Recombinant Tumor Suppressor TSC1 Differentially Interacts with Escherichia coli DnaK and Human HSP70

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ABSTRACT: Tuberous sclerosis complex (TSC) is a neurological syndrome manifested by non-cancerous tumors in several organs. Mutations in either TSC1 or TSC2 tumor suppressor gene cause the disease. In the cell, TSC1 is known to form a heterodimer with TSC2 because of which an active complex is formed that negatively regulates the mTORC1 activity during cellular stress. Hence, mutation in TSC1 or TSC2 is manifested by excess proliferation of the cells leading to the development of numerous benign tumors. The TSC1 and TSC2 complex is known to interact with several protein-binding partners. One such significant interaction of this complex is with the molecular chaperone HSP70. The role of TSC1 in that interaction is still elusive. Here, we have expressed and purified TSC1 (302–420 residues) in a bacterial expression system and have shown that this region directly interacts with HSP70. We have shown that TSC1 increases the ATPase activity of Escherichia coli DnaK, a HSP70 homologue. On the contrary, TSC1 was found to show inhibitory activity toward human HSP70. Our result suggests that TSC1 (302–420 aa) shows differential interaction between the HSP70 homologues. This points toward the evolutionary significance of chaperoning system and the importance of eukaryotic tetratricopeptide repeat domain interaction motif -EEVD. Our study shows the evidence that TSC1 interacts with HSP70 and has a role to play in the chaperoning activity to maintain cellular homeostasis.

1. INTRODUCTION

Tuberous sclerosis complex (TSC) is a genetic disorder with a variety of manifestations including neurological symptoms. The TSC patients suffer from hamartomas or benign tumor formation in several organs such as brain, kidneys, lungs, and so forth. The mortality among TSC patients is higher in case of brain and renal lesions. TSC1 and TSC2 are tumor suppressor genes that were identified in 1997 and 1993, respectively, as a genetic loci mutated in TSC. In the cell, TSC1 is known to form a heterodimer with TSC2 because of which an active complex is formed that inhibits mTORC1 activity. mTORC1 is a master regulator that enables diverse sets of both redundant and distinctive cellular pathways of growth, nutrient, and energy homeostasis. Hence, mutation in TSC1 or TSC2 is manifested by the excess proliferation of cells leading to the development of numerous benign tumors.

In addition, phosphorylation of TSC2 by LKB1-AMPK plays an important role in the cell stress pathway (hypoxia, DNA damage, and low energy). Inoue et al. have shown TSC1 interaction with HSP70 proven with far-western blot and mass spectrometry in the mammalian expression system. Also, the importance of T417 of TSC1 for interaction with HSP70 and co-localization of this protein complex on the mitochondrial membrane preventing apoptosis has also been shown in another study.

The HSP70 chaperones are known to interact with two major classes of proteins. First is the substrate or client proteins that require assistance with folding. The client’s exposed hydrophobic residues are bound specifically to the substrate-binding domain (SBD) of HSP70. Second is the co-chaperones that interact with the chaperone (HSP70) and help in the client folding, while not being clients themselves. Unlike the substrates, the co-chaperones do not bind to the SBD. Nevertheless, both substrate and co-chaperone elicit conformational changes and perturb the ATPase activity in HSP70. Mostly, the ATPase activity is enhanced during their interaction with exceptions of certain co-chaperones, which negatively regulate the ATP hydrolysis. The role of TSC1 in that interaction with HSP70 is still elusive.

DnaK, a HSP70 homologue in Escherichia coli binds to peptides or proteins and helps them to fold and stabilize. DnaK is the most studied HSP70 of all. More than two decades of research has established a well-defined structure and mechanism of action of DnaK. DnaK consists of a nucleotide-binding domain (NBD), a SBD, and a flexible linker region connecting the two domains.
interaction of DnaK with the other proteins is facilitated by the exchange of the nucleotides (ATP/ADP) in the NBD. The SBD contains a β-sheet groove (SBD-β) to bind to the peptide and α-helical lid (SBD-α) to lock the substrate.23 DnaK has a wide variety of clients causing it to be promiscuous protein. Nevertheless, there are certain factors that control the substrate-binding activity of this dynamic chaperone. The factors include SBD-β dynamics, SBD-α lid open or close status, substrate binding, nucleotide-state (ATP/ADP/apo), oligomerization activity of DnaK, and the interaction with co-chaperones.22,24

HSP70s are highly conserved in terms of sequence and mechanism. The NBD and SBD-β are conserved across the species. However, less conservation is observed in the SBD-α and the intrinsically disordered region in the c-terminal of the HSP70 proteins. The eukaryotic HSP70 has a characteristic TPR (tetratricopeptide repeats) binding motif, -EEVD. The EEVD motif plays a key role in interaction with the co-chaperones. Human HSP70 interaction with the co-chaperones HIP (Hsc70 Interacting Protein) and HOP (HSP70-HSP90 Organizing Protein) is also mediated using this motif. Mostly, the ATPase activity is enhanced during their interaction with exceptions of certain co-chaperones, which negatively regulate the ATP hydrolysis. For example, HSP90 is inhibited or decelerated by its co-chaperones HOP and Cdc37.35 Notably, HOP is a co-chaperone for both HSP70 and HSP90 involved in the transfer of clients between them. Woodford et al. have observed that TSC1 acts as an inhibitory co-chaperone of HSP90.52 Here, we also show that TSC1 negatively regulates HSP70. Our finding in synergy with the earlier reports highlight the role of TSC1 as a co-chaperone of HSP70 and HSP90. Here onward, the term DnaK will be used for denoting E. coli HSP70 and the term HSP70/HSPA1A will represent the human heat shock protein (Figure 1).

ATP hydrolysis plays an important role in inducing conformational changes in the domains of HSP70 that are required for substrate binding or release.33 For instance, HSP70 interacts with HSP110 by triggering nucleotide release.34 Similarly, the measure of ATP to ADP conversion would validate whether a protein interacts with the heat shock protein. In a substrate-less ATP-bound DnaK, the intrinsic ATPase activity of DnaK is very low (0.02 min⁻¹ at 25 °C). However, when stimulated by a substrate or a co-chaperone, the activity of DnaK can increase by 9 to 13-fold, respectively.35 In the quest for finding the relationship between TSC1 and HSP70, we have targeted the ATPase activity of HSP70 as a readout. Here, we reveal that TSC1 (302−420 amino acids) interacts with human HSP70 using GST pull-down assay. We find that an increase in concentration of TSC1 (302−420 amino acids) enhances and inhibits the ATPase activity of DnaK and HSPA1A, respectively.

Figure 1. Sequence alignment of DnaK and HSPA1A: the different domains of HSP70 family of proteins are highlighted in three different colors denoting the nucleotide-binding domain, substrate-binding domain, and c-terminal variable intrinsically disordered region in blue, yellow and red respectively. The -EEVD motif of HSPA1A is highlighted in deep red. The symbols in clustal Omega represents the following, “*”—identical residue, “.”—strongly similar residue, and “.”—Weakly similar residue.
2. RESULTS

2.1. Cloning, Expression, and Purification. The vector containing TSC1 (302−420 aa) gene sequence was analyzed using agarose gel electrophoresis (Figure S2). TSC1 (302−420 aa) was expressed as a GST fusion protein in E. coli BL21 DE3 cells. The domain architecture of the full length TSC1 is depicted in Figure 3A. The GST-TSC1 fusion protein was not expressed in the soluble form at 37 °C. The temperature was lowered, and it was found that at 18 °C, maximum quantity of soluble protein was obtained. The IPTG concentration for induction was also varied and 0.5 mM was found to be optimum (Figure S3A). The clarified cell lysate was subjected to Glutathione resin affinity purification. At 10 mM reduced glutathione, 40 kDa GST-TSC1 along with 26 kDa GST was eluted. The presence of GST band is an indication of non-specific bacterial protease activity (Figure S3B).

2.2. Evidence of E. coli DnaK Direct Interaction with Recombinant TSC1 (302−420 aa). After GST affinity purification and the subsequent on-column cleavage, the collected fractions of TSC1 along with the co-eluted DnaK (Figure 3B) were pooled and analyzed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS−PAGE). In the gel, TSC1 has migrated to less than 15 kDa, which is closer to the actual weight of TSC1 that is 12.8 kDa as shown in Figure 3B. The molecular weight of DnaK is about 69 kDa, and the band position exactly matches 70 kDa corresponding to the ladder. The resultant GST that was bound to the resin was eluted. The presence of GST band is an indication of non-specific bacterial protease activity (Figure S3B).

2.3. MALDI/MS Analysis. The recombinant cleaved TSC1 was analyzed using matrix-assisted laser desorption/ionization (MALDI). A prominent peak at 12.8 kDa was obtained (Figure 2). In order to check the presence of TSC1 and DnaK liquid chromatography electrospray ionization−tandem mass spectrometry (LC ESI−MS/MS) was performed (Proteomics facility, IISc Bangalore, Karnataka, India). The purified proteins were run in 10% SDS−PAGE. The gel was stained in Coomassie Brilliant Blue R-250. The bands DnaK (70 kDa) and TSC1 (13 kDa) were excised and sent for LC ESI−MS/MS (Mass-Spec facility Indian Institute of Technology Bombay). The results were loaded in Mascot server (Matrix Science) using peptide mass fingerprinting. The peptide sequences were matched in NCBI database with the host organism Homo sapiens for TSC1 and E. coli for DnaK (Figures S6 and S7).

2.4. Separation of TSC1 and DnaK. TSC1 (302−420 aa) has a theoretical pI of 7.9, and DnaK’s pI is 4.7. Hence, when these proteins at buffer pH 7.2 were subjected to a positively charged anion exchange resin (Bio-Rad HighQ resin), negatively charged DnaK was bound to the resin and positively charged TSC1 (302−420 aa) was obtained as unbound fractions (Figure 3C). DnaK was eluted from the resin using 500 mM NaCl (Figure 3D).

2.5. GST Pull-Down Assay. The GST pull-down assay confirms the interaction of TSC1 (302−420 aa) with HSP70. Figure 4B shows a prominent presence of HSP70 (lane 1), which was pulled using the probed GST-TSC1. Our GST alone control did not pull down HSP70 (Figure 4B, lane 2). The positive control for the blot was the HEK293T lysate that shows the HSP70 presence. We wanted to ensure that the

Figure 2. MALDI analysis of TSC1 (302−420) showing the intact protein with the molecular weight of 12.8 kDa.
positive signal that we see in Figure 4B, lane 1 is not due to GST-TSC1 input. Therefore, we checked the GST-TSC1 input for HSP70 presence, which turned out to be negative as expected (Figure 4B, lane 4). The samples were analyzed using SDS−PAGE (Figure 4C), which shows that GST-TSC1 in lane 1 was indeed responsible for the pull-down of HSP70 from the HEK293T cell lysate.

2.6. Stimulation of DnaK ATPase Activity by TSC1.
DnaK on interaction with the substrate tends to have increased ATP-ADP exchange rates. The intrinsic ATPase activity of DnaK has been known to be stimulated by the co-chaperones in E. coli namely DnaJ, GrpE, as well as the substrates. The main function of co-chaperones is to enhance the activity of DnaK by increasing the rate of γ-phosphate cleavage and nucleotide dissociation.36 When the co-chaperones are absent, the substrate affinity is determined by the type of nucleotide (ADP/ATP) that is bound to DnaK.37 The impact of TSC1 (302−420 aa) was analyzed in terms of the ATPase activity of DnaK. We observed that with increasing concentration of TSC1, the activity of DnaK was gradually increasing (Figure 5). The same experiment showing the TSC1 and ATP only control is provided in the Supporting Information (Figure S9).

2.7. Inhibition of HSPA1A Activity by TSC1.
The commercially available full length human HSP70 was used for performing the assay. HSPA1A has shown decrease in the ATPase activity with increase in TSC1 (302−420 aa) concentration in the ADP glo ATPase assay. When the intrinsic ATPase activity of the human HSP70 is considered as 100%, a 20% reduction in activity was observed at 2.5 μM of TSC1 (Figure 6). The inhibitory activity remained almost the same even on doubling the TSC1 concentration to 5 μM. Woodford et al. have shown that TSC1 (998−1164 aa) acts as a co-chaperone and has a similar inhibitory effect on HSP90. They have also shown that the c-terminal EEVD motif in HSPA1A has a role to play in the interaction of TSC1 (302−420 aa) as well as the absence of EEVD motif in DnaK led to a contrasting increase in ATPase activity upon TSC1 interaction.

3. DISCUSSION
TSC is a genetic syndrome that is manifested as benign tumors in the brain, kidney, lungs, heart, eyes, and skin.1 Mutations in the tumor suppressor genes TSC1 and TSC2 lead to uncontrolled proliferation of the cells.2 Previous work has shown that TSC2 is a client of HSP70 and HSP90, as the inhibition of these molecular chaperones leads to TSC2 ubiquitination and proteosomal degradation.34 There are reports in the literature showing unsuccessful attempts to purify N-terminal regions of human TSC1. Hence, they had

Figure 3. Two-step purification TSC1 and DnaK. (A) Domain structure of TSC1 showing interaction regions with different proteins. (B) One-step affinity purification of GST-TSC1 followed by on-column cleavage using HRV-3C protease; M, molecular weight marker BenchMark ranging from 10 to 220 kDa in (A,B,C). Lane 1, DnaK (green arrowhead) co-eluted with TSC1 (302−420) (red arrowhead). (C) Pure TSC1 (red arrowhead) yielded from two steps of anion exchange chromatography (AEC) Lane 1−4, TSC1 (302−420) unbound fractions from AEC. (D) Pure DnaK (green arrowhead) obtained from AEC of the affinity purified DnaK-TSC1; Lane 1−4, wash fractions of AEC with wash buffer Tris-Cl (50 mM), NaCl (150 mM) at pH 7.2, Lane 5,6, pure DnaK elute fractions with Elution buffer Tris-Cl (50 mM), NaCl (500 mM) at pH 7.2.
switched to more stable homologues of TSC1 in *Schizosaccharomyces pombe*. Here, we have successfully cloned and purified TSC1 (302–420 amino acid) in the bacterial expression system.

Our results show that TSC1 enhances the ATPase activity of DnaK and inhibits the ATPase activity of HSPA1A. HSP70 chaperones throughout the species are known to have increased ATPase activity on interacting with a co-chaperone or a substrate except for few inhibiting co-chaperones. In this case, TSC1 can be a substrate or a co-chaperone to HSP70. In 2009, Inoue et al. have given the first proof that HSP70, when pulled using the control GST. Lane 3 was loaded with clarified HEK293T cell lysate (input-positive control) that was used for the assay, which also shows a prominent HSP70 band. Lane 4 is the input-negative control loaded with GST-TSC1. (C) Samples presented in the immunoblot in (B) were loaded in the same order and separated using SDS–PAGE and stained using Coomassie brilliant blue. Lane 1 shows GST-TSC1 (red asterix) at 37 kDa and GST at 26 kDa because of non-specific protease activity (Figure S3). Lane 2 shows the GST (blue arrowheads) only control. Lane 3 and 4 are the HSP70 target input (HEK293T cell lysate) and purified GST-TSC1 probe input, respectively.

![Figure 4](https://dx.doi.org/10.1021/acsomega.0c02480)

Figure 4. GST pull-down assay showing interaction of GST-TSC1 and HSP70. (A) Schematic showing the proteins used for the GST pull-down assay. GST-TSC1 is the probe protein, GST is the control, and the HSP70 is the target protein. (B) Direct interaction between TSC1 and HSP70 by GST pull-down assay. After the pull-down with GST-TSC1 and the control GST, the eluted proteins were subjected to SDS–PAGE and transferred to a PVDF membrane and immunoblotted. Anti-HSP70 was used to detect the presence of HSP70 (orange arrowheads) in the pull-down fractions and the input controls. Lane 1 shows the presence of HSP70 in the case of GST-TSC1 probe pull-down. Lane 2 shows no sign of HSP70, when pulled using the control GST. Lane 3 was loaded with clarified HEK293T cell lysate (input-positive control) that was used for the assay, which also shows a prominent HSP70 band. Lane 4 is the input-negative control loaded with GST-TSC1. (C) Samples presented in the immunoblot in (B) were loaded in the same order and separated using SDS–PAGE and stained using Coomassie brilliant blue. Lane 1 shows GST-TSC1 (red asterix) at 37 kDa and GST at 26 kDa because of non-specific protease activity (Figure S3). Lane 2 shows the GST (blue arrowheads) only control. Lane 3 and 4 are the HSP70 target input (HEK293T cell lysate) and purified GST-TSC1 probe input, respectively.
Figure 5. DnaK ATPase activity measurement in the presence of different concentrations of TSC1. It is observed that the ATPase activity of DnaK increases with the increase in the concentration of TSC1. RLU—relative light unit.

Figure 6. HSPA1A ATPase activity measurement in the presence of different concentrations of TSC1. The increase in concentration of TSC1 (302–420) has shown decrease in human HSP70 ATPase activity. RLU—relative light unit.

HSP70 interaction was not clear. Our experimental result qualifies TSC1 as a co-chaperone of HSPA1A, and we show that TSC1 (302–420 aa) directly interacts with HSP70. However, it does not completely rule out its possibility as a substrate of DnaK, which resulted in the increase in ATPase activity. We could only show the effect of TSC1 in DnaK and HSP7A1A. Future experiments are needed to further affirm the relationship between TSC1 and HSP70.

4. MATERIALS AND METHODS

4.1. Materials. The pGEX-6P1 vector along with TSC1 (302–420 aa), 354 bp gene fragment was commercially obtained from Genscript. Restriction endonucleases BamHI and EcoRI were purchased from New England Biolabs, Inc. Glutathione resin was purchased from Genscript (L00206). L-Glutathione reduced was from Sigma-Aldrich (catalog no. G4251). The plasmid isolation was performed using a mini-prep kit from Qiagen (catalog no. 27104). E. coli strains DH5α (catalog no. 18265-017) and BL21 DE3 (catalog no. 69450) were purchased from Novagen, and the glycerol stocks of these strains are stored and maintained according to the standard protocol. For the transformation, cells were chemically made competent using CaCl2 treatment. Ampicillin, phenylmethylsulfonyl fluoride (PMSF), Tris base, sodium chloride (NaCl), isopropyl β-D-thiogalactoside (IPTG), Triton X-100, glycerol, acrylamide, N,N′-methylenebisacrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate (APS), tetramethylethylenediamine (TEMED), bromophenol blue, coomassie brilliant blue R-250, glycine, and reduced glutathione were purchased from Sigma-Aldrich (Darmstadt, Germany). PreCision protease (HRV 3C protease) was purchased from GE Healthcare (catalog no.7-0843-01). Bio-Rad Macro-Prep High Q resin (catalog no. 1580040) was purchased for performing AEC. The ADP-Glo assay kit was purchased from Promega Corp (Madison, WI). The centrifugal filtration unit with 10 kDa cut-off was purchased from Merck Millipore (Amicon Merck UFC901008). The recombinant full length human HSP70 (HSPA1A) was purchased from Boston Biochem (AP 100). HEK293T cells were a kind gift from Dr. Sivapriya Kirubakaran (Indian Institute of Technology Gandhinagar). Anti-HSP70 (#4872) antibody was purchased from Cell Signaling Technology. RIPA buffer (radioimmuno-precipitation assay buffer) was purchased from Thermo scientific (catalog no. 89900).

4.2. Cloning of TSC1 (302–420) in pGEX-6P1 Vector. The protein sequence of TSC1 (302–420 amino acids) was obtained from UniProt ID Q92574. From this point, the term TSC1 will be used to represent TSC1 (302–420 aa). After the TSC1 gene was codon optimized for expression in E. coli, it was synthesized and cloned in a pGEX-6P1 vector that was commercially obtained from Genscript. The vector was designed in a way that the GST tag at the N-terminal end was separated from the gene by an HRV 3C protease sequence (Figure S1). The TSC1 gene was flanked by BamHI and EcoRI restriction sites on either side. pGEX-6P-1-TSC1 was transformed into E. coli DH5α using the standard procedure. The transformed bacteria were plated on LB agar containing Ampicillin (100 μg/mL) and left overnight at 37 °C. The following day, few colonies were picked and inoculated in 25 mL of LB media with Ampicillin (100 μg/mL) and left overnight at 37 °C at 250 rpm. The pGEX-6P1 plasmids with TSC1 inserts were isolated by using the Qiagen Mini-Prep kit (Figure S2). The presence of TSC1 insert was confirmed by double digestion of the isolated plasmids with the restriction enzymes EcoRI and BamHI. The isolated plasmid was subjected to sequence analysis to confirm the presence of the TSC1 gene insert.

4.3. Expression and Purification of TSC1. The vector pGEX-6P-1-TSC1 was transformed into E. coli BL21 DE3, and the clones were obtained by using Ampicillin as a selection marker. E. coli containing the gene of interest were grown overnight at 37 °C to 250 rpm in 30 mL of LB media containing ampicillin (100 μg/mL) as a primary culture. The secondary culture was introduced with 3% inoculum in 1 L of LB media. GST-tagged fusion TSC1 expression was induced by addition of 0.5 mM IPTG to the BL21 DE3 cells after the density reached OD600 = 0.8. The bacterial culture was shifted to 18 °C for expression and incubated for 16 h. The cells were harvested by centrifuging at 4500xg for 20 min at 4 °C. The

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presence of GST-TSC1 was initially analyzed through SDS-PAGE.

Cell pellets after the harvest were resuspended in 25 mL of lysis buffer (50 mM Tris-Cl, 50 mM NaCl, 5 mM EDTA, 0.15 mM PMSF, and 1% Triton-X100) per liter of culture. The suspended cells were snap frozen using liquid nitrogen and stored at −80 °C until further use. The cells were subjected to sonication using Vibracell VCX-130 cell disruptor (Sonics and Materials Inc., Newtown, CT, USA). The cells were disrupted on ice for 10 min (40% amplitude with 10 s ON and 15 s OFF cycle) until the cell lysate attained homogeneity. The cell lysate was clarified by centrifugation for 40 min at 14,800×g at 4 °C. For purification of GST-tagged TSC1, the supernatant containing the soluble protein was added to phosphate buffered saline (PBS)-equilibrated GST resin and was kept for binding at 4 °C for 8 h. The matrix was then packed in a gravity flow column (Bio-Rad) and subjected to washing and elution.

4.4. On-Column HRV3C Protease Cleavage. We used the on-column prescission (HRV3C) protease cleavage protocol to purify the proteins. After affinity chromatography, both TSC1 and DnaK were co-eluted. To test the interaction between the DnaK and TSC1, the proteins were separated. Washing the bound GST-TSC1 with 50 mM Tris, 2 mM ATP, and 1 mM DTT at pH 7.5. The protease (1 U/100 μg of protein) was introduced in the column and incubated overnight at 4 °C. The flow-through containing the TSC1 was collected. The bound GST was eluted, and all samples were run in SDS-PAGE in order to check if the cleavage was complete.

4.5. Anion Exchange Chromatography. After affinity chromatography, both TSC1 and DnaK were co-eluted. To test the interaction between the DnaK and TSC1, the proteins were separated. Washing the bound GST-TSC1 with 50 mM Tris, 2 mM ATP, and 1 mM MgSO4 for 30 min in room temperature (RT) did not remove the DnaK entirely. Owing to its huge difference in the pI, we used AEC to separate DnaK (pI 4.7) and TSC1 (pI 7.9). The theoretical pI of the proteins was calculated using the ExPaSp ProtParam tool.42 The affinity purified protein was applied to the anion exchange media pre-equilibrated with Tris-Cl (50 mM) and NaCl (150 mM) at pH 7.2.

4.6. GST Pull-Down Assay. To confirm the direct interaction of TSC1 with HSP70, we used GST pull-down assay.43 GST-TSC1 was not cleaved using the on-column cleavage procedure described above. Instead, the recombinant GST-TSC1 fusion protein was purified as explained in Section 4.3 (Expression and Purification of TSC1) and then eluted after enough column washes. The resultant purified fraction contained a GST-TSC1 and a GST band (Figure S3). We have observed this auto-cleavage even after adding protease inhibitor in our purification steps. After affinity chromatography, GST-TSC1 was subjected to ion exchange as described in Section 4.5 (AEC) to remove the co-eluted DnaK. The final GST-TSC1 fraction was buffer exchanged to PBS and was used for the pull-down assay. Our target protein for the pull-down was human HSP70. We used HEK293T cells to prepare our cell lysate. The HEK293T cells were grown to confluence (3 × 10⁶) in a 6 cm dish and then lysed using RIPA buffer. The extracted lysate was then clarified by centrifugation for 10 min at 4 °C. This lysate was used in the pull-down experiments.

The Glutathione agarose beads were washed and equilibrated using PBS and 50 μL of the resin was aliquoted into two clean microcentrifuge tubes. The beads were blocked overnight with 3% bovine serum albumin in PBS. After PBS washes, the beads were incubated with 150 μL of 30 μM GST-TSC1 and the same amount of GST control. Equal amount of the HEK293T clarified cell lysate was added to both the GST-TSC1 vial and the GST vial. The mixture was incubated while shaking for 4 h at 4 °C. The Glutathione agarose beads were washed with PBS four times while incubating the slurry for 5 min each time. An elution buffer containing 10 mM reduced glutathione was used to elute the proteins bound to the beads. The eluted fractions along with the input were analyzed using SDS-PAGE (Figure 4C) and immunoblotting using anti-HSP70 antibody (Figure 4B).

4.7. ADP-Glo ATPase Activity Assay. DnaK and HSPA1A ATPase reaction was performed in an opaque, white, 96-well plate with the buffer containing 50 mM Tris-Cl, 150 mM NaCl, and 20 mM MgCl2. Different concentrations of TSC1 (0.6, 1.25, 2.5, and 5 μM) were supplied to a constant 0.6 μM DnaK and HSPA1A and incubated for 40 min at RT. A final concentration of 100 μM ATP was added to the reaction mixtures with 25 μL as the final volume, and the ATPase reaction was initiated and incubated for 1 h at RT. ADP-Glo reagent (25 μL) was added and incubated for 1 h at RT. The developing reagent (50 μL) was added and kept for 40 min before measuring the reading. The luminescence reading was measured using the EnVision multilabel plate reader (PerkinElmer, Inc. MA, USA).

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02480.

pGEX-6P1 vector map containing TSC1 (302−420); agarose gel electrophoresis; expression of GST-TSC1 (302−420 aa); SDS-PAGE after glutathione affinity chromatography followed by size-exclusion chromatography; SDS-PAGE after on-column cleaving; LC-ESI MS/MS of TSC1 and DnaK; multiple sequence alignment; and DnaK ATPase activity (PDF)

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Author Contributions
V.T. and N.N. conceptualized the idea and designed the project. V.T. and N.N. designed all experiments and protocols. V.T. guided in the optimization of expression protocol. N.N. and A.S. performed ATPase activity assay. All authors contributed in parts writing this manuscript.
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