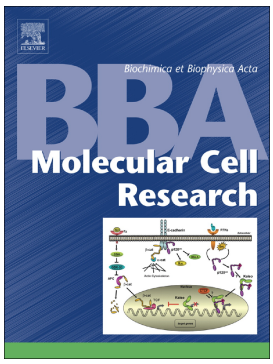

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Pallabi Mitra, Abhijit S. Deshmukh, Chinmayee Choudhury

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Molecular chaperone function of stress inducible Hsp70 is critical for intracellular multiplication of *Toxoplasma gondii*

Pallabi Mitra[1,*], Abhijit S. Deshmukh[2] and Chinmayee Choudhury[3]

[1] *Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad, India*

[2] *DBT-National Institute of Animal Biotechnology, Hyderabad, India*

[3]*Department of Experimental Medicine and Biotechnology, Post Graduate Institute of Medical Research and Education, Chandigarh, India.*

* Corresponding author

Pallabi Mitra

Department of Animal Biology,

School of Life Sciences,

University of Hyderabad,

Hyderabad- 500046,

Telangana, India

Email: pallabi_scmmjnu@rediffmail.com

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Keywords: Toxoplasma gondii; Molecular chaperone Hsp70; Co-chaperone; Autophagy; Lysosome; Pifithrin

Highlights

- *T. gondii* infection upregulates expression of host stress inducible Hsp70
- Hsp70 selective inhibitor PES attenuates intracellular *T. gondii* multiplication.
- PES disrupts molecular chaperone function and impairs cellular autophagy
- Molecular docking analysis reveals TgHsp70 as another promising target of PES
- Biotinylated-PES experimentally confirms its interaction with TgHsp70
- Simultaneous targeting of host and parasite protein(s) as a therapeutic possibility

Abstract

Intracellular pathogens like *Toxoplasma gondii* often target proteins and pathways critical for host cell survival and stress response. Molecular chaperones encoded by the evolutionary conserved Heat shock proteins (Hsps) maintain proteostasis and are vital to cell survival following exposure to any form of stress. A key protein of this family is Hsp70, an ATP-driven molecular chaperone, which is stress inducible and often indiscernible in normal cells. Role of this protein with respect to intracellular survival and multiplication of protozoan parasite like *T. gondii* remains to be examined. We find that *T. gondii* infection upregulates expression of host Hsp70. Hsp70 selective inhibitor 2-phenylethynesulfonamide (PES) attenuates intracellular *T. gondii* multiplication. Biotinylated PES confirms selective interaction of this small molecule inhibitor with Hsp70. We show that PES acts by disrupting Hsp70 chaperone function which leads to dysregulation of host autophagy. Silencing of host Hsp70 underscores its importance for intracellular multiplication of *T. gondii*, however, attenuation achieved using PES is not completely attributable to host Hsp70 indicating the presence of other intracellular targets of PES in infected host cells. We find that PES is also able to target *T. gondii* Hsp70 homologue which was shown using PES binding assay. Detailed molecular docking analysis substantiates PES targeting of TgHsp70 in addition to host Hsp70. While establishing the importance of protein quality control in infection, this study brings to the fore a unique opportunity of dual targeting of host and parasite Hsp70 demonstrating how structural conservation of these proteins may be exploited for therapeutic design.

1. Introduction

Toxoplasma gondii is an intracellular protozoan parasite which can infect both animals and humans by virtue of its ability to invade and replicate within any nucleated host cell **[1]**. An estimated 2 billion people globally are infected with *T. gondii* which often manifests as severe disease causing encephalitis and retinochoroiditis in immunocompromised or immunosuppressed people or when it is congenitally transmitted early in fetal development **[2]**. It is increasingly found to be associated with mental illnesses and neurocognitive impairment **[3, 4]**. It has been identified as one of the leading foodborne pathogens and its zoonotic transmission makes it an important area of public health interest. There is currently lack of effective therapeutics which can cure the latent infection. A better understanding of the pathways critical for its propagation would enable designing of robust anti-parasitic strategies.

Emerging data suggest that infectious organisms modulate cell survival pathways in the hosts to establish and promote its own multiplication **[5]**. Molecular chaperones encoded by the evolutionary conserved Heat shock proteins (Hsps) are vital to cell survival following exposure to any form of stress **[6]**. Proteins belonging to Hsp family are structurally and functionally diverse; and their cellular expression ranges from constitutive expression to stress induced **[6, 7]**. A key protein of this family is Hsp70 (also called Hsp72, Hsp70-1, or HspA1A), an approximately 70 KDa ATP-dependent molecular chaperone, which is stress inducible and often present at low or undetectable levels in most normal cells and tissues **[7]**. This versatile stress inducible molecular chaperone has significant roles in protein folding and maintaining proteostasis. Elevated expression of this protein has been demonstrated in various cancers and cellular

infections in addition to thermal stress, oxidative stress and nutrient deprivation **[7]**. In addition to its primary role in facilitation of folding of nascent polypeptides, formation of protein complexes and prevention of protein aggregation, it also determines cell fate due to its regulatory role in signalling pathways mediating cell survival **[8, 9].** Heightened expression of Hsp70 in various forms of cancer have been associated with increased resistance of these cells to various forms of cell death and often associate with poor patient prognosis **[7-11]**.

Heat shock proteins (Hsps) are highly conserved proteins and are found to be present across all domains of life including protozoan parasites. Several homologues of Hsps have been identified and characterized in protozoan parasites including members of Hsp40, Hsp70 and Hsp90 **[12, 13]**. In fact, several Hsp70 homologues have been identified in phylum Apicomplexa and have been demonstrated to have molecular chaperone activity and a role in maintenance of protein homeostasis in these organisms **[12-15]**. *T. gondii* Hsp70 homologue has been so far examined and described in the context of its immunomodulatory effect during host cell infection **[16, 17]** and its potential role in stage differentiation **[18]**. Proteomic analysis of host cells infected with *T. gondii* indicated an upregulation in expression of Hsps including Hsp70 **[19]**.

Molecular chaperones functions in close coordination with the two major protein degradation systems in mammalian cells, the autophagy-lysosome and proteasome pathways, which aids in the elimination of conformationally-altered, or misfolded proteins **[20]**. The close functional interaction among various molecular chaperones and with other pathways that maintain protein homeostasis helps to explain how targeting the function of Hsp70 simultaneously impacts multiple signalling

pathways that sustain cells. Thus, targeting the actions of Hsp70 offers a promising therapeutic approach and has been well studied with respect to cancer treatment [**7]** while it still remains to be fully explored with respect to infectious diseases.

There are several compounds that inhibit Hsp70 and can be used to modulate Hsp70 actions **[10, 13, 21, 22]** and assess its effects. In this context, a small-molecule 2-phenylethynesulfonamide (PES), also called Pifithrin-μ, has been identified as specific inhibitor of stress-inducible Hsp70 **[23, 24]**. PES has been well utilized to understand the varied activities of the Hsp70 protein with respect to multiple cancer-critical biological processes **[23, 24].**The specificity of this small molecule, PES, may be employed as an investigative tool to understand the significance of this molecular chaperone in host parasite relationship.

Here we report that *T. gondii* infection causes an elevated expression of stress inducible Hsp70 in host cells. Small molecule inhibitor PES which selectively targets the stress inducible Hsp70 was utilized to understand the significance of this parasite driven increase in Hsp70 expression. PES causes an attenuation in intracellular *T. gondii* multiplication. We find that PES binds Hsp70 in *T. gondii* infected host cells and acts by interfering with Hsp70 molecular chaperone activity by disrupting its key interactions with co-chaperones. PES was found to promote accumulation of key client proteins in detergent insoluble fraction consistent with aggregation and inactivation. PES treatment of the *T. gondii* infected cells further impairs the host autophagic process, consequently attenuating intracellular parasite multiplication. Silencing of Hsp70 expression using siRNA resulted in dysregulated autophagy, however, the attenuation of intracellular parasite replication was only part of what was achieved by

PES treatment of infected host cells. Notably, silencing of HSP70, only partially rescued PES-induced parasite attenuation, suggesting presence of additional intracellular targets of PES in infected host cells. We demonstrate that *T. gondii* homologue of Hsp70 (TgHsp70) is a potential target of PES. PES interacts with recombinant TgHsp70 through its substrate binding domain which was determined using deletion protein variants of TgHsp70 in PES binding assay. Molecular modelling and virtual docking analysis not only substantiated the findings but also proposed TgHsp70 to be a very good target of PES. Like recombinant TgHsp70, endogenous TgHsp70 binds PES as demonstrated by pull down assay using biotinylated PES. PES targeting of TgHsp70 abrogates its interaction with TgHsp40, a Sis1 like J domain protein known to be associated as a co-chaperone. Taken together, the observations highlights the importance of molecular chaperone mediated protein quality control for *T. gondii* multiplication in host cells and a unique possibility of dual targeting strategy to control parasite multiplication.

2. Materials and methods

2.1 Ethics statement

The animal experiments conducted for this study were approved by the University of Hyderabad Institutional Animal Ethics Committee (IAEC, Reference No. UH/IAEC/2019-I/10). All experimental protocols followed were in accordance with the guidelines approved by Institutional Biosafety committee (IBSC, Reference No.PM-N-21 -Aug 2018).

2.2 Mammalian cells and in vitro infection with *T. gondii*

Human retinal pigment epithelial cells (ARPE-19; CRL-2302, American Type Culture Collection; Manassas, VA), Human brain microvascular endothelial cells (HBEC-5i, ATCC, CRL-3245), the mouse microglia cell line NR9460 (BEI Resources, NIAID, NIH), were cultured in complete media following standard procedures. Murine microglial cells are grown in Dulbecco's Modified Eagle's Medium containing 4 mM L-glutamine, 4500 mg per L glucose, 1 mM sodium pyruvate, and 1500 mg per L sodium bicarbonate, supplemented with 10% fetal bovine serum and 10 µg/mL ciprofloxacin. Human brain microvascular endothelial cells (HBMEC) were cultured in basal medium supplemented with Endothelial Cell Growth Supplement (ECGS) and 10% fetal bovine serum (FBS). Mammalian cells were infected with tachyzoites (MOI 1:5) of the RH strains of *T. gondii*. The number of parasites per vacuole was determined using light microscopy.

2.3 Chemicals and Antibodies

In specified experiments, mammalian cells were treated with PES, lysosomal inhibitors pepstatin, pan-caspase inhibitor-Z-VAD-FMK. For treatment with PES (P0122-Sigma), stock solutions were made in DMSO and diluted in PBS; the final concentration of DMSO was less than 0.4%.Mammalian cells were infected with *T. gondii* RH parasites. PES treatment with different concentrations ranging from 1 µM to 5 µM (as indicated for each experiment) was given at 6 hours post-infection for a period of 24 hours before the number of parasites was determined using light microscopy. For treatment with pepstatin (P5318-Sigma), mammalian cells infected with *T. gondii* parasites were treated without or with PES for 24 hours in absence or presence of lysosomal protease inhibitor pepstatin (10 µM). Monolayers were examined by light

microscope at the end of the treatment. For treatment with pan caspase inhibitor Z-VAD-FMK (Sigma) cells infected with *T. gondii* parasites were treated without or with PES for 24 hours in absence or presence of Pan caspase inhibitor Z-VAD-FMK (10 µM). Monolayers were examined by light microscope at the end of the treatment. Mammalian cells infected with *T. gondii* tachyzoites were treated with 5 µM PES for 24 hours in absence or presence of Cycloheximide (CHX) (25 µg/ ml) (Sigma -C7698) for last 12 hours. Monolayers were examined by light microscope to determine parasites per vacuole. All experiments were repeated at least on three separate occasions.

The following primary antibodies were used in this work: anti-HSP70 (4873), antiHSP90 (4874), anti-CHIP (2080), anti-HSP40 (4869), anti-p62/SQSTM1 (5114), anti-LC3 (3868) and Beta tubulin (2146) (Cell Signaling Technology, Inc., Danvers, MA); anti-HSC70 (sc-7298) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The peroxidase-conjugated secondary antibodies (i.e., donkey anti-rabbit, donkey anti-mouse, and donkey anti-rat) and streptavidin-HRP were purchased from Cell Signaling Technology. TgHsp70 antibody is generated in- house following a protocol as described previously **[25]**. TgIMC1 was generated in-house for a previous study **[26]**. TgHsp40 antibodies were also raised in-house for this study. Recombinant human Hsp70 (11660-H07B) and Hsc70 (11329-H07E) proteins were procured commercially (Sino-Biological Inc.).

2.4 Preparation of Biotin-Conjugated PES (B-PES)

Sulfo-NHS-SS-biotin (biotin) and PES (2-phenylethynesulfonamide or pifithrin µ) were purchased from Pierce Biotechnology, Inc. (Rockford, IL) and Sigma respectively. The biotinylated form of PES (B-PES) was produced as indicated by the

manufacturer (Pierce). Briefly, 10 mM solution of Sulfo-NHS-SS-biotin was mixed with 1.5 mg of PES, followed by a 30 min incubation at 25℃. Subsequently, 50 mM Tris-HCl (pH 8.0) was added to the biotin-PES (B-PES) mixture to quench any non-reacted biotinylation reagent.

2.5 B-PES-Pull down

5-10 µM biotin or B- PES was incubated with 10–20 mg of clarified (centrifugation at 11000 x g for 10 min at 4) whole-cell lysates obtained from infected cells prepared using buffer (200 mg/L KCl, 200 mg/L KH_2PO_4, 8000 mg/L NaCl, 2160 mg/L Na_2HPO_4 7H2O, 0.5% Triton X-100, 0.5% NP-40 [pH 7.4]) supplemented with protease inhibitor cocktail. 100–200 µl of prewashed NeutrAvidin resins were used to capture biotin and B-PES associated proteins by incubating the mixtures at 4℃ for 1 hr on a rocker. Following incubation, NeutrAvidin resins with bound B-PES complex were washed three times using the indicated buffer. The captured B-PES proteins bound to the NeutrAvidin resins were either resuspended directly in SDS-loading buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 10% 2-mercaptoethanol) or eluted using 100 mM DTT, which cleaves the disulphide bond in the spacer arm of the biotin attached to PES. The associated proteins were resolved by SDS- PAGE.

In vitro binding experiments were performed by mixing biotin or B-PES with 2µg of recombinant protein in 1X PBS for 2 hr at 4 followed by pull-down using NeutrAvidin resins. The captured B-PES-TgHSP70 complexes bound to the NeutrAvidin resins were washed extensively and eluted using 100 mM DTT. The associated proteins were resolved by SDS-PAGE.

2.6 Immunoprecipitation and Immunoblot

The IP was undertaken using IP kit (26149, Thermo Fisher Scientific) following protocol detailed by the manufacturer. Cells were washed in PBS and lysed in IP lysis buffer supplemented with protease inhibitor on ice for 1 h. Total protein extract was obtained by centrifugation at 21,000 ×g, 10 min at 4 °C. Lysates were immunoprecipitated using appropriate antibody crosslinked to agarose for 1 h at 4 °C. The immunoprecipitated protein complexes on the beads were washed three times with lysis buffer and eluted. Elutes were then boiled in the Leamilli sample buffer (SDS loading buffer) at 95 °C for 10 min and analysed by western blotting. Protein samples from lysed cells were loaded at equal concentrations in an SDS-PAGE gel and transferred to a PDVF membrane. The membranes were probed with antibodies of interest. Detergent (1% NP 40) soluble and in-soluble fractions were prepared as described previously [23]. Briefly, the cells were lysed in 1% NP40 lysis buffer containing 50mM Tris (pH7.5), 100mM KCl, 2mM EDTA supplemented with protease inhibitor cocktail followed by centrifugation at 10,000 x g for 25 minutes at 4°C. The resultant pellet, the detergent insoluble fraction, was resuspended in Leamilli sample buffer while the supernatant was further centrifuged to remove the debris to obtain the detergent soluble fraction. Fractions thus obtained were subjected to western blot analysis using requisite antibodies. Images were generated using Photoshop. When needed, change in brightness and/or contrast was applied equally to the whole image. Densitometry analysis was done using ImageJ (NIH) and normalized against untreated controls and background signals.

2.7 Indirect Immunofluorescence assay (IFA)

IFA was essentially done as described previously **[25]**. Briefly, cells were grown in confluent monolayers on coverslip in 6-well plates and infected with *T. gondii* RH parasites followed by PES treatment as detailed above. The infected cells were fixed post 10 to 18 h of infection in 4% paraformaldehyde, followed by permeabilization with 0.25% Triton X-100. The samples were further blocked in 1% BSA and incubated sequentially with primary and secondary antibodies. The following primary antibodies were used at the indicated dilutions: anti- LC3II (1:100), anti-P62 (1:100), mouse anti-IMC1 (1:1000) and rabbit anti-IMC1 (1:1000). Alexa-conjugated secondary antibodies (Alexa Fluor-488 or Alexa Fluor-594) were used at a dilution of 1:1000. The coverslips were mounted with Vectashield medium (Vector Laboratories) with DAPI (4′, 6-diamidino-2- phenylindole) on glass slide and images were captured with a Leica Confocal microscope with 100X objective. Images were processed using LAS X software.

2.8 Apoptosis assay

Apoptosis analysis of cells treated with PES for 24 h was carried out by dual labelling of cells with Annexin-fluorescein and propidium iodide (556547, BD Biosciences) followed by flow cytometry measurements.

2.9 Purification of recombinant *T. gondii* proteins

Recombinant TgHsp70, TgHsp40, TgBiP and deletion mutants of TgHSP70 were made as described earlier **[27]** using primers detailed in Supplementary data (Supplementary Table 2.). The constructs were prepared using a pET21a/pET28a plasmids (Novagen, EMD Millipore) followed by expression in the Rosetta Escherichia coli strain or BL21 DE3 cells (Stratagene). Expressions of the fusion protein was

induced by adding 0.3 mM IPTG and harvested after overnight culture at 18℃ (for deletion constructs and TgBiP) or 0.5mM IPTG at 37℃ overnight (for TgHsp70 full length and TgHsp40). The cells were collected by centrifugation and lysed using lysis buffer as described previously **[26]**. The fusion protein incorporating a hexa-histidine tag was purified by bench top chromatography using a nickel-nitrilotriacetic acid resin (QIAGEN).

2.10 Transfection

siRNA designed for human Hsp70 gene (HspA1A) and negative control siRNA were purchased from Eurofins Genomics. The negative control siRNA was an oligonucleotide with no homology to any known human gene. The cells were transfected with indicated siRNAs using the Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RPE cells were transfected with siRNA (10 nM), and immunoblot or infection with *T. gondii* was performed after 2 days as described earlier **[28]**. The decrease in Hsp70 expression was monitored by western blotting.

2.11 Homology modelling and molecular docking analysis

As the 3D structures of Heat shock protein Hsp70 of Toxoplasma gondii and related homologue BiP (TgBiP) have not been solved, it was modelled using the available homologous structures in protein data bank. ClustalΩ was used for the target template alignments and Modeller 9.24 **[29]** was used to generate the homology model of TgHsp70 and TgBiP using structures of their human homologues available in protein data bank. The first 385 residues of TgHsp70 were modelled from 3ATV **[30]**, while the residues from 386-615 were modelled from 4PO2 **[31]**. The C-terminal residue stretch

615-674 was modelled with I-TASSER web server [**32**] using ab initio modelling/threading as no homologous template was available for this region. The model with the best confidence score was chosen along with the other homologous templates 3AVT and 4PO2 for building a consensus model of the full length TgHsp70 protein. Another model of TgHsp70 was generated without the GGMP repeats, where residues 620-674 were deleted from the full model. Structure of TgBiP was modelled from the human BiP protein (PDB-ID 6ASY) and the C-terminal 28 residues were modelled using I-TASSER server. Although, the structures of HsHsp70 and HsHsc70, were available in parts, the stretches of C-terminal regions were not solved for them. These terminal residue stretches were also modelled using the I-TASSER server. Thus, we have a total of five structures, i.e., TgHsp70 with GGMP repeats, TgHsp70 without GGMP repeats, TgBiP, HsHsp70 and HsHsc70. These five structures were subjected to loop refinement followed by 10000 steps of steepest decent energy minimization in a TIP3P water box to avoid any steric clash using DESMOND [***33***]. The stereo chemical qualities of the models generated for the full length TgHsp70 and TgBiP proteins were verified using the PROCHECK module of PDBSUM server **[34]** before using them for docking calculations.

The modelled structures were pre-processed using Protein Preparation Wizard (PPW) module of Schrödinger software package, version 2019-2[**35**]. Missing hydrogens were added, and appropriate bond orders were assigned to the structures. The protonation states of the polar residues were optimized with the protassign module of PPW, which uses PROPKA to predict pKa values (pH 7.0±2.0) and side chain functional group orientations. The structure was then subjected to restrained

minimization (cut-off RMSD 0.3 Å) with impref to avoid steric clashes. The prepared structure was further used for preparation of grids and molecular docking. PES was subjected to preparation in LigPrep **[35]**, generating their ionization states at pH 7.0 (± 2.0) using Epik ionizer.

Three energy grids were generated on the three distinct domains (regions) of the modelled structure of Hsp70 of *T. gondii*. The three regions are i) the ATP binding region (residue 1-383), ii) residues 384-510 and iii) the C-terminal region (residues 545-674). The grid centres were defined as the respective centroids of all the constituent residues and the grid box size was defined as 25 Å in order to cover the whole region for blind docking. For the second model of TgHsp70 without GGMP repeat, grid was generated taking residues 545-614. For the other proteins i.e., TgBiP, HsHsp70 and HsHsc70, only the C-terminal domains were used for grid generation. Glide module of Schrodinger suite **[36]** was used for docking the prepared structure of PES to the above energy grids. 20 best poses were generated for each docking calculation using OPLS_2005 force field, with all default parameters. Complexes with the best scoring poses of PES with TgHsp70 (with and without GGMP repeats), TgBiP, HsHsp70 and HsHsc70 were further subjected to implicit water (dielectric constant 80.4) molecular dynamics simulations for 20 ns using OPLS_2005 force field. 10 cluster representatives were sampled from each trajectory and then Molecular Mechanics-Generalized Born Surface Area (MM/GBSA) based binding free energy (ΔG_{bind}) of PES were computed for each cluster representative using Prime module of Schrodinger.

2.12 Circular dichroism spectroscopy

Far-UV CD spectra for all protein variants of TgHsp70 were recorded using a Jasco J-1500 spectropolarimeter using a 0.1 cm path length cuvette. Typically, samples contained 20 μM protein in 15 mM Tris buffer at pH 7.4, containing 50 mM NaCl. The far-UV CD spectra of all the variants were recorded from 190 to 250 nm, and the spectrum of the buffer was systematically subtracted from the spectra of all protein samples.

2. 13 Statistics

Statistical analysis, as indicated in figure legends, were done using GraphPad Prism (GraphPad Software Inc., CA, USA).All numerical data were collected from three independent experiments unless stated otherwise. Results were expressed as mean ± SD (standard deviation bars in graphs) or as mean ±SE (standard error bars in graphs). P values *< 0.05; **<0.01; and ***<0.001 were considered statistically significant.

3. Results

3.1 Elevated expression of stress inducible Hsp70 in *T. gondii* infected host cells

Heat shock proteins (Hsps) were originally determined as a group of heat inducible proteins, but was later found to be differentially expressed in response to a variety of exogenous factors including physiological stress and infections **[6, 7]**. In the light of the cited observations, we wanted to determine the expression level of Hsp70 in infected cells with respect to uninfected control at different time points post-infection. Human retinal pigment epithelial (RPE) cells were infected with *T. gondii* RH (type I) parasites and infected cells were checked for the expression of Hsp70 and other member of the highly conserved family of Hsps including Hsc70 and Hsp90 using western blot analysis at various time points post-infection. We observed a clear upregulation in

expression of host Hsp70 with respect to uninfected cells and a progressive increase in expression as a function of time post-infection (**Fig. 1A**). In contrast, under the same experimental condition expression of a related Heat shock protein, Hsc70 remained largely unchanged (**Fig. 1A**). Another prominent member of the family, Hsp90, did not display consistent noteworthy changes under our experimental conditions. Beta tubulin also remained unaltered across the various time point tested and acted as the loading control. The antibodies utilized for the experiments were found to be specific for host proteins and did not cross-react with purified parasite lysate (**SI Fig. S1A-C**). The upregulation of host Hsp70 was also observed in Human brain microvascular endothelial cells (EC) and the mouse microglia cell line (MG) infected with *T. gondii* RH parasites (**Fig. 1B, C**). Altogether, *T. gondii* infection causes elevated expression of host stress inducible Hsp70 in various mammalian cells.

3.2 Selective targeting of Hsp70 using small molecule inhibitor PES attenuates intracellular parasite multiplication

In order to understand the significance of the elevated expression of host Hsp70, we attempted to selectively target the host Hsp70 using a small molecule inhibitor PES, also called phenylacetylenylsulfonamide or Pifithrin-μ. This small molecule inhibitor which has been originally identified in a screen of drug like small molecule inhibitors affecting P-53 mediated apoptosis **[37]** and has been demonstrated to be highly selective for Hsp70 and interferes with its protein quality-control activities mediated through the substrate binding domain **[23, 24]**. This direct inhibitor of the stress inducible Hsp70 has been demonstrated to be preferentially cytotoxic to a broad range of solid tumor cell types **[23, 38]**. We tested whether inhibition of Hsp70 using PES affect parasite survival

and replication. Host cells were treated with PES for 24 hrs post tachyzoites infection (6 hours post-infection). Monolayers were examined using light microscopy to determine the number of parasites per vacuole. PES caused a dose dependent attenuation of parasite multiplication in all the cell types infected with *T. gondii* RH parasites including RPE, EC and MG (**Fig. 2 A- C**). The same concentration of PES did not produce any visible apoptotic changes in the uninfected host cells or infected cells tested (**SI Fig. S2A, B**). This anti-*T. gondii* effect were observed at concentrations much lower than those reported to have potent cytostatic activity against various cancer cell lines. The results indicate that pharmacologic inhibition of Hsp70 manifests anti-*T. gondii* effect by reduction in intracellular multiplication.

To verify whether stress inducible Hsp70 is indeed an intracellular target in *T. gondii* infected host cells we synthesized a biotinylated form of PES (Biotin-PES or B-PES) using a thiol cleavable amine reactive reagent as described in the experimental procedures. We utilized this Biotin-PES to capture PES interacting proteins from lysates prepared from *T. gondii* infected cell, which were then pulled out using Avidin resins. DTT was used to elute PES interacting proteins which were then resolved using SDS PAGE and subjected to western blot analysis using specific antibodies. The analysis revealed that in *T. gondii* infected cells including RPE, EC and MG, PES selectively interacts with stress inducible Hsp70 and not Hsc70 or Hsp90 (**Fig. 2D-F**). While Hsp70 and Hsc70 are highly homologous and PES targeting of Hsc70 has been reported previously **[24]**, under our experimental condition we could not detect any interaction of PES with Hsc70. This may be attributed to the lower concentrations of PES used for the study as against the much higher concentrations used in previously reported studies.

The observations indicate that the intracellular attenuation of *T. gondii* is potentially mediated through PES targeting of Hsp70 activities.

3.3 PES interferes with molecular chaperone action of Hsp70

In order to better understand the molecular basis for these observations, we sought to examine if PES interferes with Hsp70 molecular chaperone activity which involves proper folding of nascent proteins, refolding of misfolded proteins and formation and disassembly of multi-protein complexes **[6, 39]**. Most of these actions and their regulation is mediated through its association with a range of co-chaperones including Hsp40 and CHIP **[40, 41]**. We used immunoprecipitation followed by western blot analysis to determine if PES alters the interactions between Hsp70 and co-chaperones Hsp40 and CHIP. PES treatment aggregates the association of Hsp70 with these co-chaperones (**Fig. 3A-C**). All the *T. gondii* infected cells used for this analyses generated consistent results (**Fig. 3A-C**).

We further find that PES mediated parasite attenuation displays a partial rescue upon treatment with inhibitor of nascent protein synthesis Cycloheximide (CHX) (**Fig. 3D**), indicating that the accumulation of misfolded nascent protein may be one of the factors affecting parasite multiplication in the host cells evaluated. Cellular proteins tend to display increased aggregation following PES treatment owing to its effect on Hsp70 chaperone function [**24**]. Therefore, to further examine if PES mediated alteration of chaperone function of Hsp70 affects client proteins, we examined two well established client partners of Hsp70, EGFR and AKT. We observed increased abundance of EGFR and AKT in the detergent- insoluble fraction consistent with aggregation of these proteins in PES treated RPE cells.

3.4 Targeting Hsp70 using PES leads to autophagy dysregulation

Considering the PES mediated interference of Hsp70 molecular chaperone function in *T. gondii* infected host cells, we further investigated if there is any altered expression of autophagy markers given the close functional co-ordination between chaperones and autophagy lysosomal pathway [**42, 43**]. Addition of PES to host cells post-infection resulted in accumulation of autophagosome marker, the proteolytically processed form of microtubule associated Light Chain 3 (LC3-II) (**Fig.4 A-C**).The accumulation of LC3-II in presence of PES was also observed in immunofluorescence analysis (**Fig. 4D**). Further, attenuating effect of Hsp70 targeting by PES was partly abrogated in presence of lysosomal protease inhibitor pepstatin A, indicating contribution of lysosome mediated cellular autophagy (**Fig. 4 E**), however, it does not completely explain the observed attenuation in parasite progression. An inhibition of caspase activation and apoptosis can induce autophagy **[42, 43]**. However, the observed effect remained unaltered upon treatment with pan-caspase inhibitor Z-VAD-FMK suggesting that effect may be caspase independent under the given experimental conditions (**Fig. 4F**). Infected cells did not register any apoptotic changes upon treatment with PES (**S1 Fig. S2B**). We further examined another marker of autophagy, protein p62 or SQSTM1 (sequestosome-1) which is often upregulated in response to stress and mediates transport of poly-ubiquitinated protein aggregates for degradation **[44, 45]**. Autophagy regulates the steady state level of p62 and its accumulation is indicative of a blockade or defect in autophagic degradation **[46-49]**. PES treatment of *T. gondii* infected mammalian cells showed an accumulation of p62 autophagic adaptor as demonstrated by western blot analysis (**Fig. 5A-C**). Also, immunofluorescence

analysis were in line with this observation where prominent p62 puncta and aggregates could be visualized in the PES treated *T. gondii* infected cells (**Fig. 5D**).

3.5 Silencing of host Hsp70 affects parasite multiplication

Pharmacologic inhibition of stress inducible Hsp70 using small molecule inhibitor PES interfering with its molecular chaperone activity, resulted in attenuation of intracellular propagation of *T. gondii* mediated through the cellular autophagy machinery. In the light of the findings, it was imperative to examine if depletion of the host Hsp70 manifests similar reduction in parasite load. RPE cells were transfected with siRNA targeting stress inducible Hsp70 or control siRNA followed by infection with *T. gondii*. We found that knockdown of Hsp70 in RPE cells reduced parasite load (**Fig. 6A**). We checked if Hsp70 protein expression was effectively depleted following siRNA treatment and observed Hsp70 specific siRNA treatment efficiently downregulated Hsp70 expression while its expression remained unaltered in control siRNA (non-specific) treatment (**Fig. 6B**). In order to examine if the intracellular attenuation of *T. gondii* is mediated through the autophagy machinery upon Hsp70 knockdown, we checked the level of autophagy marker LC3-II and P-62. Both LC3-II and P-62 are upregulated in infected RPE cells where Hsp70 has been knocked down (**Fig. 6B**). Interestingly, despite the ablation of the Hsp70 protein in *T. gondii* infected cells transfected with specific siRNA, the attenuation of parasite multiplication was only partial when compared to the effect observed with the highest concentration of PES used in the experiments. The results indicate that while molecular chaperone activity of stress inducible Hsp70 is critical for intracellular multiplication of *T. gondii*, there could be other intracellular targets of small molecule inhibitor PES which

abrogates multiplication of *T. gondii*. Notably, the attenuating effect of PES on intracellular parasite propagation remains even after downregulation of its target Hsp70 using specific siRNA further supporting the possibility of other intracellular targets of PES (**Fig. 6C**).

3.6 Virtual docking suggests that PES is able to bind within substrate binding region of TgHsp70.

We found that that TgHsp70 shares nearly 74% identity with the stress inducible mammalian counterpart (**SI Fig S3**). We examined the region implicated for PES binding in stress inducible Hsp70 **[50]** and found a high degree of homology in this region in the TgHsp70 counterpart. Therefore, we wanted to determine if TgHsp70 is also a molecular target of this well characterized small molecule inhibitor. To test this hypothesis, we modelled the TgHsp70 protein structure (**Fig. 7A**) using two best resolution structures of human-Hsp70 as templates, the first one was the ATP binding region, 3ATV, with a resolution of 1.58 Å, which covered residues 1-385 and the second one ,4PO2 at 2Å resolution, which covered residues 386 to 615 of the target sequence. For GGMP repeats (residues 620-674), which are present in TgHsp70 and is absent in its human counterparts, the best scoring I-TASSER model was used along with the above stated templates. A second model of TgHsp70 was generated without the residues 620-674 in order to understand the role of the GGMP repeats (**SI Fig S4 A**) by comparing the PES binding to TgHsp70 with and without this region. In addition, another closely related Hsp70 homologue present in *T. gondii*, (TgBiP), was considered for comparison (**SI Fig S4 B**). Structure of TgBiP was modelled from the human BiP protein (PDB-ID 6ASY) and the C-terminal 28 residues were modelled using I-

TASSER server. Although, the experimental structures of HsHsp70 and HsHsc70, were available in parts, the stretches of C-terminal regions were not solved for them. These terminal residue stretches were also modelled using the I-TASSER server for a fair comparison of their interactions with PES. Energy minimization of the structures in TIP3P water box was performed in order to relax the protein structures in realistic aqueous environment. The PROCHECK analyses showed that all the structures were of good stereochemical quality as expressed by the G-factors and percentage of residues in the most favoured regions of the Ramachandran Plot (**SI Fig S5**). All the structures showed good G-factors, which is a measure of unusualness in a structure and more than 90% residues of each structure were in the most favoured region of Ramachandran plot (**SI Fig S5 and Table S1**).

We used the predicted structures for docking analyses with PES ligand (**Fig. 7B**). In order to confirm the most favourable binding site of PES in TgHsp70 through computational modelling, PES was blindly docked to three different grids, generated at three different regions viz. 1-383 or the ATP binding domain, 384-510 (first part of the substrate binding domain) and 545-674 (C-terminal substrate binding domain). The best energy poses of PES bound to each region, along with their XP docking scores and binding free energies (ΔG_{bind}) were worked out **(Fig. 7C)**. The comparison of these scores at different regions shows that, the C-terminal domain (residues 545-674) is the most favourable site for PES binding. We tried to verify the role of the GGMP repeats in PES binding by comparing the docking scores, interactions and binding free energies of PES with TgHsp70, with and without GGMP repeats **(Fig. 7D)**. In the TgHsp70 model without GGMP repeats, -NH2 group of PES makes H-bond interactions with the

main chain oxygen of L612 and the sidechain oxygen of Q614 [Fig. 7D]. The aromatic ring of PES also makes aromatic H-bonds with the main chain oxygen of I608 and sidechain of D568 (**Fig. 7D**). When bound to the C-terminal domain TgHsp70 with the GGMP repeats, PES shows best docking score of -5.83 and the MMGBSA ΔG_{bind} significantly improves to -43.76 kcal/mol (**Fig. 7D**). Interestingly, the binding cavity remains the same, however, PES changes its orientation by 90 degrees anticlockwise rotation and the -NH2 group of PES makes H-bonds with the side chain carboxylic oxygens of S655, E567 and D568, while the terminal oxygen atoms attached to the S atom of PES make H-Bonds with K611 (**Fig. 7D and SI Fig S6A**). The aromatic ring makes aromatic H-bonds with the main chain oxygens of K563 and G616 residue (Fig. **7D and SI Fig S6A**). This comparison shows that although the GGMP repeats are not directly involved in ligand binding, they have a significant influence on the orientation of the ligand in the binding site and the overall free energy of binding.

In addition, *PES was docked to HsHsp70, TgBiP and HsHsc70 proteins followed by implicit water dynamics in order to compare its binding affinities with these homologous proteins.* Docking of PES with the HsHsp70 confirm that the predictions generated by the docking program used in our studies were in agreement with those generated in previous studies. *The docking score and* ΔG_{bind} *of PES with HsHsp70 were also calculated and were compared to those of* TgHsp70 (**Fig. 7E**). The *-NH2 group of PES makes H-bond interactions with conserved residues L610 (main chain oxygen) and Q612 residues of HsHsp70 and its aromatic ring makes aromatic H-bond with the side chain of E556 (**Fig. 7E and SI Fig S6B)**.

Docking score of PES with TgHsp70 was also found to be better than those with HsHsc70 (-4.56) and TgBiP (-5.32) ***(SI Fig S7 A, B)****. PES makes H-bond interactions with the K561 and L610 of HsHsc70 and aromatic H-bond with I607, which are conserved residues among these four proteins (****SI Fig S7 A****). Similarly, PES makes H-bond interactions with K604 and E607 of TgBiP (****SI Fig S7 B****). MMGBSA binding energy of PES complexed with these proteins over 20 ns of molecular dynamics was compared. The MMGBSA binding energy of 10 structures sampled from each 20ns MD trajectory shows that PES demonstrates best binding with TgHsp70 in presence of GGMP repeats as compared to all the other homologues (****SI Fig S8****). It is interesting to note that due to presence of the GGMP repeats, the binding pattern and orientation of PES is strikingly altered.*

3.7 T. gondii homologue of Hsp70 is also a target of PES

Encouraged by our computational findings, we proceeded to validate the observations experimentally. Towards this end, we first checked if PES directly binds to recombinant purified TgHsp70 using Biotinylated PES (B-PES) in a pull down assay as described previously **[23]**. Full length recombinant purified His tagged TgHsp70 was used in this assay (**Fig. 8A**). B-PES was able to pull down TgHsp70 consistently (**Fig. 8B)**. This interaction was efficiently competed away with excess of un-biotinylated PES providing evidence for its specificity (**Fig. 8B**). We performed the same experiment using recombinant human Hsp70 and Hsc70 as positive controls (**Fig. 8B)**. Both these proteins showed specific interaction (**Fig. 8B)**, consistent with the previous study **[24]**. In addition, B-PES was also found to interact with a related *Toxoplasma* Hsp70

homolog TgBiP recombinant protein (**Fig. 8B**) consistent with the computational findings.

To further map the region of TgHsp70 involved in the interaction with PES, we created several deletion constructs which included amino- terminal domain (1-383 aa), domain including the C-terminal substrate binding domain (193-674 aa), domain excluding the potential substrate binding region (193-545) and the potential substrate binding region (439-674) (**Fig. 8C**). Circular dichroism spectroscopy indicated that all the recombinant TgHsp70 proteins were properly folded (**SI Fig S9**). We found that biotinylated PES (B-PES) could efficiently pull down the carboxy-terminal domain containing the substrate binding region (193-674) but not the amino terminal domain (1-383) or the carboxy terminal domain devoid of the latter part of substrate binding region (**Fig. 8D**). The protein carrying the substrate binding region (439-674) was also consistently pulled down by B-PES (**Fig. 8D**). The positive interaction with both the domains were competed away by untagged PES (**Fig. 8 E, F**). Together the data indicate that the C-terminal substrate binding region is critical for PES interaction which is in line with the findings for the mammalian counterpart of this protein. Summary of the independent B-PES binding experiments implicates the C-terminal region of TgHsp70 as the probable binding site of PES (**Fig. 8G**). We further demonstrate that endogenous TgHsp70 was effectively pulled out of *T. gondii* infected cell lysates using biotinylated PES while another highly expressed *T. gondii* protein SAG1 was not (**Fig**. **9A**) , attesting to the specificity of PES towards TgHsp70 and further validating the results obtained using recombinant TgHsp70 protein. In order to further establish specificity of PES targeting in *T. gondii* infected cells, we performed

western blot analyses using streptavidin-HRP antibody which would identify only the proteins which interact with Biotin –PES. In cellular lysates treated with increasing concentrations (2 and 5 µM) of Biotin-PES, streptavidin-HRP showed two signals corresponding to human and TgHsp70 respectively under the given experimental conditions (**Fig. 9B)**. To examine the functional effect of PES targeting of TgHsp70 in *T. gondii* infected cells, we tested if PES treatment alters its interaction with Sis1-like J-domain protein (TgHsp40) which has been demonstrated previously to be a cytosolic co-chaperone associated with TgHsp70 [**51**]. We found that PES treatment of *T. gondii* infected cells abrogates this functional interaction as demonstrated by co-immunoprecipitation experiment (**Fig. 9C)**.

4. Discussion

Here we report that small molecule inhibitor 2-phenylethynesulfonamide (PES) targeting stress inducible Hsp70 in host cells affects its molecular chaperone activity and leads to dysregulation of host autophagy resulting in attenuation of intracellular parasite multiplication. In addition, we demonstrate that the same inhibitor also targets parasite Hsp70 homologue bringing to the fore a possibility of dual targeting of host and parasite protein(s) using a small molecule impairing parasite multiplication. Thus far, essential role of Hsp70 was demonstrated in the context of viral replication **[52, 53]**. This study provides considerable evidence for the first time that Hsp70 activity is critical for *T. gondii* intracellular multiplication and upregulation of its expression upon infection is a pro-survival measure by the parasite.

It is well demonstrated that lowering Hsp70 levels in cells is relatively safe and do not manifest any pathological changes in mice where Hsp70 is knocked out **[54, 55]**.

Therefore, a range of Hsp70 modulators have been studied, mostly in the context of tumor biology **[56]**, in particular, several small molecule inhibitors are now available which could aid to assess the potential of Hsp70 as a therapeutic target **[13, 22]**. PES is one such inhibitor whose selectivity towards stress inducible Hsp70 has been well documented in the context of tumor biology **[23, 24, 50]**. Moreover, the effectiveness of PES has been recently demonstrated in abrogating viral multiplication **[57]**. In this study, PES has uncovered the importance of Hsp70 mediated protein quality control for *T. gondii* propagation. Importantly**,** anti-*T. gondii* activity of PES was observed in a range of mammalian cells of both human and murine origin indicating the potential of molecular chaperones like Hsp70 as a valuable therapeutic target and a possible conserved mechanism of action of PES.

Coordinated action of Hsp70 along with several of its co-chaperones including Hsp40 and CHIP is critical for conformational maturation, stability and overall activity of a wide variety of proteins including signalling molecules. Consequently, any interference with Hsp70 activity may affect multiple pathways critical for intracellular parasite survival. Addition of PES to infected cells interferes with the molecular chaperone activity of Hsp70 by abrogating its interaction with co-chaperones which may potentially lead to proteotoxic stress and consequently overwhelm the autophagy lysosomal pathway resulting in its impairment. The observations concur with previous findings that parasite utilizes host cell autophagy machinery to promote its own growth **[58]**, consequently, any block in that pathway would result in impairment of its propagation. While growth restrictive role of autophagy in case of intracellular pathogens have been demonstrated previously **[59, 60]** observations in this study are in line with the observations which demonstrates *T. gondii's* dependence on host cell

autophagy to promote its survival by exploiting its nutritive function **[58]**. In fact, the current study highlights pro-pathogen role of autophagy in case of *Toxoplasma* which was thus far well elucidated for RNA viruses **[61, 62]**.

While utilizing host cell autophagic machinery for its benefit, *T. gondii* also ensures that it avoids getting targeted by this machinery itself by activating appropriate host cell signalling pathways **[28]**. It is interesting to note here that EGFR and AKT which are known modulators of autophagy in *T. gondii* infected cells **[28]**, are important client proteins, which have been reported to be affected by the PES-mediated inhibition of Hsp70 leading to their misfolding and aggregation **[24]**. Our findings are consistent with this study as we find progressive accumulation of these proteins in detergent insoluble fractions in PES treated infected cells. Consequently, deficiency in activity of these proteins may also in turn affect parasite multiplication. Further, Akt activation is also linked to inhibition of apoptosis of *T. gondii*-infected cells **[63],** which may also be affected upon Hsp70 modulation. Taken together, it appears likely that any interference with Hsp70 activity may affect overall parasite progression through multiple pathways.

Hsp70 has been demonstrated to be important for lysosomal membrane stability **[64, 65]**. PES induced lysosomal membrane destabilization may have contributed to parasite attenuation directly and may also explain the autophagic block that was observed due to disruption of lysosomal pH as demonstrated previously **[66]**. Subcellular localization of host cell autophagy apparatus, in particular, the clustering of host cell lysosome around the parasitophorous vacuole **[67]** may also be noted in this context and may help explain the observations in our study. Moreover, abrogation of the autophagy-lysosome pathway may also compromise the function of the proteasome

system, as the cellular abundance of misfolded or aggregated proteins exceeds the capacity of the cells to process these molecules **[68].**It requires to be investigated if proteosomal pathway is also compromised upon PES inhibition of Hsp70 in *T. gondii* infected cells.

It has been demonstrated previously that Hsp70 inhibitor PES binds to the substrate-binding domain of Hsp70, and requires the C-terminal helical 'lid' of this protein (amino acids 573-616) in order to bind **[50]**. Sequence alignment data indicated that the *Toxoplasma* homologue of Hsp70 could also be a potential intracellular target of PES. Structural modelling of TgHsp70 followed by molecular docking analysis revealed that *PES binds to* TgHsp70 with a better docking score and binding energy as compared to human homologues, HsHsp70, HsHsc70 and closely related *T. gondii* homolog TgBiP evaluated in this study. *The C-terminal domain (residues 545-674) emerged as the most favourable site for PES binding in TgHsp70. Importantly, the modelling data indicated that the GGMP repeats (residues 620-674), which are unique to TgHsp70 and are absent in human and T. gondii homologues, may have critical bearing on PES binding. In fact, presence of GGMP repeats at the C-terminal region significantly enhances PES binding as compared to the other human and T. gondii homologous proteins, which lack this repeat. While the residues in this repeat region were not found to be directly interacting with PES but contribute to alter the conformations of binding site residues thereby ensuring optimal binding affinity with TgHsp70.* The PES binding assay using recombinant TgHsp70 deletion variant proteins, highlighted the crucial role of the C-terminal domain for PES binding. *Thus, the*

computational findings correlate with the observations from experimental binding assay.

We found that TgHsp70 is indeed an intracellular target in *T. gondii* infected cells and PES treatment abrogates its interaction with co-chaperone TgHsp40. While it cannot be ruled out that PES may have multiple targets given the conservation of Hsp70 family of proteins in both host and parasite, the current study provides evidence of PES targeting of host stress inducible Hsp70 and its parasite counterpart TgHsp70. Therefore, it is reasonable to speculate that it may have significantly contributed to the PES mediated parasite attenuation under the given experimental conditions. Nonetheless, since PES mediated parasite attenuation is yet to be fully understood, future biophysical studies should provide much needed insights into the molecular mechanisms by which PES interferes with Hsp70 actions and the consequence of PES binding to TgHsp70 and other cellular targets in *T. gondii* infected cells.

Conflict of interest

The authors declare no conflicts of interest.

Competing interests

The authors declare no competing financial interests.

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Author contributions

PM: Conceptualization, Data Curation, Funding Acquisition, Project Administration, Resources, Investigation, Methodology, Formal Analysis, Visualization, Writing Original Draft, Writing-Review & Editing. ASD: Investigation, Methodology, Resources, Data Curation. CC: Investigation, Methodology, Formal Analysis.

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Figure legends

Fig. 1. Upregulation in expression of stress inducible Hsp70 in T. gondii infected host cells. **(A)** Human retinal pigment epithelial (RPE) cells were infected with *T. gondii* tachyzoites and infected cells were checked for the expression of Hsp70 (~72KDa), Hsc70 (~70 KDa) and Hsp90 (~90 KDa) using western blot analysis at indicated time points post-infection (0, 2, 6 and 18 hours). **(B)** Similarly, Human brain microvascular endothelial cells (EC) infected with *T. gondii* tachyzoites were monitored for the expression of Hsp70, Hsc70 and Hsp90 using western blot analysis at indicated time points post-infection. **(C)** Similarly, mouse microglia cell line (MG) infected with

T. gondii tachyzoites were assessed for the expression of indicated proteins using western blot analysis. Relative density of Hsp70 signal was obtained by normalisation relative to the uninfected control samples (0 min time point) in each case. Beta-Tubulin was used as a loading control in all the experiments. In each case, data shown are representative of three independent experiments. Densitometry data represent mean ±SEM of three independent experiments.

Fig. 2. Small molecule inhibitor PES which selectively targets Hsp70 attenuates intracellular parasite progression. **(A)** RPE cells were infected with *T. gondii* tachyzoites followed by incubation without or with PES using indicated concentrations beginning at 6 hours post tachyzoites infection. Monolayers were examined by light microscope at 24 hours following treatment with PES to determine parasites per vacuole. **(B)** Similarly, EC cells infected with *T. gondii* tachyzoites were treated without or with PES as indicated. Monolayers were assessed to determine parasites per vacuole by light microscope at 24 hours following treatment. **(C)** Similarly, *T. gondii* infected MG cells treated with PES were examined by light microscope at 24 hours post treatment to determine number of parasites per vacuole. Results are shown as the mean ± SD and are representative of 3 independent experiments (*P≤0.05, **P≤ 0.01, and ***P≤ 0.001; two-way ANOVA with Bonferroni's post-test). **(D)** Biotin-PES (B-PES) was used to capture PES interacting proteins from lysates prepared from RPE cells infected with *T. gondii* (6-8 hours post-infection), which were then pulled out using Avidin resins. PES interacting proteins eluted from the resin were subjected to western blot analysis using indicated antibodies. **(E)** Similarly, B-PES was used to capture PES interacting proteins from lysates prepared from *T. gondii* infected EC cells followed by

immunoblotting using indicated antibodies. **(F)** B-PES was used to examine PES interacting proteins from lysates prepared from *T. gondii* infected MG cells using western blot analysis as indicated. In each case, results shown are representative of three independent experiments.

Fig. 3. PES interferes with molecular chaperone activity of Hsp70. **(A)** RPE cells infected with *T. gondii* tachyzoites were either treated without or with 5 µM PES for 24 hours, beginning at 6 hours post-infection, followed by immunoprecipitation using Hsp70 antibody and immunoblot analysis using indicated antibodies to examine interaction with co-chaperones. **(B)** Similarly *T. gondii* infected EC cells treated with or without PES, were subjected to immunoprecipitation using Hsp70 antibody followed by immunoblot analysis as indicated. **(C)** MG cells infected with *T. gondii* and treated with or without PES were subjected to immunoprecipitation and immunoblot analysis as indicated. In each case, results shown are representative of three independent experiments. **(D)** RPE, EC and MG cells infected with *T. gondii* tachyzoites were treated with 5 µM PES for 24 hours in absence or presence of CHX (25 µg/ ml) for last 12 hours. Monolayers were examined by light microscope to determine parasites per vacuole. Results are shown as the mean ± SD and are representative of 3 independent experiments. (**P≤ 0.01, and ***P≤0.001; two-way ANOVA with Bonferroni's post-test). (E) RPE cells were treated without or with increasing concentration of PES as indicated. Cells were harvested and fractionated into detergent soluble and detergent in-soluble preparations, and assayed by western blot for the proteins indicated.

Fig. 4. PES effect is mediated through autophagy-lysosomal pathway. (A) Uninfected RPE cells or RPE cells infected with *T. gondii* tachyzoites were either

treated without or with 5 µM PES for 24 hours and examined for the expression of autophagy marker LC3II using western blot analysis. **(B)** Similarly, uninfected or *T. gondii* infected EC cells treated with PES were assessed for the expression of autophagy marker LC3II as indicated. **(C)** *T. gondii* infected MG cells treated with PES was examined using immunoblotting analysis as indicated. Beta- Tubulin was used as a loading control in each case. Relative density of LC3II signal was obtained by normalisation relative to the uninfected and untreated control samples in each case. **(D)** RPE cells infected with *T. gondii* tachyzoites were either treated without or with 5 µM PES for 24 hours and subjected to immunofluorescence analysis using indicated antibodies to examine accumulation of LC3II protein in the infected cells. DAPI was used as nuclear stain. The images were captured using Leica confocal microscope. Scale bar: 5µm. **(E)** RPE, EC and MG cells infected with *T. gondii* parasites were treated without or with PES for 24 hours in absence or presence of lysosomal protease inhibitor pepstatin (10 µM). Monolayers were examined by light microscope at the end of the treatment. **(F)** RPE, EC and MG cells infected with *T. gondii* parasites were treated without or with PES for 24 hours in absence or presence of pan-caspase inhibitor Z-VAD-FMK (10 µM) and examined by light microscope at the end of the treatment. Results are shown as the mean ± SD and are representative of 3 independent experiments (**P≤ 0.01, and ***P≤ 0.001 and ns- not significant; two-way ANOVA with Bonferroni's post-test). RH is an indication of *Toxoplasma* addition.

Fig. 5. PES treatment leads to autophagic dysregulation in *T. gondii* infected host cells. **(A)** Uninfected RPE cells or RPE cells infected with *T. gondii* tachyzoites were either treated without or with 5 µM PES for 24 hours and examined for the expression

of P62, a determinant of autophagic flux, using western blot analysis. **(B)** Similarly EC cells infected with *T. gondii* tachyzoites were treated with PES and assessed for the expression of P62 using western blot analysis. **(C)** T. *gondii* infected MG cells treated with PES was similarly examined for the expression of P62 as indicated. Beta- Tubulin was used as a loading control in each case. Relative density of P62 signal was obtained by normalisation relative to the uninfected and untreated control samples in each case **(D)** RPE cells infected with *T. gondii* tachyzoites were either treated without or with 5 µM PES for 24 hours and subjected to immunofluorescence analysis using indicated antibodies to examine accumulation of P62 protein in infected cells. DAPI was used as nuclear stain. The images were captured using Leica confocal microscope. Scale bar: 5µm. RH is an indication of *Toxoplasma* addition.

Fig. 6. siRNA mediated knock down of Hsp70 expression impedes parasite progression. **(A)** RPE cells were transfected with control siRNA or Hsp70 siRNA. Cells were then infected with *T. gondii* tachyzoites 48 hours after transfection. Monolayers were examined microscopically 30 hours post challenge. Results are shown as the mean ± SD and are representative of 3 independent experiments (***P≤ 0.001 and ns- not significant; two-way ANOVA with Bonferroni's post-test). **(B)** RPE cells were infected with *T. gondii* tachyzoites 48 hours following siRNA transfection. Cell lysates were prepared and examined for the expression of indicated proteins using western blot analysis. Beta-Tubulin was used as a loading control. **(C)** siRNA treated RPE cells infected with *T. gondii* tachyzoites were subjected to PES treatment for 24 hours (starting 6 hours post infection). Monolayers were examined by light microscope at the end of the treatment. Results are shown as the mean ± SD and are representative

of 3 independent experiments (*P≤0.05, **P≤ 0.01, and ***P≤ 0.001 and ns- not significant; two-way ANOVA with Bonferroni's post-test). RH is an indication of *Toxoplasma* addition.

Fig.7. *In silico* docking analysis of PES to TgHsp70 structural model.

(A) Structural alignment of the model of TgHsp70 (orange) with HsHsp70 template (yellow). **(B)** Chemical representation of PES. **(C)** Best energy poses of PES bound to different regions of TgHsp70 model along with the respective docking scores and binding energies. **(D)** PES (green) interaction with C terminal region of TgHsp70 in absence (blue) and presence (orange) of the unique GGMP repeats along with the respective docking scores and binding energies . **(E)** PES interaction with C terminal region of human Hsp70 (yellow) as indicated.

Fig. 8. TgHsp70 binds small molecule inhibitor PES. (A) Coomassie stained gel showing purified recombinant histidine tagged full length TgHsp70 protein. **(B)** Pull-down assay of His-tagged recombinant full length TgHsp70 with biotinylated PES (B-PES). Purified TgHsp70 protein was incubated with B-PES, in the absence or the presence of untagged PES. Pull down using Avidin followed by western blot analysis with anti-His antibody. Similarly, pull down assay with B-PES was performed using recombinant His-tagged proteins, human Hsp70 (bottom left panel), human Hsc70 (top right panel) and TgBiP (bottom right panel). Input is shown in the bottom panel in each case. **(C)** Coomassie stained gels showing purified recombinant histidine tagged deletion variants of TgHsp70 protein with indicated amino acid co-ordinates. **(D)** Pull down assay of indicated deletion variants of His –tagged TgHsp70 recombinant proteins performed using B-PES and precipitation using Avidin followed by western

blot analysis with anti-His antibody to map interaction domain. Input is shown in the bottom panel. **(E)** Pull-down assay of His-tagged recombinant TgHsp70 domain (193-674 aa) with B-PES as indicated. **(F)** B-PES Pull down of recombinant TgHsp70 domain (439-674 aa), in absence or presence of untagged PES, to demonstrate specificity. **(G)** TgHsp70 domain organization and summary of the results of multiple independent B-PES binding assays performed as in B, D and E.

Fig. 9 PES specifically abrogates TgHsp70 function in *T. gondii* infected cells. (**A**) Cell lysates prepared from RPE cells infected with *T. gondii* tachyzoites were incubated with B-PES followed by pull down using Avidin. B-PES associated proteins eluted from Avidin resins were subjected to western blot analysis using indicated antibodies. (**B**) Proteins bound to biotynilated PES in *T. gondii* infected cell lysate were identified by virtue of their biotin tag by streptavidin– horseradish peroxidase in western blot analysis. B-PES was used to capture PES interacting proteins from lysates prepared from *T. gondii* infected cells under the same experimental condition followed by western blot analysis as indicated. 2µM (+) and 5µM(++) of B-PES was used in each case.(**C**) RPE cells infected with *T. gondii* tachyzoites were either treated without or with 5 µM PES for 24 hours, beginning at 6 hours post-infection, followed by immunoprecipitation using TgHsp70 antibody and immunoblot analysis using indicated antibodies to examine interaction with co-chaperone TgHsp40.

Author contributions

PM: Conceptualization, Data Curation, Funding Acquisition, Project Administration, Resources, Investigation, Methodology, Formal Analysis, Visualization, Writing Original Draft, Writing-Review & Editing. ASD: Investigation, Methodology, Resources, Data Curation. CC: Investigation, Methodology, Formal Analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

- *T. gondii* infection upregulates expression of host stress inducible Hsp70
- Hsp70 selective inhibitor PES attenuates intracellular *T. gondii* multiplication.
- PES disrupts molecular chaperone function and impairs cellular autophagy
- Molecular docking analysis reveals TgHsp70 as another promising target of PES
- Biotinylated-PES experimentally confirms its interaction with TgHsp70
- Simultaneous targeting of host and parasite protein(s) as a therapeutic possibility

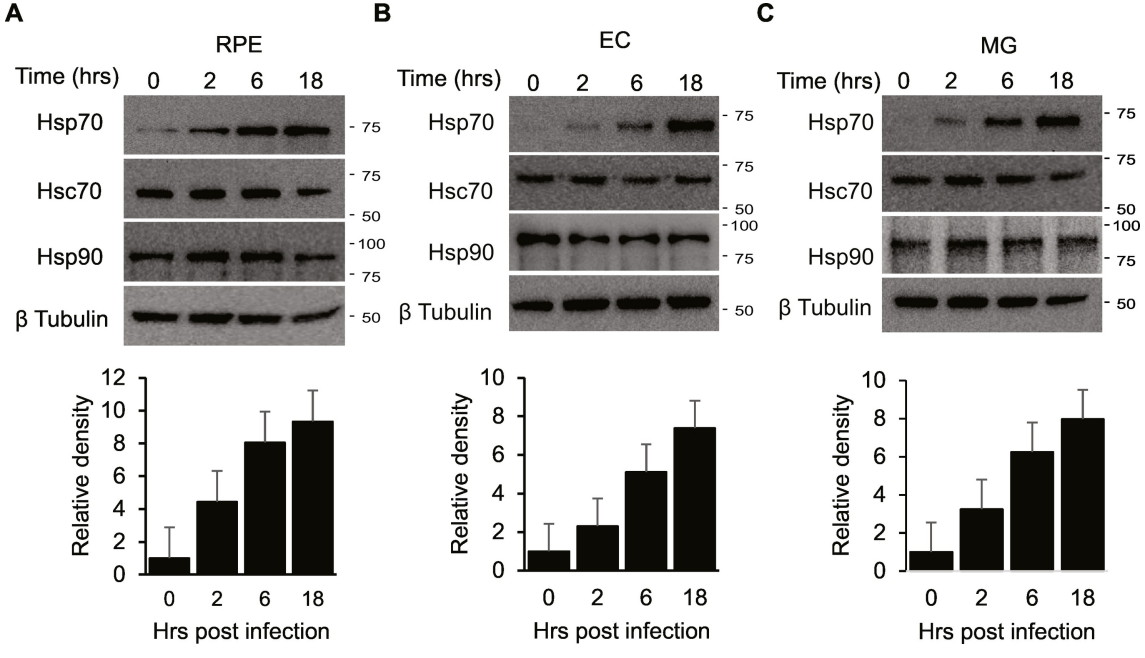

A
RPE
Time (hrs) 0 2 6 18
Hsp70
Hsc70
Hsp90
β Tubulin
75
75
50
100
75
50
Relative density
Hrs post infection
B
EC
Time (hrs) 0 2 6 18
Hsp70
Hsc70
Hsp90
β Tubulin
75
75
50
100
75
50
Relative density
Hrs post infection
C
MG
Time (hrs) 0 2 6 18
Hsp70
Hsc70
Hsp90
β Tubulin
75
75
50
100
75
50
Relative density
Hrs post infection

Figure 1

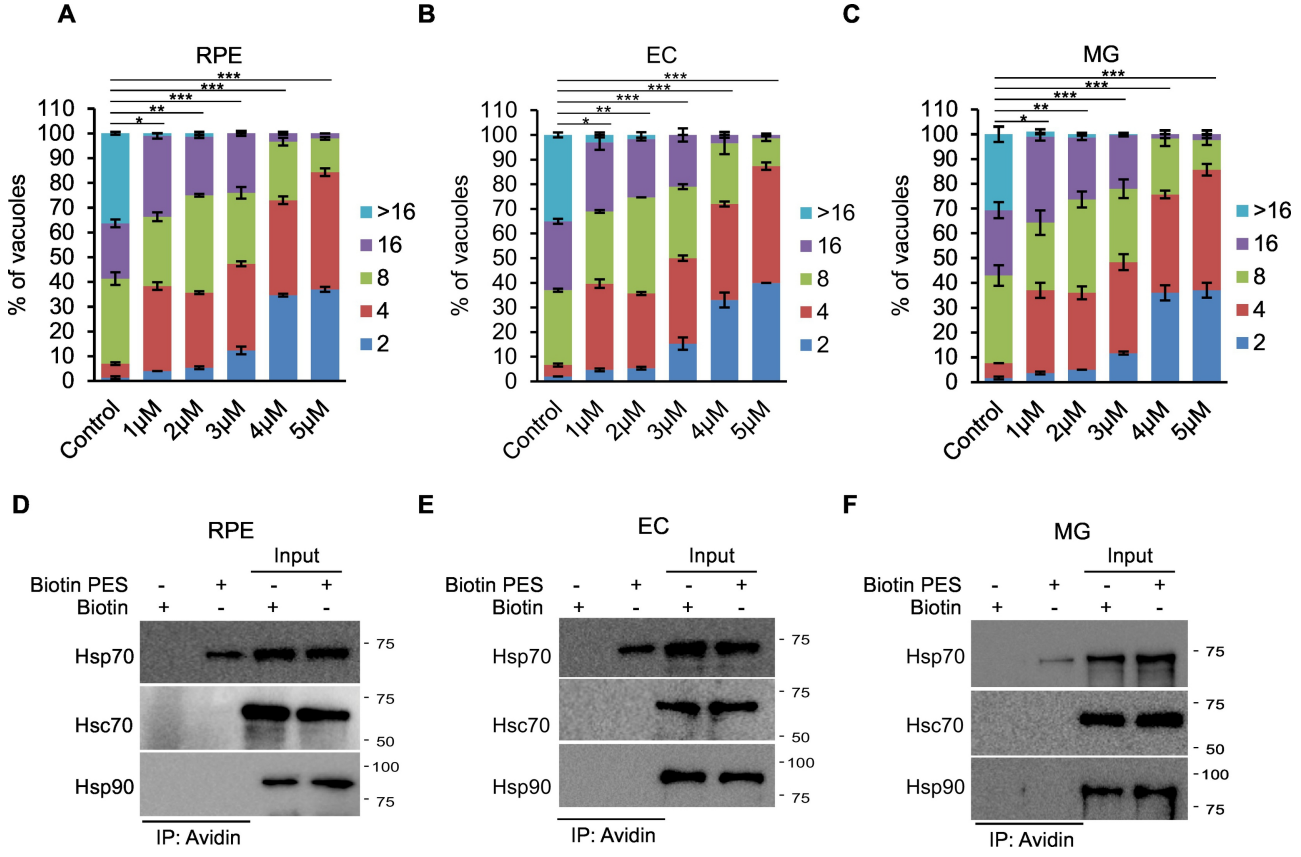
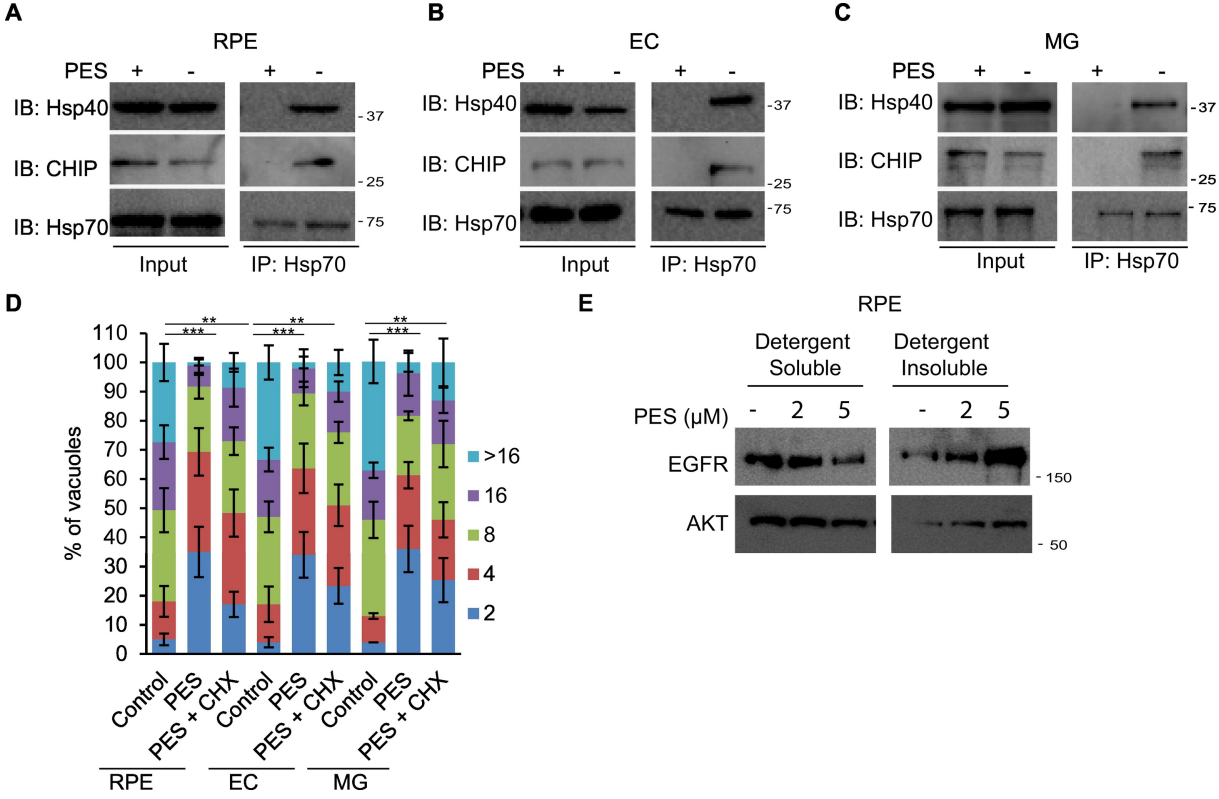

A
RPE
B
EC
C
MG
% of vacuoles
>16
16
8
4
2
Control
1μM
2μM
3μM
4μM
5μM
D
RPE
E
EC
F
MG
Input
Biotin PES
Biotin
Hsp70
Hsc70
Hsp90
IP: Avidin

Figure 2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RPE
PES + - + -
IB: Hsp40
IB: CHIP
IB: Hsp70
37
25
75
Input
IP: Hsp70
B
EC
PES + - + -
IB: Hsp40
IB: CHIP
IB: Hsp70
37
25
75
Input
IP: Hsp70
C
MG
PES + - + -
IB: Hsp40
IB: CHIP
IB: Hsp70
37
25
75
Input
IP: Hsp70
D
% of vacuoles
110
100
90
80
70
60
50
40
30
20
10
0
**
***
>16
16
8
4
2
Control
PES
PES + CHX
RPE
EC
MG
E
RPE
Detergent Soluble
Detergent Insoluble
PES (μM) - 2 5 - 2 5
EGFR
AKT
150
50

Figure 3

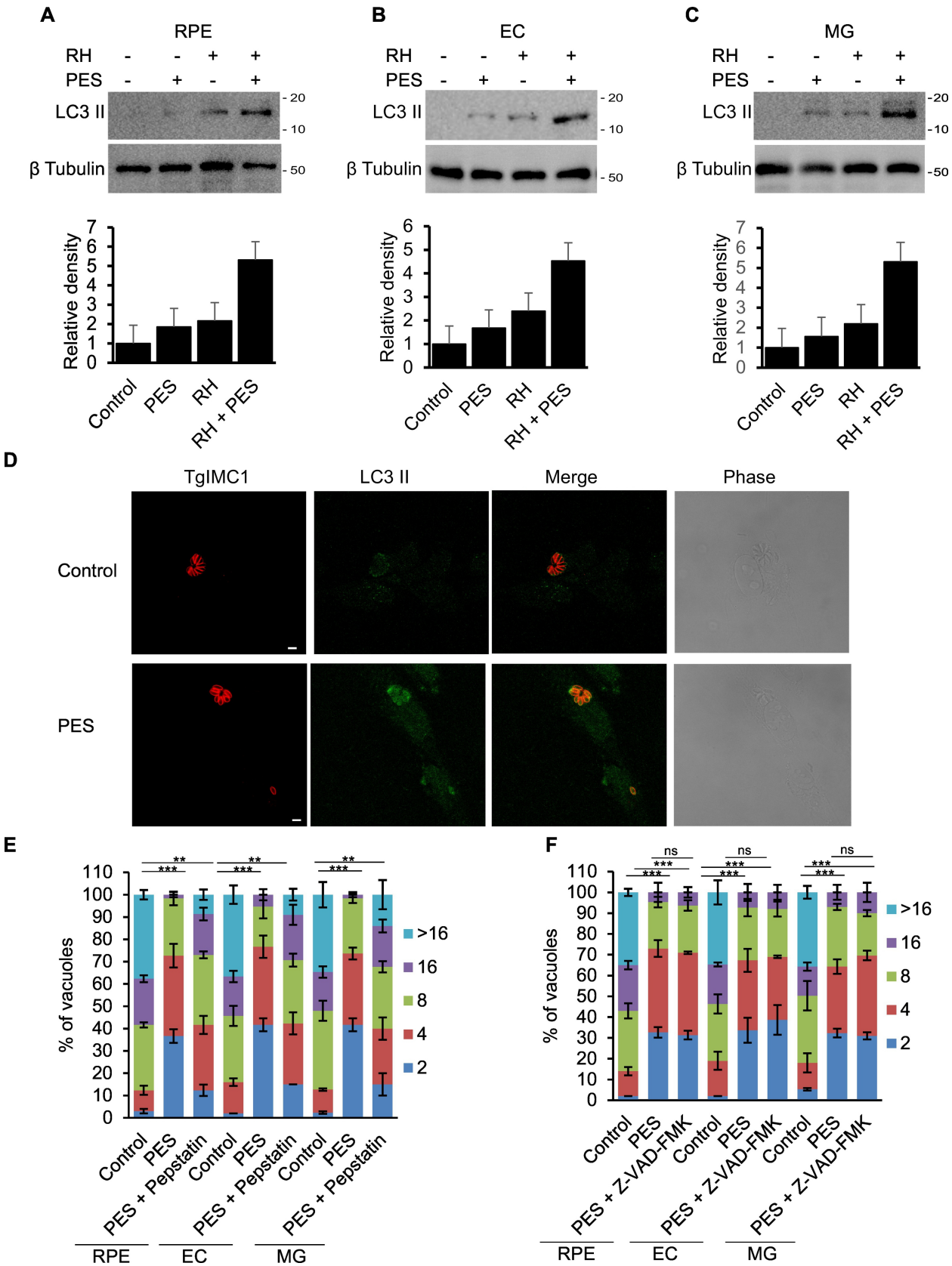
A
RPE
RH - - + +
PES - + - +
LC3 II
β Tubulin
20
10
50
Relative density
Control
PES
RH
RH + PES
B
EC
C
MG
D
TgIMC1
LC3 II
Merge
Phase
Control
PES
E
% of vacuoles
>16
16
8
4
2
Control
PES
PES + Pepstatin
RPE
EC
MG
F
ns
PES + Z-VAD-FMK

Figure 4

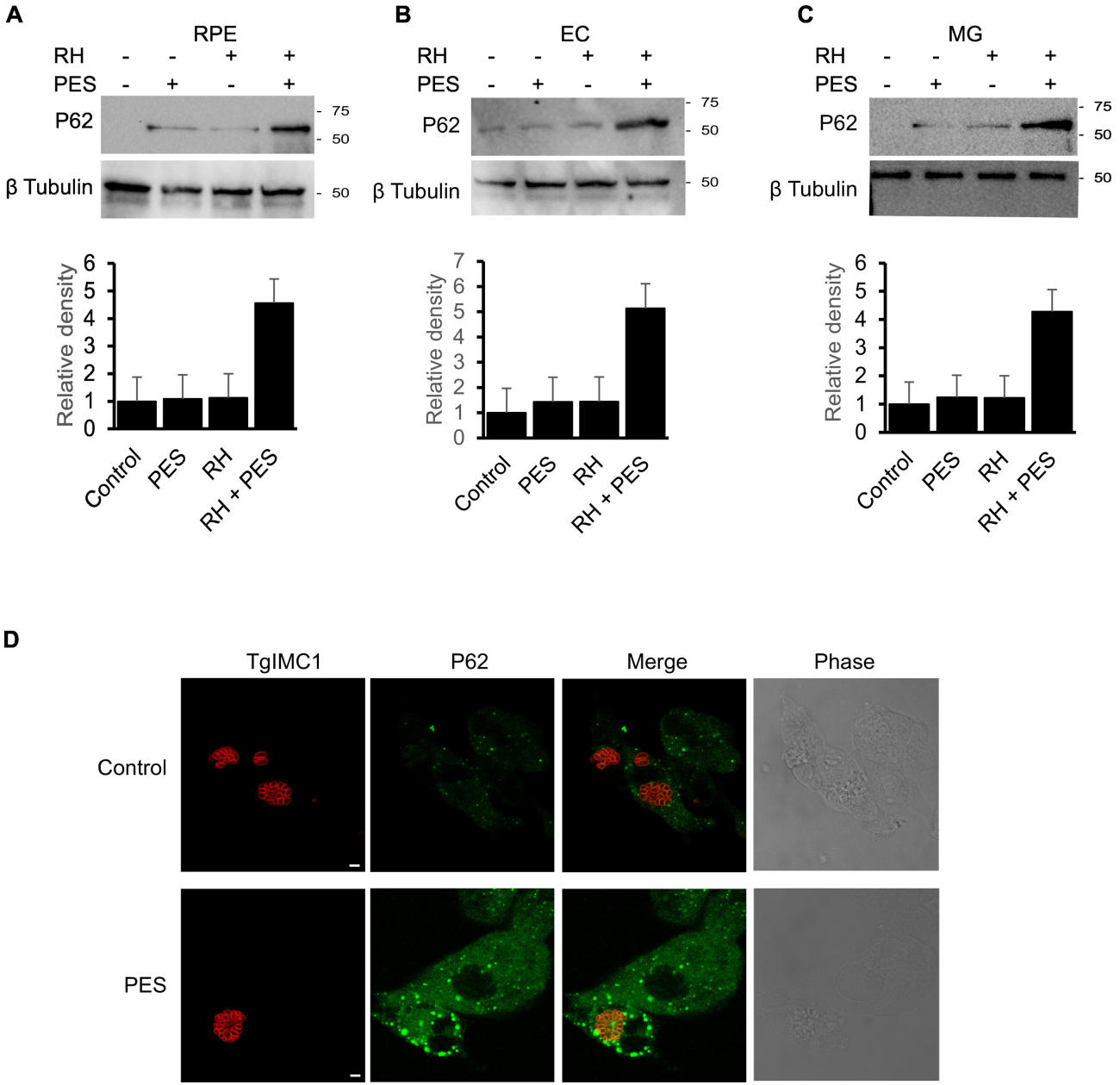
A
RPE
RH - - + +
PES - + - +
P62
β Tubulin
75
50
50
Relative density
Control
PES
RH
RH + PES
B
EC
C
MG
D
TgIMC1
P62
Merge
Phase
Control
PES

Figure 5

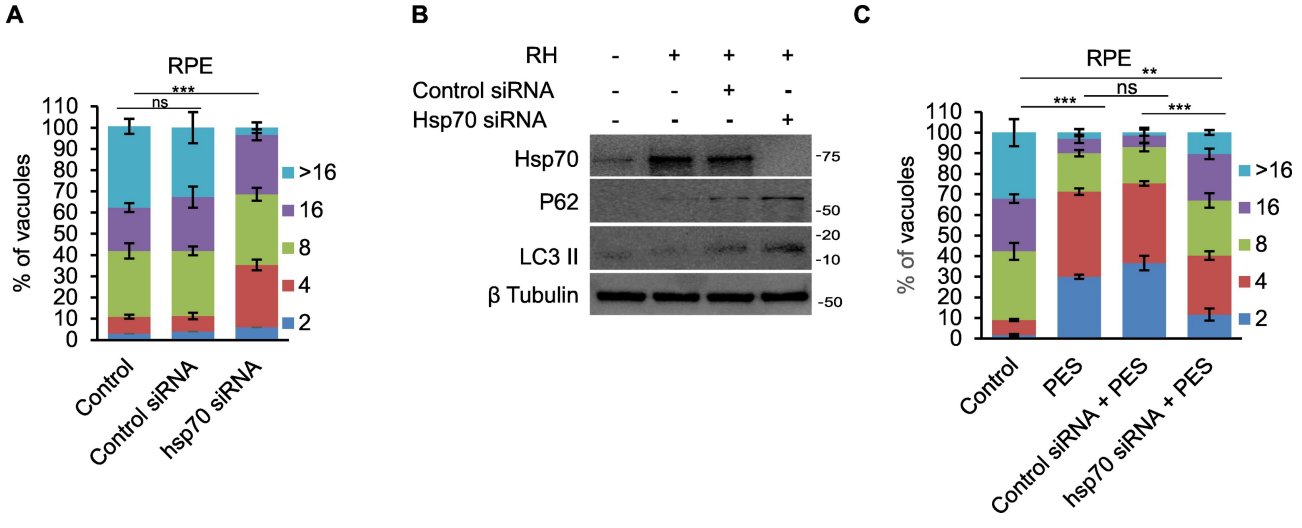
A
RPE
***
ns
% of vacuoles
110
100
90
80
70
60
50
40
30
20
10
0
>16
16
8
4
2
Control
Control siRNA
hsp70 siRNA
B
RH - + + +
Control siRNA - - + -
Hsp70 siRNA - - - +
Hsp70
P62
LC3 II
β Tubulin
-75
-50
-20
-10
-50
C
RPE
**
ns
***
***
% of vacuoles
>16
16
8
4
2
Control
PES
Control siRNA + PES
hsp70 siRNA + PES

Figure 6

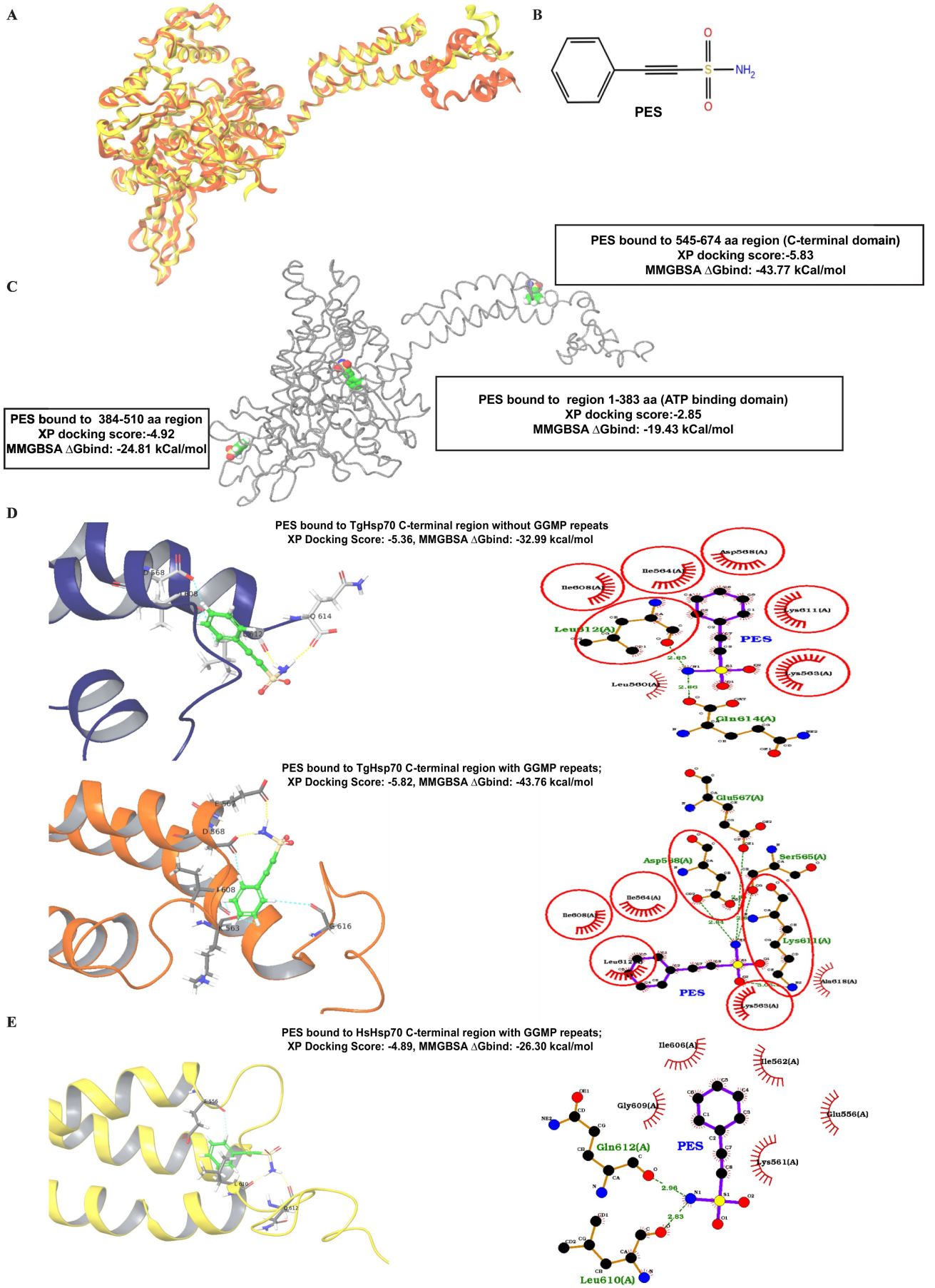
A
B
PES
PES bound to 545-674 aa region (C-terminal domain)
XP docking score:-5.83
MMGBSA ΔGbind: -43.77 kCal/mol
C
PES bound to 384-510 aa region
XP docking score:-4.92
MMGBSA ΔGbind: -24.81 kCal/mol
PES bound to region 1-383 aa (ATP binding domain)
XP docking score:-2.85
MMGBSA ΔGbind: -19.43 kCal/mol
D
PES bound to TgHsp70 C-terminal region without GGMP repeats
XP Docking Score: -5.36, MMGBSA ΔGbind: -32.99 kcal/mol
PES bound to TgHsp70 C-terminal region with GGMP repeats;
XP Docking Score: -5.82, MMGBSA ΔGbind: -43.76 kcal/mol
E
PES bound to HsHsp70 C-terminal region with GGMP repeats;
XP Docking Score: -4.89, MMGBSA ΔGbind: -26.30 kcal/mol

Figure 7

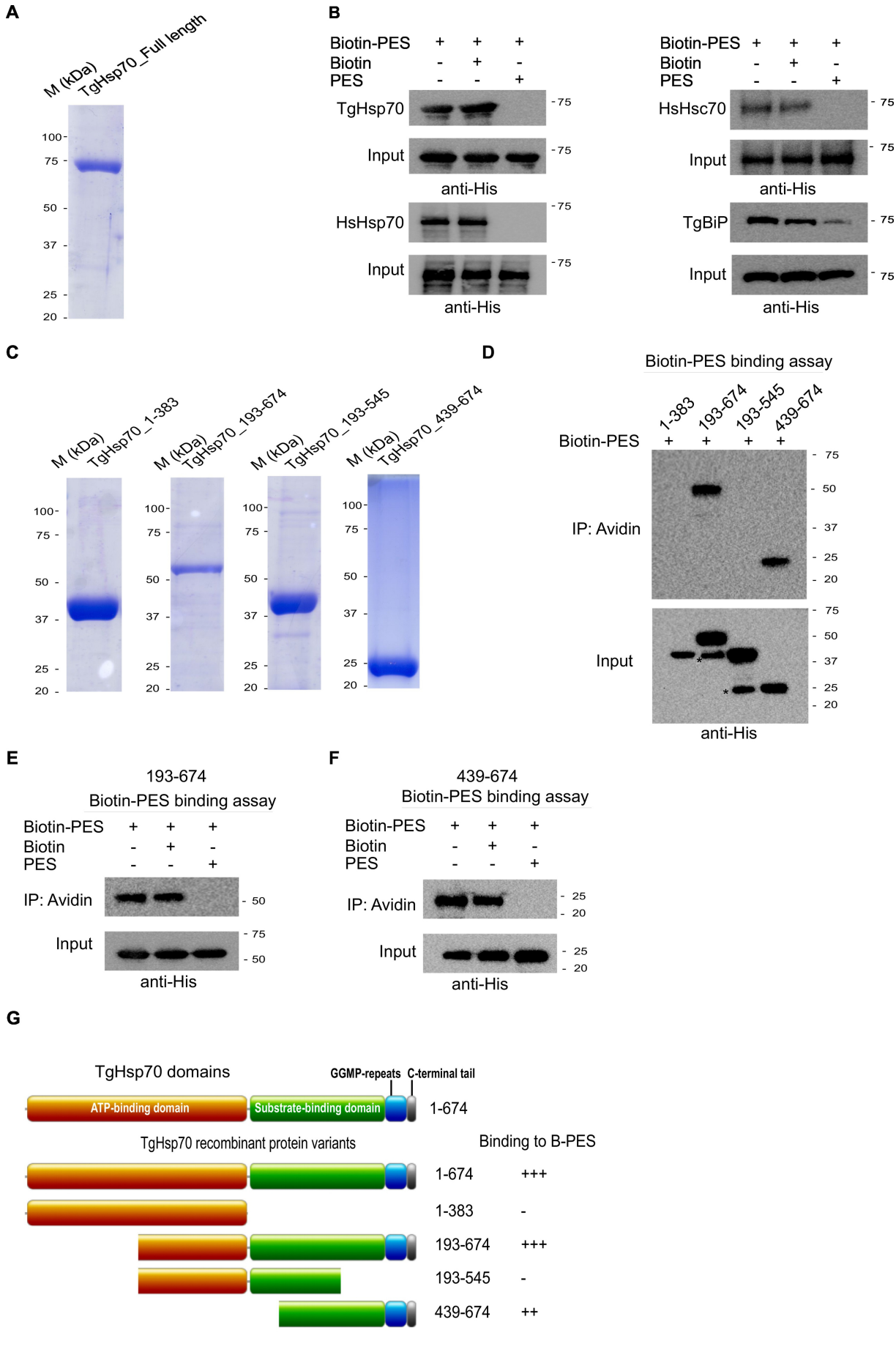
A
M (kDa)
TgHsp70_Full length
100
75
50
37
25
20
B
Biotin-PES + + +
Biotin - + -
PES - - +
TgHsp70
Input
anti-His
HsHsp70
Input
anti-His
HsHsc70
Input
anti-His
TgBiP
Input
anti-His
75
C
M (kDa)
TgHsp70_1-383
TgHsp70_193-674
TgHsp70_193-545
TgHsp70_439-674
D
Biotin-PES binding assay
1-383
193-674
193-545
439-674
Biotin-PES
IP: Avidin
Input
anti-His
E
193-674
Biotin-PES binding assay
Biotin-PES + + +
Biotin - + -
PES - - +
IP: Avidin
Input
anti-His
F
439-674
Biotin-PES binding assay
Biotin-PES + + +
Biotin - + -
PES - - +
IP: Avidin
Input
anti-His
G
TgHsp70 domains
GGMP-repeats
C-terminal tail
ATP-binding domain
Substrate-binding domain
1-674
TgHsp70 recombinant protein variants
Binding to B-PES
1-674 +++
1-383 -
193-674 +++
193-545 -
439-674 ++

Figure 8

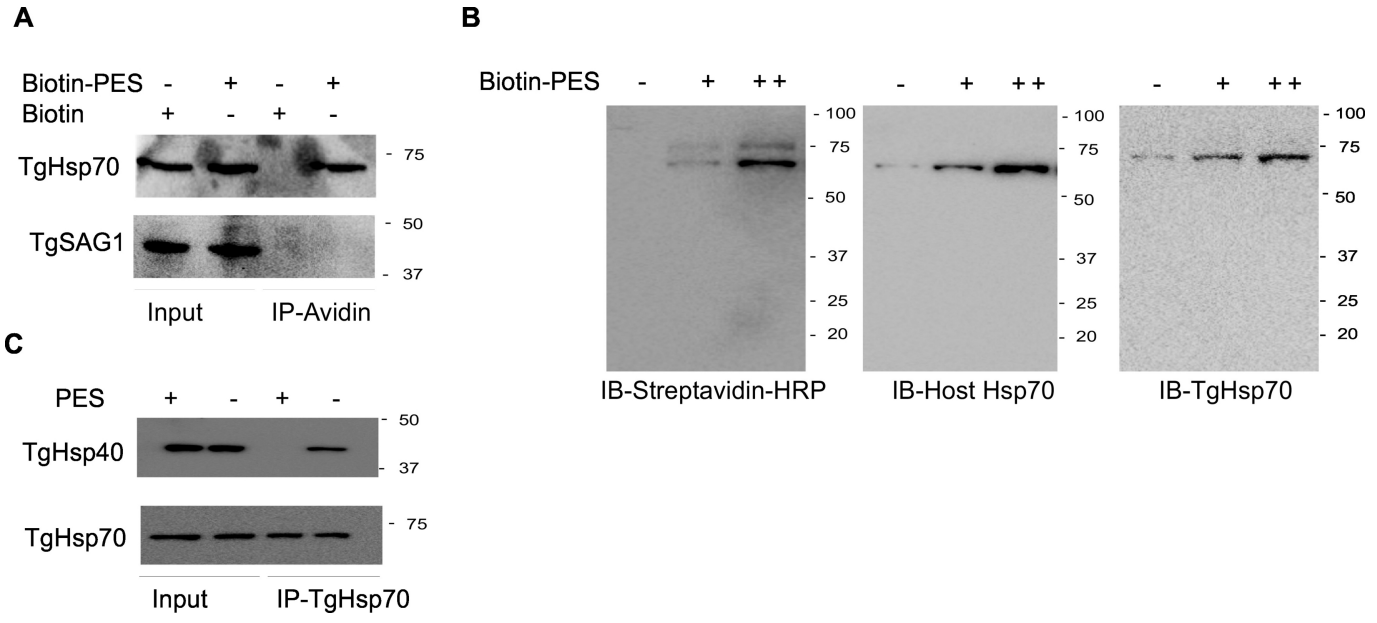
A
Biotin-PES - + - +
Biotin + - + -
TgHsp70
TgSAG1
75
50
37
Input
IP-Avidin
B
Biotin-PES - + ++ - + ++ - + ++
100
75
50
37
25
20
IB-Streptavidin-HRP
IB-Host Hsp70
IB-TgHsp70
C
PES + - + -
TgHsp40
TgHsp70
50
37
75
Input
IP-TgHsp70

Figure 9