Supporting Information

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SI Materials and Methods

Materials. 8-Anilinonaphthalene-1-sulphonic acid (ANS), bis-ANS, citrate synthase (CS), Accu Taq polymerase oxaloacetic acid, BSA, tunicamycin (tn), thapsigargin (tp), FITC-labeled anti-mouse IgG, cy3-labeled anti-rabbit IgG, and acetyl CoA were obtained from Sigma Aldrich. TALON was purchased from Clontech Laboratories; guanidinium chloride (GdnHCl) was procured from USB; Nde1 and Dpn1 were New England Biolabs; and Protein G Sepharose was purchased from Amersham Biosciences. Amicon concentrators with a 3.5-kDa cutoff were purchased from Millipore; anti-calreticulin antibody was from Imgenex, and anti-GAPDH antibody was from Genscript.

Purification of Recombinant Wild-Type and Mutant Human Resistin Protein. *Escherichia coli* M15 cells were transformed with plasmids pQE30 human resistin (hRes) (Fig. S7) and pQE30F49YhRes containing wild-type and mutant genes, respectively. Recombinant human resistin proteins (rhRes and F49YrhRes) were purified by affinity chromatography as described (1). The purity of the protein was checked by Western blot analysis and also demonstrated to be pure and free from any detectable bacterial contaminants as evident from FPLC and mass spectrometry analyses (1).

ANS Fluorescence of Recombinant Wild-Type and Mutant hRes. Fluorescence of ANS in the presence of rhRes and F49YrhRes was measured by exciting at 390 nm and following the emission between 450 and 550 nm. Different concentrations of rhRes/F49YrhRes (0.1, 0.5, and 1 mg/mL) were incubated with 50 μM ANS for 30 min at room temperature, and the fluorescence of protein-bound dye was measured. The spectra were corrected with appropriate buffer and protein blanks.

Photoincorporation of bis-ANS into rhRes. rhRes (0.3 mg/mL) was incubated with 50 μM bis-ANS in 1 mL of 50 mM Tris·HCl, pH 8.0. Samples were placed on ice and cross-linked at 254 nm for 1 h in a Hoefer UV cross-linker. Unbound bis-ANS was removed by dialysis against 50 mM Tris, pH 8.0. Photolabeled proteins were electrophoresed on SDS/PAGE and visualized on a UV transilluminator.

Homology Modeling. The monomer sequence of hRes comprises 92 amino acid residues. The 3D structures of the trimer and hexamer models of both wild-type and mutant resistin were constructed based on the mouse resistin structure (Protein Data Bank ID code 1RFX) (2) by comparative modeling methods in InsightII (Accelrys Inc.). Compatibility of the model structure with its amino acid sequence was analyzed by the program Verify 3D (3), and the stereo chemical quality of the structures was examined using Ramachandran plot (4).

Molecular Dynamic Simulations. Molecular dynamic (MD) simulations of rhRes and F49YrhRes structures in trimer and hexamer forms were carried out using GROMOS96 53a6 force fields in GROMACS-4.0.7 (5–7) and analyzed using trajectory files obtained during 20 ns of MD simulations at 298 and 333 K with an integration step of 2 fs, and the results were compared at both temperatures. The proteins were solvated using a 1.5-nm box edge from the protein molecules with explicit Solvent-Simple Point charge models (SPC216 water models). The size of the box generated for trimer was 73.378 × 109.901 × 72.147, and it was 78.939 × 169.815 × 84.598 for hexamer. The total system was minimized using the conjugate gradient method for 5,000 steps and 500 ps of position-restrained equilibration to distribute water molecules throughout the system. This equilibrated system was subjected to MD simulations up to 20 ns. The particle mesh Ewald (PME) summation method was used for the calculation of the electrostatics with a real space cutoff of 10 Å and a PME order of 6 and a relative tolerance between long- and short-range energies of $10e^{-6}$. Short-range interactions were evaluated using a neighbor list of 14 Å updated every 100 steps; the Lennard–Jones interactions and the real space electrostatic interactions were truncated at 14 Å. The V-rescale thermostat was used to maintain the temperature, and the Parrinello–Rahman algorithm was used to maintain the pressure at 1 atm. The protein system MD simulations were performed at 298 and 333 K for trimer (Figs. S3 and S4 and at 298 and 350 K simulation for hexamer (Fig. S5). The hydrogen bonds were constrained using the LINCS algorithm. The system coordinates were saved after every 100 ps for further analysis. The rmsd, the root-mean-square fluctuations of the protein Cα trace, and the potential energy curves were plotted and analyzed using the trajectory files generated during the MD simulation. The output structures and trajectories were analyzed using VMD, PyMOL, and Discovery Studio 2.5 visualizers. All of the graphs were generated using "Grace" (Figs. S3–S5).

Site-Directed Mutagenesis. The forward primer used for site-directed mutagenesis (5′-CCCGAGGCTACGCCGTCAC-3′) carried a replacement of thymidine by adenine. For PCR, a 50-μL reaction mix contained 100 ng of plasmid pQE30hRes, 0.2 mM dNTP, 1 U of Acu Taq polymerase, and the supplied reaction buffer. The PCR product was treated with 5 U of DpnI for 2 h. DH5α cells were transformed with the PCR product. Mutation was confirmed by DNA sequencing (ABI prism 3100 DNA analyzer).

Aggregation Assays. Chaperone activity of rhRes and F49YrhRes was investigated in terms of its ability to prevent aggregation of the enzymes CS and Nde1 using reported protocol (8). CS (0.15 μM) or Nde1 (5 U) in 50 mM Tris (pH 8.0) in the absence or presence of 0.15 μM of rhRes or F49YrhRes or GroEL or lysozyme (negative control) was mixed at room temperature, and light scattering was measured at 320 nm against a blank (50 mM Tris, pH 8.0). All of the samples were incubated at 45 °C for 50 min. The experiment was carried out at 45 °C in a controlled temperature peltier block.

Immunocytochemistry. U937 (2×10^5) cells were cultured and treated with 5 μg/mL of tunicamycin or thapsigargin in 0.05% dimethyl sulfoxide (DMSO) for 24 h. DMSO to a final concentration of 0.05% was added to the cells for the indicated times as a control. After treatment, all cells were harvested and fixed with 4% paraformaldehyde (Sigma) in PBS for 15 min at room temperature, washed with PBS, and permeabilized with chilled methanol (Qualigens) for 20 min at 4 °C. After blocking the cells in 2% BSA (Sigma) in PBS for 30 min at room temperature, cells were incubated with monoclonal human anti-calreticulin antibody and raised in mouse (1:100) for staining of the endoplasmic reticulum (ER) and human anti-resistin antibody and raised in rabbit (1:10,000) to localize resistin. This was followed by incubation with FITC-labeled anti-mouse IgG (1:32) and cy3-labeled anti-rabbit IgG (1:100), respectively. Coverslips were mounted onto slides using 50% glycerol (Qualigens). Images were captured using confocal microscope (LSM 510 META; Zeiss).

Residual Activity of Denatured Nde1. Nde1 (10 U) was incubated at 60 °C for 20 min in the absence or presence of different concentrations (10, 15, and 20 µg) of rhRes or F49YrhRes. Nonspecific proteins, adiponectin (20 µg), and BSA (20 µg) were used as controls. The residual enzyme activity was assessed by digestion of 150 ng of circular pUC18 at 37 °C for 1 h. The digestion mixture was electrophoresed on 1% agarose gel, stained with ethidium bromide, and visualized under UV light in a Gel doc system (Bio-Rad). The residual activity of Nde1 denatured in the presence of bis-ANS rhRes was also tested similarly.

Refolding of GdnHCl Denatured CS. CS (20 µM) was denatured with 6 M GdnHCl, 3 mM DTT, 2 mM EDTA, 50 mM Tris (pH 7.4) for 30 min at room temperature. The denatured CS was diluted 100-fold in 20 mM potassium phosphate (pH 7.4), 10 mM $MgCl_2$, and 1 mM oxaloacetic acid with rhRes (1 µM), F49YrhRes (1 µM), or GroEL (1µM + 2 mM ATP + 2 µM GroES) as a positive control. A ratio of 1:1 resistin/F49YrhRes or GroEL:CS was used. All reactions were incubated at 37 °C for 1 h. Refolding to the native form was confirmed through the attainment of CS activity, which was monitored by the disappearance of acetyl-CoA at 233 nm (Jasco/PerkinElmer spectrometer). The residual activity was expressed as the percentage of activity of native CS.

Coimmunoprecipitation Assays. Immunoprecipitation was carried out to detect the interaction of rhRes, if any, with denatured CS enzyme and Late Expression Factor 4 (LEF4) protein. CS and LEF4 were denatured with 8 M GdnHCl. Ten micrograms of native and denatured CS or native and denatured LEF4 were incubated with 10 µg of rhRes separately for 15 min. rhRes was immunoprecipitated using 5 µL of polyclonal antisera against hRes in RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, aprotinin, and leupeptin) for 2 h at 4 °C. Twenty microliters of protein G Sepharose beads was added to the reaction mix and incubated for 1 h at 4 °C with gentle shaking. After washing the beads with RIPA buffer, immunoprecipitated fraction was boiled in SDS loading dye, electrophoresed on SDS/PAGE, and stained with Coomassie Blue.

Growth Rescue of *E. coli* from Thermal Shock. *E. coli* M15 cells, transformed either with pQE30 plasmid vector alone or with recombinant plasmids, pQE30hRes, or pQE30F49YhRes were grown overnight at 37 °C in LB containing kanamycin (25 µg/mL) and ampicillin (100 µg/mL). Primary cultures were diluted 10-fold in LB containing antibiotics as secondary culture and were allowed to grow at 37 °C until the OD reached 0.6. Cultures were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside for 2 h. Uninduced and induced cultures were diluted to 1:1,000 and incubated at either 37 °C or 50 °C for 45 min; then 10 µL of each sample was spread on LB agar plates with appropriate antibiotics and grown at 37 °C overnight, and the colonies were counted. Cells survival was calculated as a ratio of the cfu after heat shock (50 °C) and without heat shock (37 °C), and this value was then normalized taking the vector (pQE30) control as one fold.

Western Blot Analysis. U937 cells were treated with tn or tp at 0, 2, 5, 10, and 15 µg/mL for 24 h and harvested for Western blot. Cells were lysed using lysis buffer (20 mM Tris, pH 7.5, 0.1% Nonidet P-40, 0.1 mM EDTA, 2 mM PMSF). Cells were disrupted by sonication and centrifuged to separate the cell debris. Total protein concentration was measured in duplicate by Bradford using BSA as a standard. Proteins were resolved on 10% SDS polyacrylamide gel using the Tris-tricine buffer system and then transferred to PVDF membrane using Bio-Rad mini gel electro transfer. After blocking, blots were incubated with anti-human resistin or anti-GAPDH (Sigma) for 1 h at room temperature followed by washing with phosphate buffered saline with 1% (vol/vol) Tween-20 three times. Blots were hybridized with horseradish peroxidase- conjugated goat anti-rabbit IgG (Sigma 1:10,000) or anti-mouse IgG (1:10,000) and developed using an ECL Western blot detection kit (Millipore) according to the manufacturer's instructions.

Quantitative PCR Analysis. U937 cells were treated with either tn or tp at 5 µg/mL for 6, 12, and 24 h, and cells were harvested at the respective time points. RNA was isolated using TriZol (Sigma). cDNA synthesis was performed using SuperScript III kit (Invitrogen), and quantitative PCR (qPCR) analysis was carried out using SYBR premix (Takara) on a Realplex qPCR machine (Eppendorf).

ELISA. U937 cells were treated with either tn or tp (5 µg/mL) for 6, 12, and 24 h. hRes secretory levels were checked in the supernatant collected at each time duration of incubation. To quantify the secreted hRes concentrations from untreated and treated cells, a commercial resistin (human) ELISA kit (AdipoGen) was used per manufacturer's instructions.

SI Results

Homology Modeling of hRes Displayed Surface-Exposed Hydrophobic Patches. Each monomer has a 24-amino-acid N-terminal helical tail and a C-terminal disulfide-rich β-sandwich head domain. Three monomeric units associate at the N terminus to form a coiled coil in the trimer state with several hydrophobic interactions. The head domains in trimer are stabilized by electrostatic, hydrophobic, and hydrogen bond interactions. The two trimers associate to form the hexamer via disulfide bond linkage of Cys4 on the helix. The space-filled model of the hexamer (Fig. S2) reveals that it is a compactly folded structure with no internal cavity.

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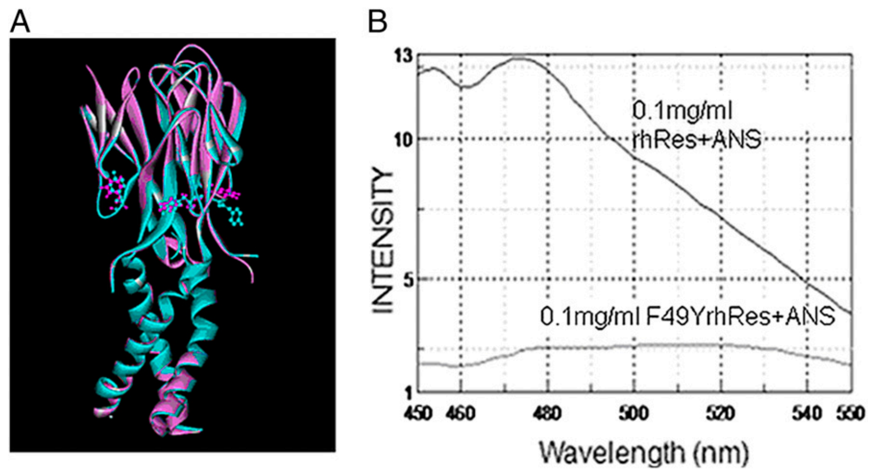

Fig. S1. hRes exists in a trimeric form and exhibits surface hydrophobicity. (*A*) Structural superimposition of native and mutant hRes. hRes trimer is depicted in magenta and mutant hRes trimer in cyan. The side chain of native phenylalanine and mutant tyrosine are shown in a ball-and-stick model. (*B*) ANS fluorescence of rhRes and F49YrhRes was measured to determine surface-exposed hydrophobic residues available for binding to nonnative proteins.

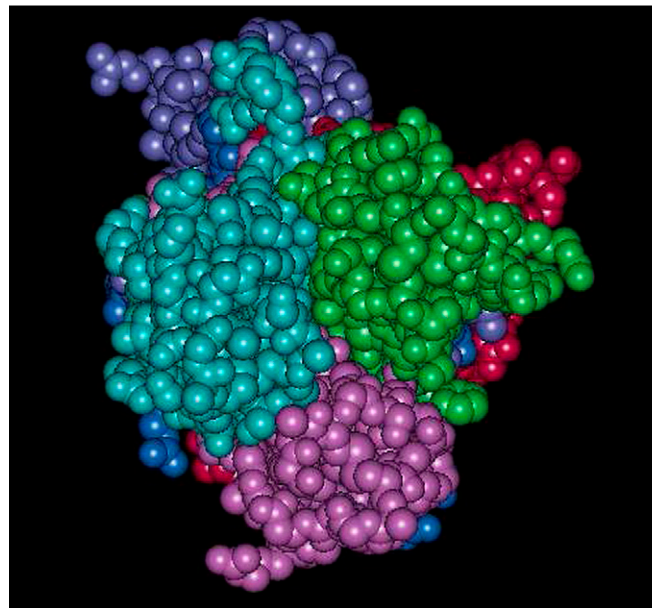

Fig. S2. Top view of the space-filled model of human resistin hexamer. The six chains are indicated in different colors.

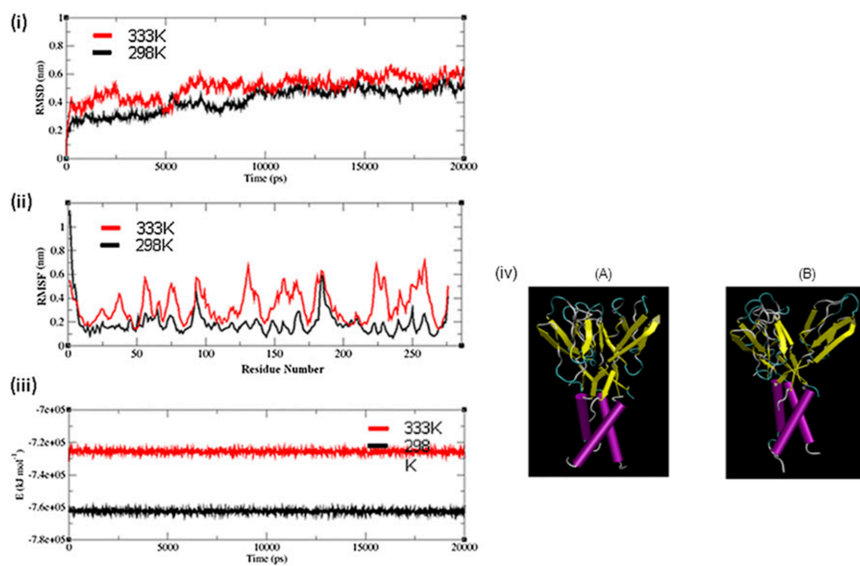

Fig. S3. MD studies of hRes reveal profiles typical for chaperones. During MD simulations hRes trimer undergoes molecular dissociation and association and has greater stability at 298 K compared with 333 K. (i) Protein Cα atoms rmsd (nm) as a function of time (ps). (ii) Root-mean-square fluctuations (nm) of protein Cα atoms. (iii) Potential energy (kJ/mol) of total system plotted as a function of time (ps). Red and black rmsd plots at 333 and 298 K, respectively. (iv) Initial closed conformation before MD simulations (*A*) and final open conformation after MD simulations (*B*) of the hRes trimer.

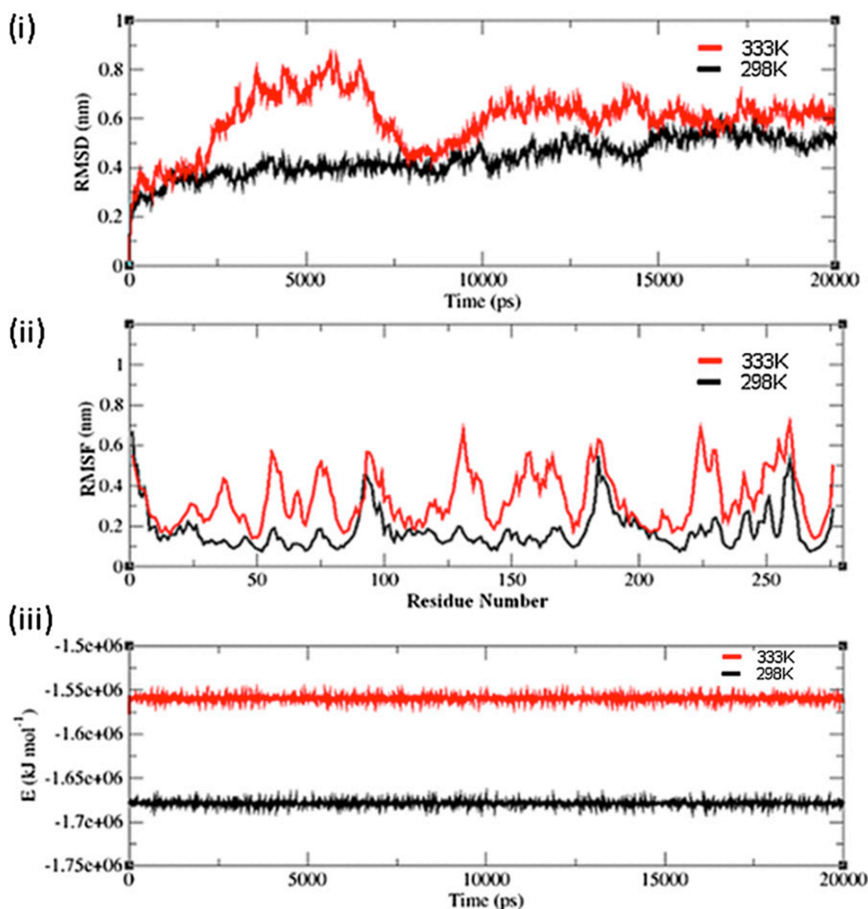

Fig. S4. During MD simulations F49YhRes trimer undergoes less molecular dissociation compared with hRes. (i) Protein Cα atoms rmsd (nm) as a function of time (ps). (ii) Root-mean-square fluctuations (nm) of protein Cα atoms. (iii) Potential energy (kJ/mol) of total system plotted as a function of time (ps). Red and black rmsd plots at 333 and 298 K, respectively.

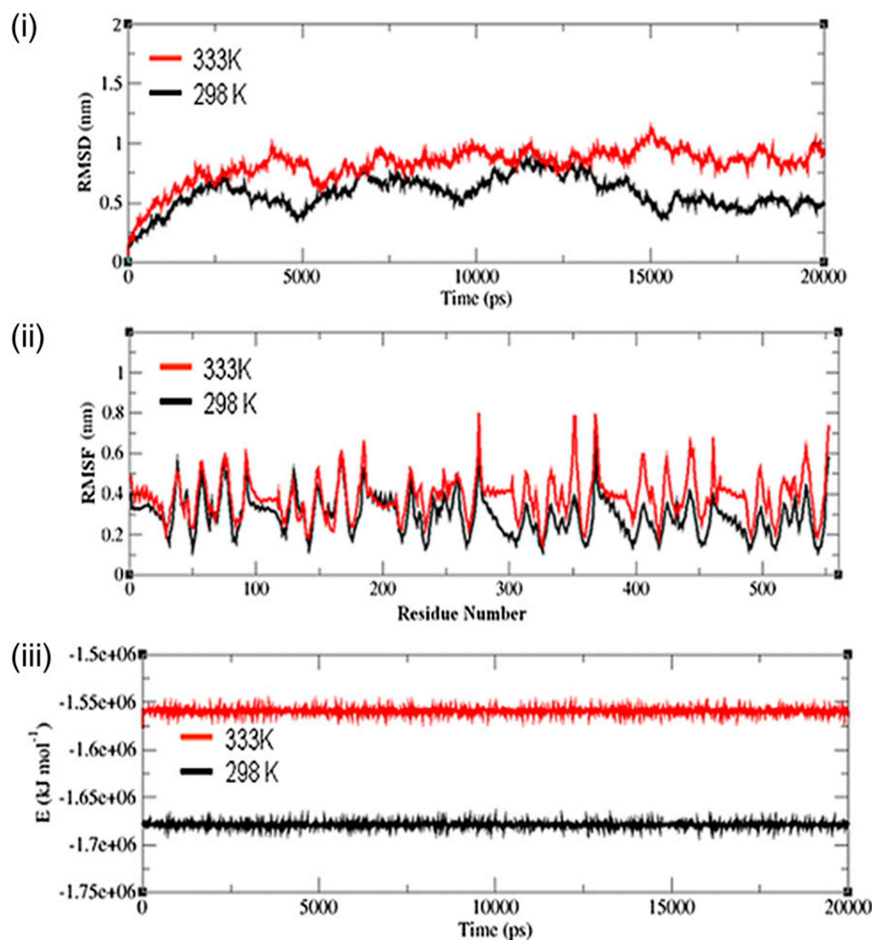

Fig. S5. hRes hexamer does not undergo molecular dissociation. (i) Protein Cα atoms rmsd (nm) as a function of time (ps). (ii) Root-mean-square fluctuations (nm) of protein Cα atoms. (iii) Potential energy (kJ/mol) of total system plotted as a function of time (ps). Red and black rmsd plots at 333 and 298 K, respectively.

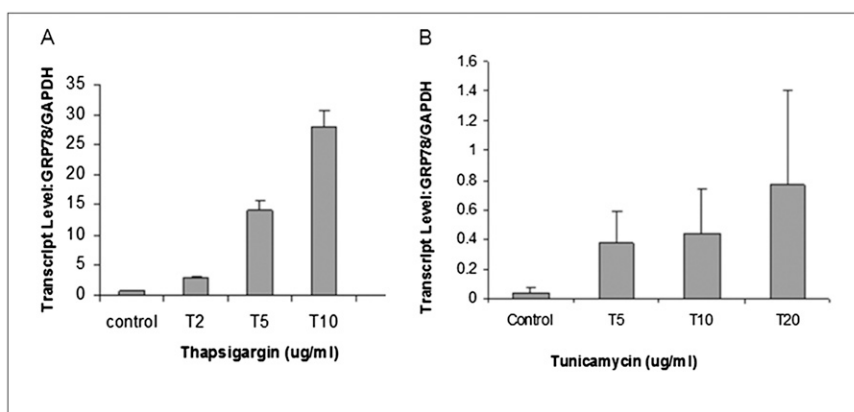

Fig. S6. Grp-78 levels in U937 cells confirm ER stress after treatment with tunicamycin and thapsigargin. Differentiated U937 cells were treated with 5 μg/mL tunicamycin or thapsigargin for 24 h. Grp-78 (ER stress marker) mRNA levels were checked to confirm the ER stress under respective treatment conditions. (*A*) With increasing concentrations of thapsigargin (0, 5, 10, and 20 μg/mL), a gradual increase in Grp-78 levels was observed. (*B*) Tunicamycin (0, 2, 5, and 10 μg/mL) treatment also induced the Grp-78 levels, indicating the occurrence of ER stress. Each experiment was carried out in triplicate. The error bars represent SEM.

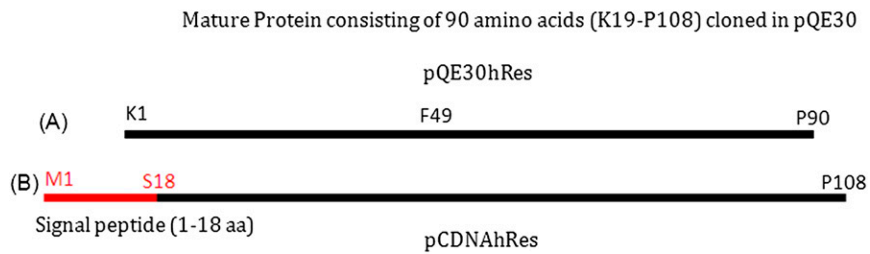

Fig. S7. Cloning and expression of human resistin gene in bacterial and mammalian expression systems. (*A*) Human resistin gene, excluding secretory signal (18 aa), was cloned in pQE30 vector to create pQE30hRes. The mature protein is 90 amino acids starting with "K" at the N-terminal end and with "P" at the C-terminal end. The phenylalanine at 49th position, with respect to K in the mature peptide, was mutated to tyrosine. (*B*) For mammalian expression, the full-length polypeptide consisting of 108 amino acids, including the 18-amino-acid secretory signal, was cloned in pCDNA to create pCDNAhRes and used to transfect HeLa cells.

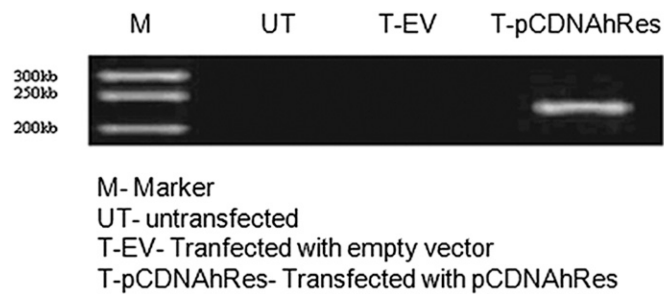

Fig. S8. HeLa cells transiently transfected with empty plasmid vector (T-EV) or pCDNAhRes or UT (untransefected) were grown for 24 h, and expression of resistin was confirmed by RT-PCR using resistin specific primers. Figure is a representative gel picture of two independent experiments.

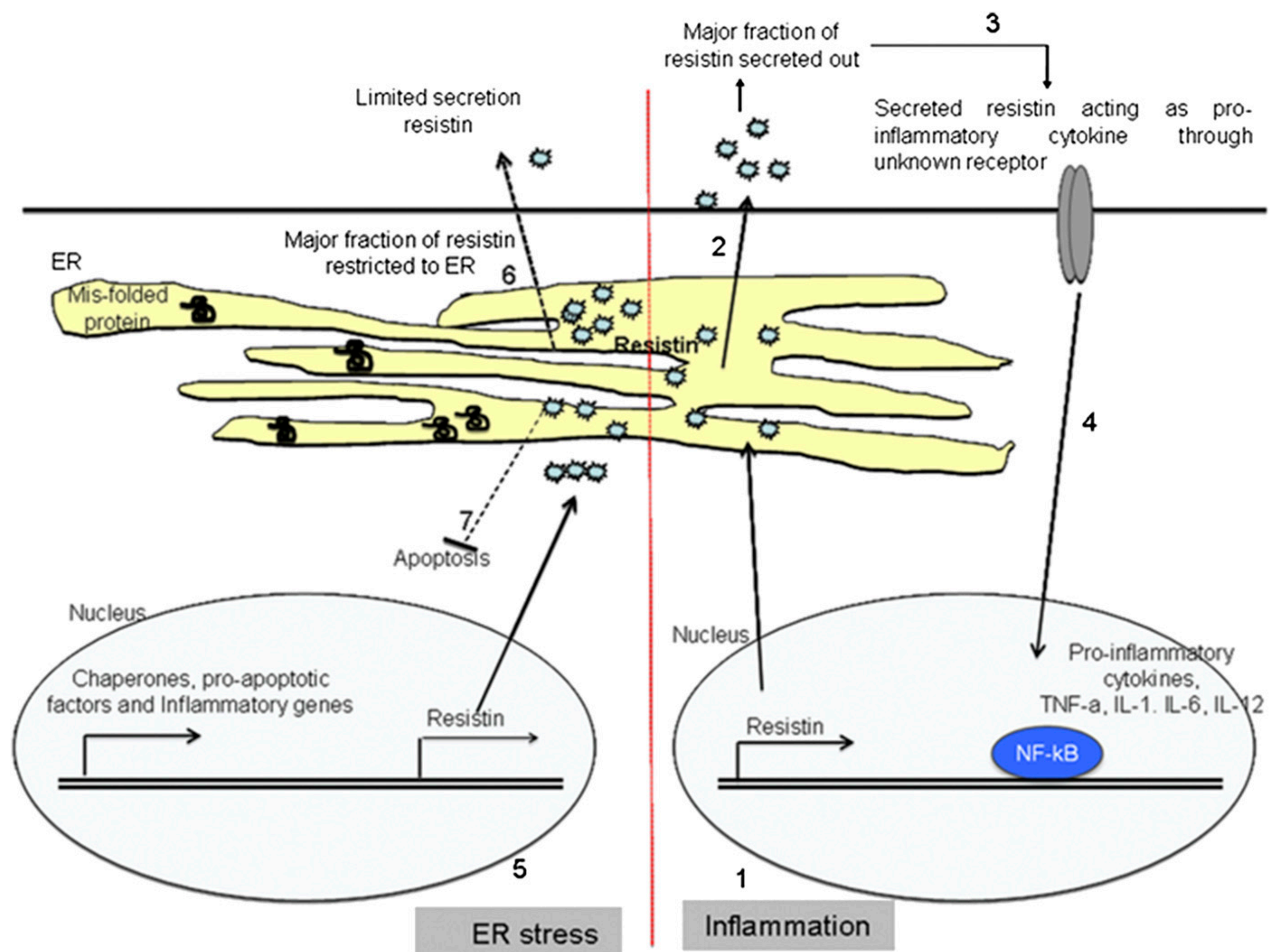

Fig. S9. Proposed model describing the dual nature of hRes. Expression of human resistin is up-regulated during inflammatory stimulation (1), such as infection, where it is predominantly secreted outside the cell (2). Secreted resistin is received by unknown receptors on the cell surface (3) where it functions as a proinflammatory molecule, promoting secretion of proinflammatory cytokine, possibly through NF-κB–mediated pathways (4). Upon ER stress (5), resistin is retained inside the ER of stressed cells (6) where it functions like a chaperone, helping in refolding of misfolded proteins and thereby rescuing the cells from stress-induced apoptosis (7).