Critical role of proteostasis-imbalance in pathogenesis of COPD and severe emphysema
Taehong Min, Manish Bodas, Steven Mazur and Neeraj Vij
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Supplementary Methods

Immunoblotting, immunoprecipitation and metabolic labeling- Cells were washed three times in ice-cold PBS and lysed directly on plates using M-PER (Pierce Biotech, Inc., Rockford, IL) protein lysis buffer containing protease inhibitor cocktail (Pierce). While human lung tissues were lysed in T-PER (Pierce) containing protease inhibitors using a sonicator (three 10s pulses). The protein extracts (100μg) were suspended in Laemmli's sample buffer (30 μl; Invitrogen) containing β-mercaptoethanol (Invitrogen), resolved by 10% SDS-PAGE and transferred to a 0.45-mm (β-actin, VCP) or 0.2-mm (UCH-L1) pore size nitrocellulose membrane (Invitrogen). The UCH-L1 antibody was purchased from Abcam Inc (Cambridge, MA), β-actin and GAPDH from Sigma (St. Louis, MO) while VCP antibody was from Santa Cruz Biotech (Santa Cruz, CA). The anti- mouse or rabbit horseradish peroxidase secondary antibodies were from Amersham (Piscataway, NJ). To detect ubiquitin accumulation in the insoluble protein fractions, we used protein pellets from equal amount of COPD lung tissue lysates and separated these on 10% SDS-PAGE followed by immunoblotting for ubiquitin. The lung tissue total-protein lysates from Gold 0-control and Gold IV-COPD subjects were immunoprecipitated with gp78 (using Agarose A/G beads, Santa Cruz Biotechnology), followed immunoblotting for VCP. For metabolic labeling, HBE cells were pulsed with 250μCi/well Trans-^{35}S-cys/met (ICN Biomedicals Inc, Irvine CA) for 30 min. After the 30 min pulse, the cells were washed with 1X PBS and 1 ml selective media (MEM) was added followed by chase for the indicated time points. The lysates were immuno-precipitated with mouse monoclonal Ub antibody as described above and run on SDS-PAGE. The gel was dried and exposed to Kodak BioMax MR film.

Murine experiments- Age- and sex- matched C57BL6 mice (n=3, 8 to 10 week old) were exposed to cigarette smoke (CS) using the TE-2 cigarette smoking machine (Teague Enterprises, Davis, CA). The CS was generated by burning research grade cigarettes (3R4F, 0.73 mg nicotine per cigarette) purchased from the Tobacco Research Institute, University of Kentucky, Lexington, KY) for acute [5 hours/day for 3- (immunostaining) or 5- days (TUNEL)] or sub-chronic (4 weeks, VCP western blot). An average total particulate matter (TPM) of 150 mg/m^3 was recorded in real time during the smoking protocols. The control group of mice was exposed to filtered room air and the mice were sacrificed 2 hours after the last exposure. In another experiment, the mice were treated with 1 mg/kg salubrinal (i.t, Tocris Bioscience) for the last 24 hours of the 3 day smoking protocol. The control mice were treated with PBS as vehicle. The lungs were used to isolate total protein extract for immunoblotting or fixed in 10% buffered formalin phosphate (Fisher Scientific Inc., Fair Lawn, NJ) for immunostaining. For evaluating the effect of inflammation on proteostasis, we induced acute lung injury in mice by intra-tracheal (i.t) instillation of *Pseudomonas aeruginosa* lipopolysaccharide (*Pa*-LPS) (20µg) for 24 hours, as indicated by a loss of approximately 1 g body weight. The lungs from control and *Pa*-LPS-treated (n=3) mice were fixed in 10% buffered formalin phosphate (Fisher Scientific Inc., Fair Lawn, NJ) and longitudinal sections (5 micron thick) were used for immunostainings.

TUNEL assay- The paraffin-embedded murine lung sections were used to detect apoptotic cells in the lungs by quantifying DNA fragmentation by TdT-mediated dUTP Nick-End Labeling by TUNEL assay (Apoptosis Detection System, Fluorescein, Promega) using our previously standardized method . Hoechst dye (Molecular probes) was used for nuclear staining. Images were captured as above with appropriate filter settings for FITC and

Hoechst. TUNEL-positive cells were counted per microscopic field in three animals from each group and plotted as number of apoptotic cells in air *versus* smoke samples to calculate the statistical significance.

ER stress activity assay – The CS extract (CSE) induced secretory ER stress activity was quantified using the Gaussia based ER Stress Monitoring Kit (Targeting systems) for a total of 24 hours. After 6 hours of transfection, cells were treated with 200 μg/ml CSE (Murty Pharmaceuticals) and/or with 50 μM salubrinal. The supernatant was harvested and plated (50 μl/well) in a 96 well flat bottom white opaque plate. The secretory ER stress activity was assayed by using Dual Luciferase Assay Kit (Promega).

Supplementary Figure Legends

Supplementary Figure 1. Interaction of VCP to specific E3 ubiquitin ligase. The paraffin-embedded longitudinal human lung sections of control (GOLD 0) and COPD lung tissue at GOLD I-IV levels of emphysema (n=8 to 10, each group), were immunostained with primary rabbit polyclonal CHIP and secondary anti-rabbit-FITC conjugated antibody. Although the data shows some increase in CHIP expression in the tissue sections with severe emphysema (A), it is not as significant relative to the increase seen in Rma1 and gp78 expression (B). The data indicates the very significant increase in VCP associated E3-E4 ubiquitin (Rma1-gp78) ligases as compared to other very commonE3 ubiquitin ligase such as CHIP. Nuclear (Hoechst) staining of the same area is shown in the bottom panel. Scale = 50 μm.

Supplementary Figure 2. The induction of VCP expression in *Pa*-LPS treated murine lungs correlates with stress response and apoptosis. The age- and sex- matched WT mice

(n=3, each group) were treated intra-tracheally with 20 μg/mouse *Pa*-LPS following the JHU ACUC approved animal handling protocol. The longitudinal lung sections present significant increase in *Pa*-LPS-induced VCP expression, and its correlation to elevated NFκB, NOS2, Nrf2, p-eIF2α levels(ER stress) and accumulation of polyubiquitinated proteins (Ub & UCH-L1). The *Pa*-LPS treated mice lungs also show an increase in the number of apoptotic cells (TUNEL staining). The data verifies the induction of VCP expression with *Pa*-LPS induced lung injury and its correlation to changes in inflammatory-oxidative stress markers. Scale = 50 μm.

Supplementary Figure 3. Decreased proteasomal activity induces VCP-gp78 co-localization. The HBE cells were treated with DMSO (control) or 10 μM MG-132 and immunostained with VCP (Red) and gp78 (Green). The data demonstrates an increase in VCP-gp78 co-localization under conditions of low proteasomal activity (arrows). n=3, Scale = 20 μm.

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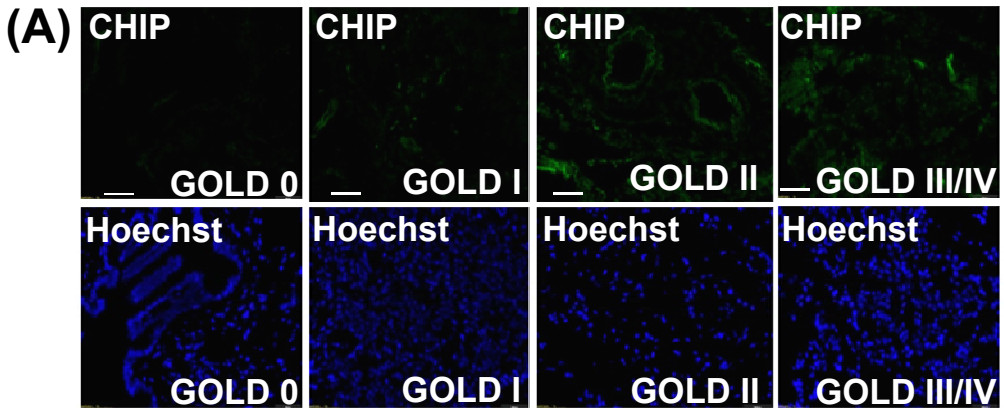
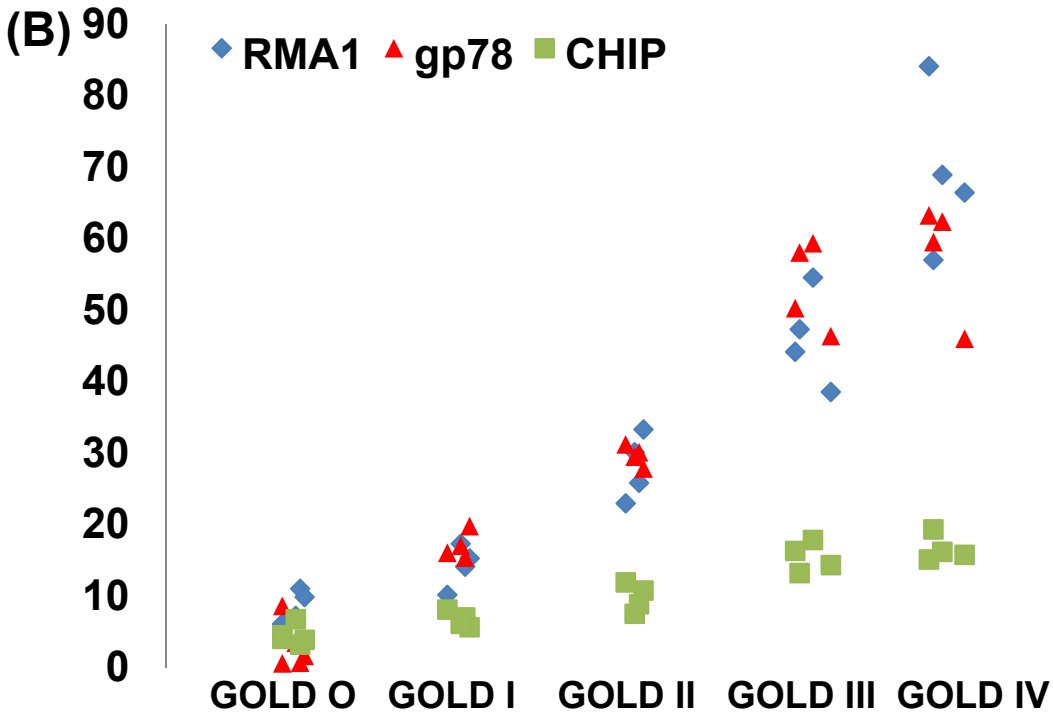
Supplementary Figure 1.





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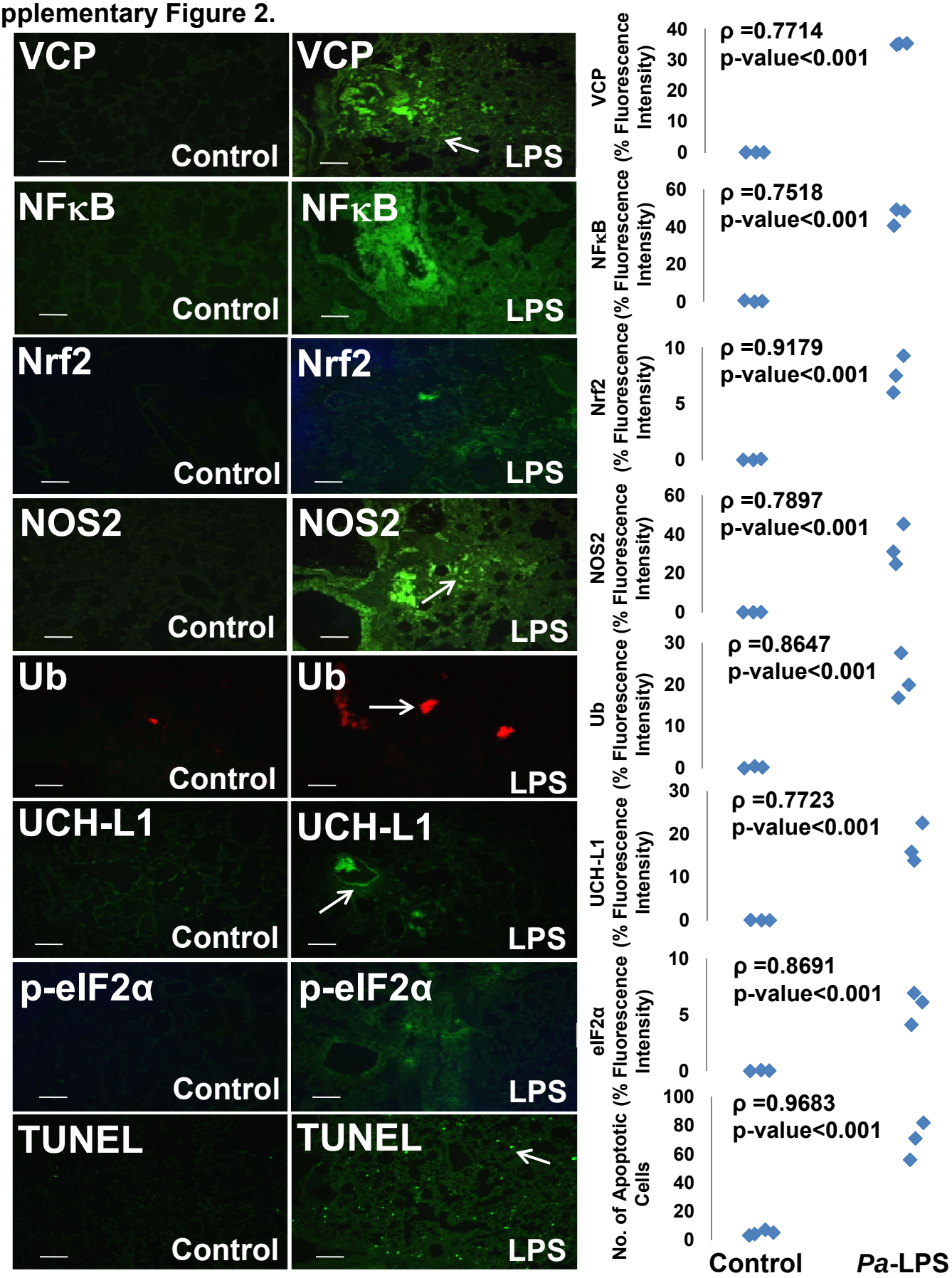
Supplementary Figure 2.



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