicated siRNAs were stimulated with 20 ng/ml TNFα for 5, 30 and 60 min. respectively. Then the raft fractions (combination of fractions 2–4) were examined for the distribution of indicated molecules by Western blot. Raft marker caveolin-1 and soluble fraction marker Rab5 were examined to display the efficiency of raft isolation and the equal loading. (**C, D**) Silenced HT29 cells were treated with 20 ng/ml TNFα for 30 min. Then TRAF2 and RIP1 in cell lysates (100 μg) were immunoprecipitated (IP) with TRAF2 Ab (**C**) or RIP1 Ab (**D**), respectively. Polyubiquitination was examined by immunoblotting (IB) with ubiquitin Ab and HRP-linked pre-adsorbed anti-mouse IgG. Control IgG was also used in the IP processes (data not shown). (**E**) ELISA assays of phosphorylated IκBα (p-IκBα) in 100 μg lysates derived from silenced HT29 cells that were treated with

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20 ng/ml TNFα as indicated. Results were presented as mean OD at 450 nm ± S.D. of triplicate samples. *, $P < 0.05$; **, $P < 0.01$ (ANOVA). (**F**) IKKβ kinase activity assay. IKKβ was immunoprecipitated (IP) with IKKβ Ab or IgG (as control). The in vitro kinase assay was performed by using recombinant IκBα as substrate. *, the relative signal intensity of p-IκBα to input IKKβ in HSP70-silenced cells 30 min. after TNFα treatment (20 ng/ml) was about 1.35 folds to that in Ctrl siRNA-transfected cells; **, the relative signal intensity of p-IκBα to input IKKβ in HSP70-silenced cells 60 min. after TNFα treatment (20 ng/ml) was about 7.48 folds to that in Ctrl siRNA-transfected cells. (**G**) NFκB gene reporter assay. HT29 cells were transiently co-transfected with pNFκB-Luc, pTA-Luc vectors or siRNA duplexes for 48 hrs. Then cells were treated with 20 ng/ml TNFα for 4h. The luciferase activity was determined by dual-luciferase assays of the lysates. Data are expressed as fold increase relative to untreated mock cells, and presented as mean ± S.D. of triplicate samples. **, $P < 0.01$ (Student's t-test). (**H**), ELISA assays of phosphorylated JNK1/2 (p-JNK1/2) in 100 μg lysates derived from siRNA-silenced cells that were treated with 20 ng/ml TNFα as indicated. Results were presented as mean ± S.D. of triplicate samples. *, $P < 0.05$; **, $P < 0.01$ (ANOVA). (**I**) HT29 cells were transiently silenced with siRNA duplexes specific for HSP70i, HSC70 or corresponding scrambled control siRNA duplexes (Ctrl siRNA) for 48 hrs. Then HSP70i and HSC70 contained in cell lysates were examined by Western blot. (**J**) Apoptosis assay. HT29 cells were silenced with siRNAs for HSP70i, HSC70 or HSP70 (both HSP70i and HSC70) for 48 hrs, treated with 20 ng/ml TNFα as indicated, stained with annexin V/PI and analyzed by FACS. The apoptotic cells (the annexin V–positive cells) were indicated as the percentages of whole populations. Results were presented as percentage of annexin $V^+$ cells to that of whole populations, and expressed as mean ± S.E.M. of three independent experiments. ▲, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (ANOVA).

activation of JNK was significantly inhibited in HSP70-silenced cells (Fig. 7H).

### Silence of HSP70 inhibits TNFα-induced apoptosis of human colon cancer cells

Transient over-expression of HSP70i-Flag in human colon cancer cells could promote TNFα-induced apoptosis (Fig. 2A–D). The above data also showed that silence of HSP70 could inhibit TNFα-induced activation of JNK but promote TNFα-induced activation of NFκB (Fig. 7E–H). To examine the roles of HSP70 silence in TNFα-induced apoptosis, we transiently transfected HT29 cells with siRNAs specific for HSC70 and/or HSP70i (Fig. 7A and I). We found that 48 hrs after TNFα stimuli, the annexin $V^+$ cells were significantly less in HSP70i-, HSC70- and HSP70-silenced cells, as compared to the corresponding scrambled control siRNA-transfected cells (Fig. 7J). It should be noted that the decrease of apoptotic cells in HSP70i-silenced cells was relatively less significant than that of HSC70- and HSP70-silenced cells (Fig. 7J), which may be due to different abundance between endogenous HSP70i and endogenous HSC70. These data suggested that HSP70 may play a pro-apoptotic role in TNFα-induced apoptosis of human colon cancer cells.

## Discussion

HSP70 up-regulation in response to stress stimuli is a universal phenomenon in mammalian cells, which may confer to the resistance of cells to subsequent apoptotic stress stimuli [29–31]. The roles of HSP70 in protection of cells from stress-induced cell death have been attributed to the roles of HSP70 in chaperoning and re-naturizing of denatured proteins, and stabilizing the mitochondrial membrane, and the inhibitory effects of HSP70 on caspases activity, apoptosome formation and JNK1/2 and ASK1 activation [29–31]. However, the effects of HSP70 over-expression in TNFα-induced apoptosis have come to controversial conclusions [29–31].

In this article, we show that HSP70 over-expression in human colon cancer cells (*e.g.* HT29 and LoVo cells) can inhibit TNFα-induced activation of NFκB but promote the activation of JNK/p38, leading to increased sensitivity of human colon cancer cells to TNFα-induced apoptosis. We provide a new explanation for the pro-apoptotic roles of HSP70 in TNFα signalling by showing that HSP70 over-expression can sequester TRAF2 in detergent-soluble fractions, increase the polyubiquitination of TRAF2, disrupt the recruitment of RIP1–TRAF2–IKK/TAK1 complex within lipid rafts and promote the association of TRAF2 with ASK1 and TAK1 in soluble fractions. Our data suggest that the exclusion of TRAF2 from lipid rafts by HSP70 over-expression may facilitate the oligomerization of TRAF2 and the activation of TRAF2-associated MKKKs in detergent-soluble fractions in response to TNFα stimuli, leading to increased JNK activation and JNK-dependent potentiation of apoptosis. Meanwhile we provide evidence that HSP70-mediated sequestration of TRAF2 in soluble fractions may interfere with the complex formation between RIP1, IKK and TAK1 in lipid rafts after TNFα stimuli, leading to the inhibition of NFκB activation. Therefore, our study suggests that lipid raft vs soluble distribution of TRAF2 may help cells distinguish the effects of TNFα in activation of NFκB and/or JNK, thus providing insights into the application of TNF-related apoptotic drugs in treatments of human colon cancer.

Both antiapoptotic and pro-apoptotic roles of HSP70 have been suggested by various findings. Under stress conditions, HSP70 can protect cells from cell death by preventing Bid cleavage and Bax translocation upstream mitochondria, preventing release of cytochrome c and smac/Diablo from mitochondria, preventing the activation of caspase 3 and the nuclear import of AIF, which are all largely dependent on the suppression of JNK activation and activity [29–31]. On the contrary, HSP70 has also been implicated in promoting apoptosis. For instance, over-expression of HSF1 (heat shock transcription factor 1, one


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transcription factor that controls the heat shock response) enhances Fas-induced cell apoptosis [32]. Elevated Hsp70 promotes TCR/CD3- and Fas/Apo-1/CD95-mediated Jurkat T-cell apoptosis [33]. Heat shock also sensitizes AML cells and endothelial cells to apoptosis [34, 35]. Moreover, it is also well established that heat shock or elevated Hsp70 alters the regulation of signalling cascades and transcription factors and potently sensitizes tumours to radiation [34–37]. These results imply that heat or Hsp70 could disable a cell survival signal under the appropriate circumstances. The most convincing evidence are raised by several findings that HSP70 inhibits the activation of NFκB [36–42], which is critical for survival of most cells through the induction of antiapoptotic genes [22]. It has been suggested that HSP70 can interfere with the IKK complex formation and the phosphorylation and degradation of IκBα [36–42], which strongly supports an apoptotic role for HSP70 in TNFα-induced apoptosis. In our studies, we confirm that HSP70 over-expression inhibits the activation of IKK-IκBα-NFκB and the expression of anti-apoptotic molecules (*e.g.* Bcl-xl and c-IAP1) induced by TNFα. We provide another explanation for the mechanisms of HSP70-mediated inhibition of NFκB by showing that HSP70 can sequester TRAF2 in the soluble fractions, leading to the impaired recruitment of IKK complex and TAK1 in lipid rafts, which finally contributed to the inhibited NFκB activation. Our studies, together with the other studies, suggest that HSP70 can exert inhibitory effects on TNFα-induced NFκB activation at multiple levels of TNFα signalling, such as impairment of the RIP1–IKK/TAK1 complex formation within lipid rafts by sequestering TRAF2 in detergent-soluble fractions, impairment of the IKK complex formation by interacting with IKKγ/NEMO and inhibition of IκBα degradation by ensuring the stability of IκBα [36–42].

However, one of the inconsistency in our study is the observation that HSP70 over-expression in human colon cancer cells promoted the activation of JNK1/2, which may be due to our findings that HSP70 can sequester and promote the oligomerization and ubiquitination of TRAF2 in soluble fractions, leading to ASK1 and TAK1 activation [15–19, 53–58]. JNK1/2 activation, in contrast to NFκB activation, may play a positive role in TNFα-induced apoptosis by promoting the phosphorylation of Bcl-2, cleavage of Bid, translocation of Bax to mitochondria and effects of reactive oxygen species [1, 2, 23–27]. Previously, ASK1 has been found to physically interact with HSP70 [15, 16, 59], facilitating the inhibition of JNK activation and the protection of cells from $H_2O_2$-induced cell death. We show in our study that HSP70 over-expression can increase the TRAF2-associated ASK1 and TAK1 kinase activity in soluble fractions, thus facilitating the activation of JNK1/2. The observed promotion of ASK1 activity by HSP70 may be due to the different cells and stimuli used in the studies and the different molecular context examined. However, our study convincingly demonstrates that HSP70 negatively regulates TNFα-induced NFκB activation but promotes TNFα-induced JNK activation, which synergistically sensitizes human colon cancer cells to TNFα-induced apoptosis.

Lipid rafts can serve as a platform for the assembly of TNFα-signalling components, which may be essential for the signal transduction from the plasma membrane to the nucleus [43–50]. Previously, it has been demonstrated that TNFR1–TRADD/FADD as well as the intermediators like RIP1, TAK1 and IKK complex can be included in lipid rafts upon TNFα treatments for the activation of IKK and TAK1, which is essential for the activation of NFκB [48–50]. However, whether the lipid raft–mediated assembly of the mentioned components is required for the activation of JNK/p38 MAPK has not been elucidated. Actually, it may be difficult to distinguish the activation of JNK/p38 MAPK from NFκB activation because TRAF2, RIP1 and TAK1 are shared molecules in both MAPK and NFκB signalling pathways [1, 2]. It has been suggested that RIP1 and TAK1 are both essential for TNFα-induced NFκB and MAPK activation [1, 2, 6–10, 13, 14, 48]. The proposal that TRAF2 may be the bifurcate of the two signalling pathways is supported by the findings that TRAF2 knockout cells are normal in TNFα-induced NFκB activation, whereas the JNK activation by TNFα is impaired [11, 28]. The explanation for this effect of TRAF2 has been largely attributed to the redundant roles of other TRAF2-like molecules, such as TRAF5. However, these studies cannot exclude the effects of TRAF2-mediated assembly of signalling intermediators in TNFα signalling because TRAF2 has been shown previously to interact with many substrates and the oligomerization of TRAF2 may facilitate the ubiquitination and activation of the TRAF2-associated molecules [1, 2, 53–58]. We show that HSP70 over-expression can lead to increased levels of TRAF2 in soluble fractions, promoted oligomerization and ubiquitination of TRAF2 and enhanced interaction of TRAF2 with ASK1/TAK1 and activation of ASK1/TAK1, suggesting that the distribution of TRAF2 in soluble fractions may facilitate the oligomerization and ubiquitination of TRAF2 and the activation of JNK upstream MKKKs (*e.g.* ASK1 and TAK1). Previously it has been suggested that TRAF2 oligomerization is sufficient for the activation of both NFκB and JNK [57, 58]. However, it remains to be determined where the oligomerization occurs and whether the difference in the oligomerization sites may affect the activation of either NFκB or JNK. Oligomerized TRAF2 within lipid rafts may be possibly serve as a scaffold for the complex formation between RIP1, TAK1 and IKKs, whereas TRAF2 oligomerization in soluble fractions may favour the ubiquitination and activation of ASK1 and TAK1. Future investigations may be needed to determine the exact roles of the relative distribution of TRAF2 in lipid rafts *versus* soluble fractions in the differential activation of NFκB and JNK by TNFα.

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