

## **SUPPLEMENTARY FIGURES AND METHODS**

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## SUPPLEMENTARY METHODS

### Recombinant IL-33 protein expression and purification

Splice variants of IL-33 protein were cloned as per previous (1) into pCDH lentiviral vectors or into pHLSEC (2) for expression in Expi293 system (Invitrogen). For mammalian expression, Flag-IL-33<sup>Δ34</sup>-His in pHLSEC was transfected into Expi293 cells using Hype293 (OZ Biosciences) and protein was purified by tandem anti-Flag (GenScript) and NiNTA resins according to protocol. Recombinant protein and co-purified bands were visualized on SDS-PAGE and western blot performed for confirmation of bands. Co-purified HSP70 and HSC70 were confirmed by Mass Spectrometry (methods per MTAC core, see below). Recombinant IL-33<sup>Δ34</sup> for binding experiments was cloned into pET21a (Novagen) with an N-terminal hexahistidine (6XHis) tag, followed by an N-terminal AviTag™ sequence (GLNDIFEAQKIEWHE) and (GGG)<sup>2</sup> linker. The IL-33<sup>Δ34</sup> construct was co-transformed with BirA ligase for in-situ biotinylation as needed. HSP70 was cloned with an N-terminal hexahistidine (6XHis) tag into pETDuet vector (Novagen). Mutants were generated using the Agilent QuickChange II XL kit per manufacturer protocol. Constructs were transformed into Rosetta2(DE3) *E. coli*, (Novagen) under antibiotic selection and bacteria were grown in Luria Broth (LB) at 37°C to an OD<sub>600</sub> of ~0.8 before induction with 0.5mM of IPTG at 20°C overnight.

Protein purification from cell pellets was performed by lysis with sonication in 50 mM Na<sub>2</sub>HPO<sub>4</sub> pH 8.0, 300mM NaCl, 5mM imidazole, 10mM β-ME and 10% glycerol with 50 mg/ml Lysozyme (bacterial produced protein), 1 mg DNase, 1 mM MgCl<sub>2</sub>, 1mM phenylmethylsulfonylfluoride (PMSF). Following centrifugation at 7,000 x g, supernatants

were passed over NiNTA superflow resin (Qiagen) with increasing imidazole concentration according to manufacturer protocols based on buffer: Na<sub>2</sub>HPO<sub>4</sub> pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 5 mM DTT and 10% glycerol supplemented with protease inhibitor cocktail (Sigma). Proteins further purified by size exclusion chromatography (SEC) using a Superdex™ 75 Increase column for IL-33 and a Superdex™ 200 Increase column for HSP70 purifications.

### **Mass Spectrometry Proteomics**

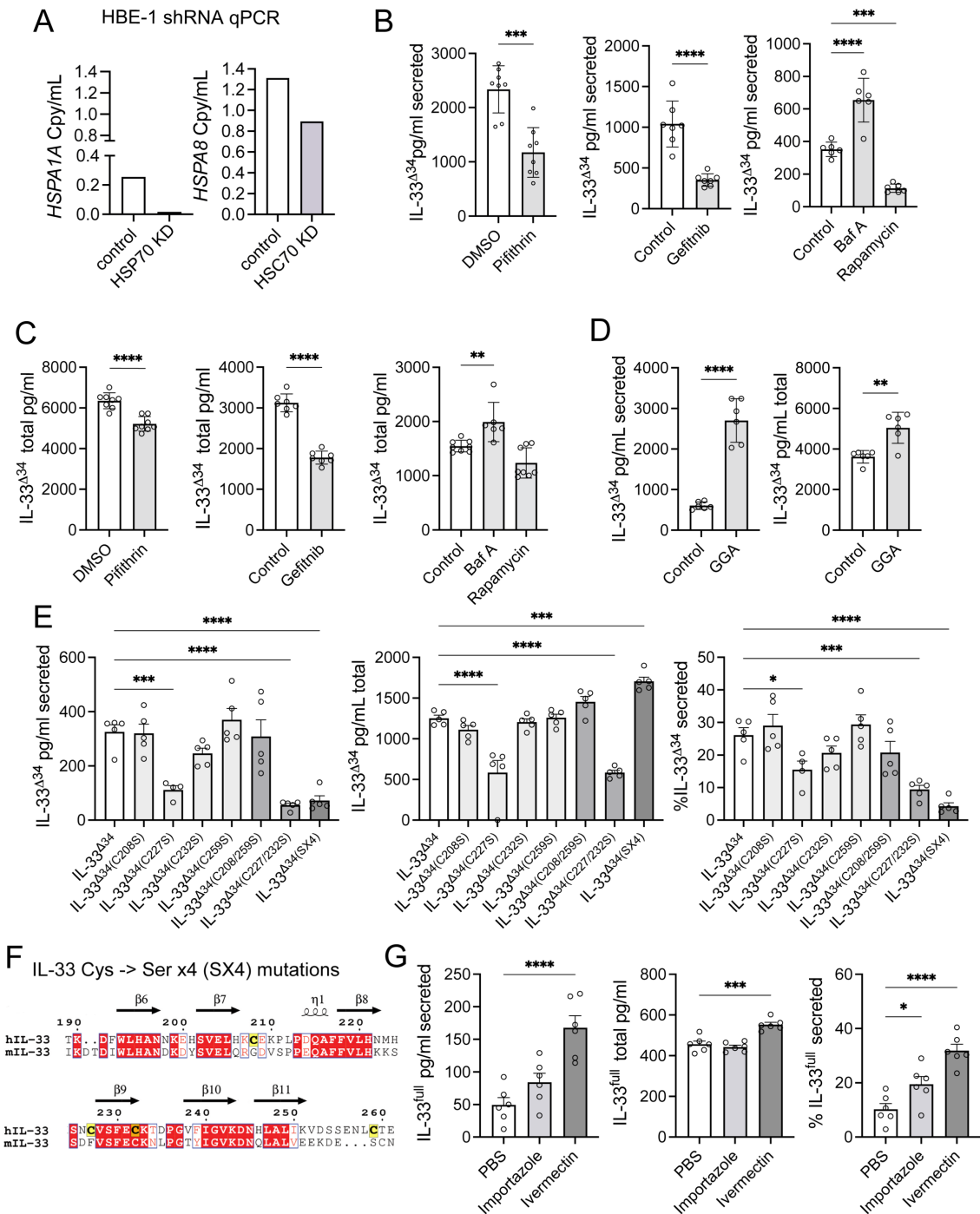
Mass Spectrometry analyses were performed by the Mass Spectrometry Technology Access Center at McDonnell Genome Institute (MTAC@MGI) at Washington University School of Medicine. Briefly, samples were reduced, alkylated, and digested with trypsin according to core facility protocols. Digested peptides were desalted on C18 spin columns and analyzed by mass spectrometry. Data was searched against a Human database using MaxQuant search engine and then performed label-free quantification (LFQ) based on the MS1 peptide intensity.

Extracellular vesicle-derived protein was filtered for >1 unique peptide, which yielded 577 proteins, including several EV marker proteins such as ANXA1, ANXA2, ACTN4, CD81, CD9. Obtained LFQ intensities were Log2 transformed, and data were grouped into groups: MTS15 (asthma), MTS16 (nonsmoker), COPD34 (COPD I-III), and COPD53 (COPD IV). These were filtered to retain proteins with at least 60% intensity values in at least one group which resulted in 565 proteins. Imputation was applied to fill in missing values based on normal distribution, which is recommended for label-free quantification.

Finally, *t*-test was carried out and results visualized by volcano plot to determine significant differences in 6 comparison sets.

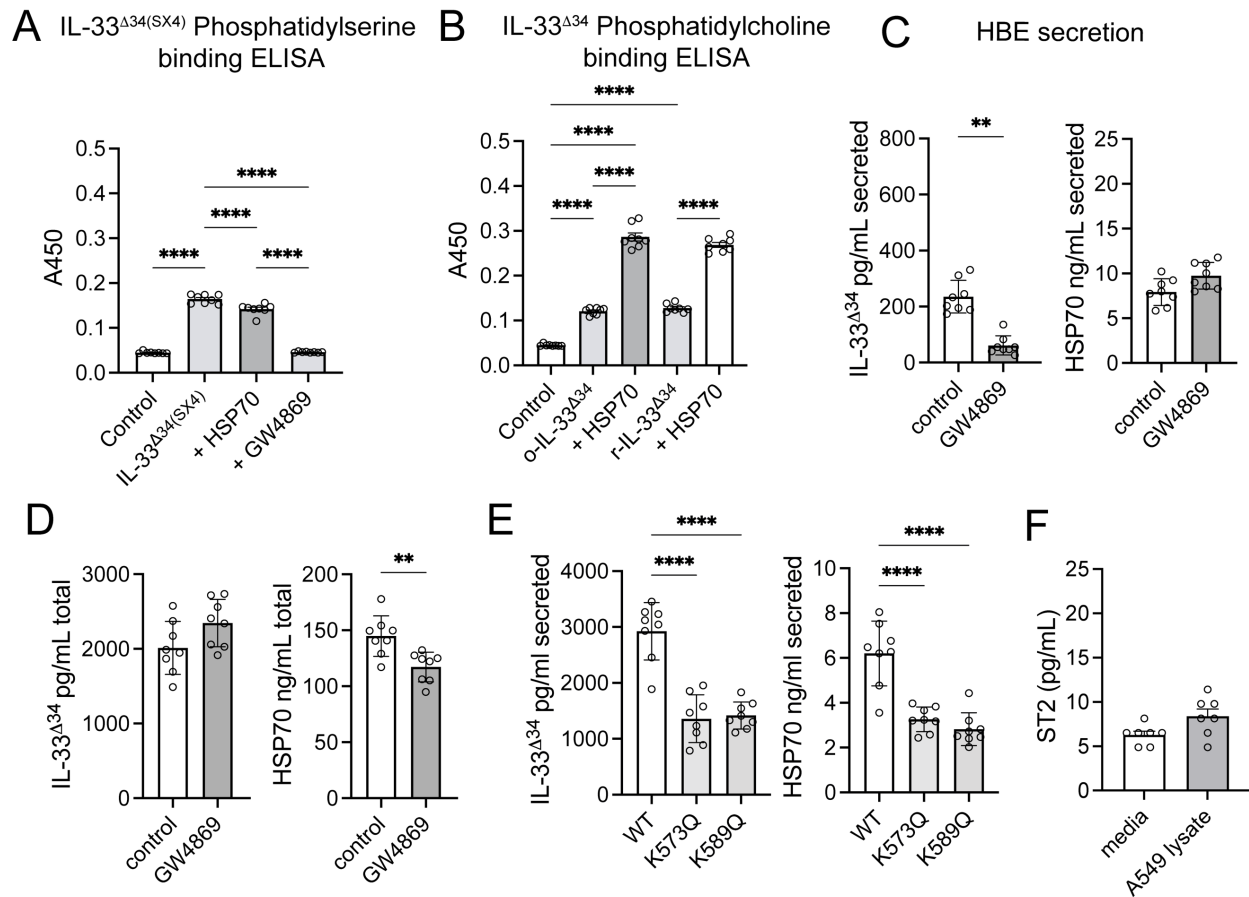
MiniTurboID labeled proteins were in-gel digested with trypsin using core facility optimized protocol. Peptides were subject to mass spec analysis and the data searched against a human database. In total, 238 proteins were identified including the presence of several naturally occurring biotin proteins that are used as positive controls for biotinylated enrichment experiments (ACACA, PC, MCCC1, MCCC2, PCCA). Results were visualized using Scaffold 5.0.

SUPPLEMENTARY FIGURES



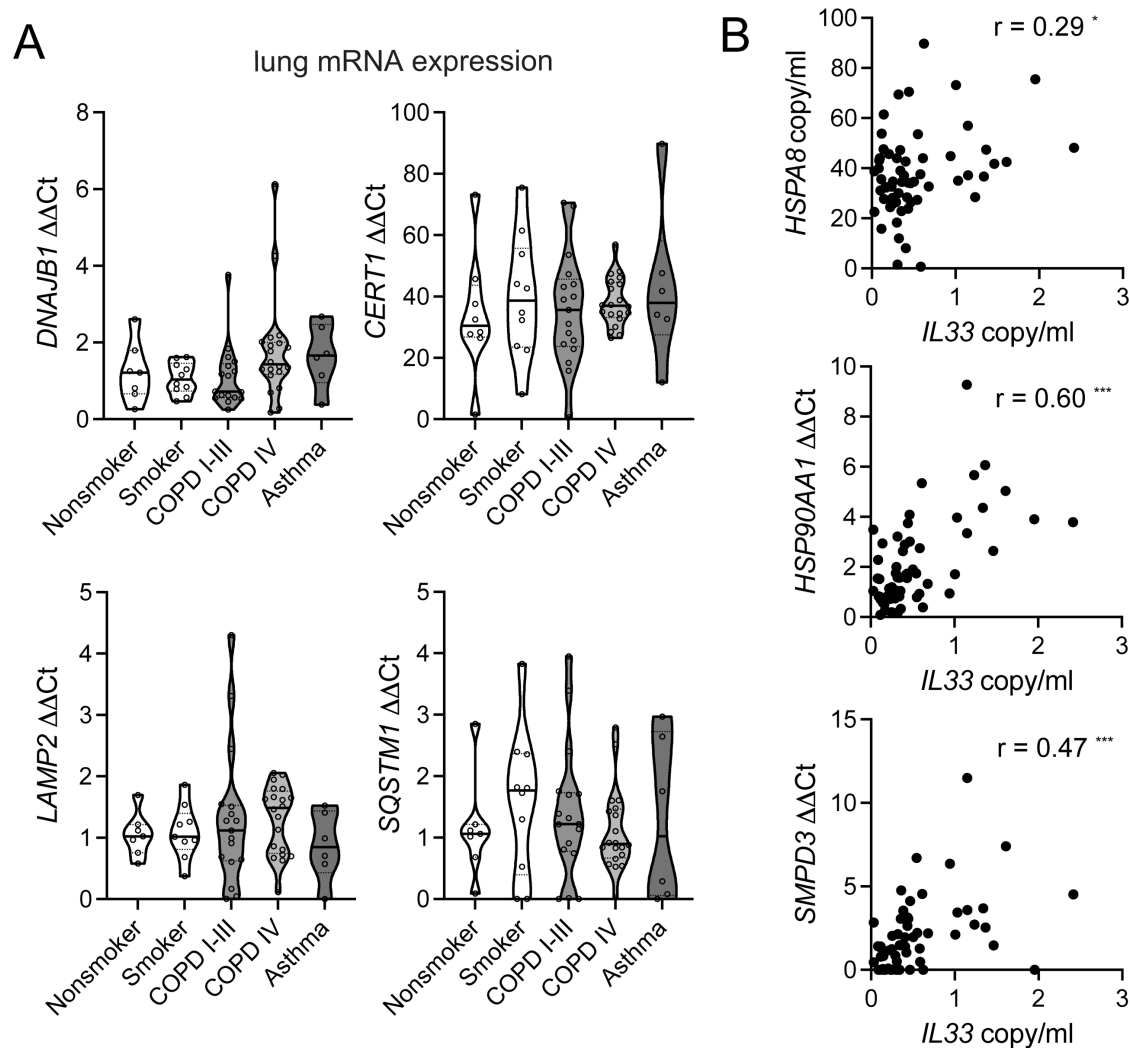
**FIGURE S1. Supporting data for Figure 2.** A) Validation of shRNA knockdown for HSP70 (*HSPA1A*) and HSC70 (*HSPA8*) in HBE-1 airway cell line (HBE) by qPCR represented as copies/ml (Cpy/ml) based on plasmid standard. B, C, D) Lentiviral-

expressed Flag-IL-33<sup>Δ34</sup>-His secreted (B) and total protein (C) measured by ELISA from HBE cell supernatant under conditions of inhibitors as shown (Pifithrin (PES) 1 μM, (*n* = 8), Gefitinib 1 μM (*n* = 7), Bafilamycin A (Baf A) 100 nM (*n* = 6), Rapamycin 100 nM (*n* = 8), and (D) GGA 20 μM, (*n* = 6)); control condition indicates PBS treatment unless otherwise indicated (i.e. DMSO). E) Flag-IL-33<sup>Δ34</sup>-His secretion data under conditions of single or double Cys to Ser mutants as indicated, inclusive of the 4 Cys to Ser mutant (SX4) and wildtype residues C208, C227, C232 and C259 (*n* = 5, for C227S *n* = 4). F) Sequence alignment of human (hIL-33) and mouse (mIL-33) segment of C-terminal domain highlighting the relevant cysteines mutated to serine in E) (bold, yellow); secondary structure of this region in human cytokine indicated above sequence based on PDB ID 4KC3. G) Full-length IL-33 (Flag-IL-33<sup>full</sup>-His) secretion efficiency in HBE cells under conditions of inhibitors Importazole 20 μM, (*n* = 6) and Ivermectin 10 μM (*n* = 6). Secretion experiments in B, C, D, E and F were repeated 3 times. Data points displayed with mean ± SEM. Statistical analysis: 1-way ANOVA (B, C, E, F) and *t* test (B, C, D). *P*-value limits: \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001.

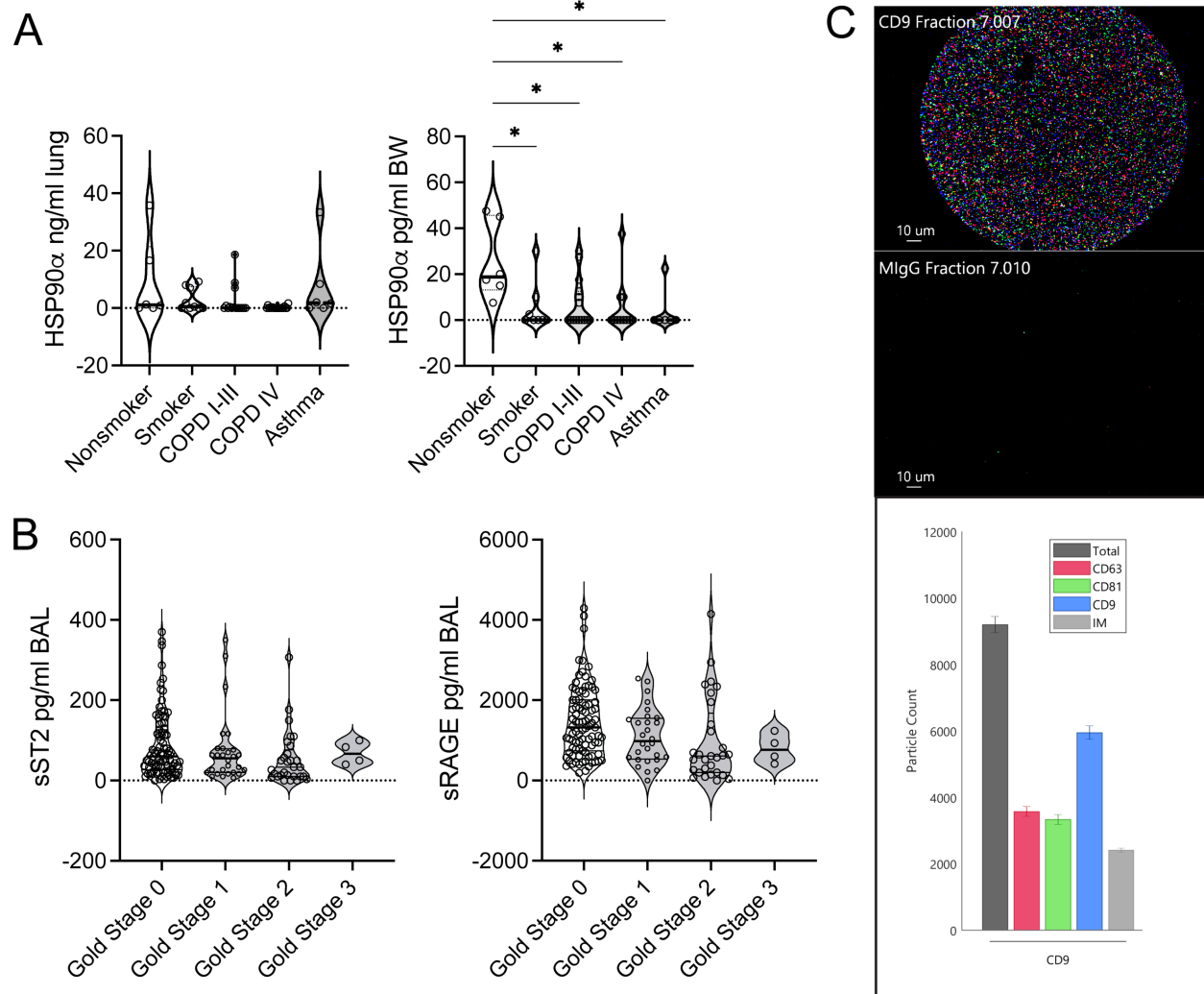


**FIGURE S2. Supporting data for Figures 3 and 4.** A) Phosphatidylserine (PS) binding for IL-33<sup>Δ34(SX4)</sup> mutant (C208S, C227S, C232S, C259S) binding ELISAs under conditions of HSP70 (500 ng/ml) and GW4869 (20  $\mu$ M) in competition. B) phosphatidylcholine (PC) for oxidized (o-IL-33<sup>Δ34</sup>) and reduced (r-IL-33<sup>Δ34</sup>) binding ELISAs under conditions of HSP70 (500 ng/ml) in competition. Detection was performed using biotinylated anti-IL-33 secondary antibody (R&D Systems) and Streptavidin HRP, and TMB substrate followed by A450 absorbance measurements. C, D) Secreted and total Flag-IL-33<sup>Δ34</sup>-His and endogenous HSP70 in HBE-1 cells under conditions of GW4869 (20  $\mu$ M) treatment, detection using anti-IL-33 and HSP70 Duosets (R&D Systems) and TMB substrate. E) Secreted Flag-IL-33<sup>Δ34</sup>-His and HSP70 in conditioned media detected after overexpression of WT or PS-binding deficient mutants K573Q and K589Q. F) ST2 ELISA

performed on A549 lysates to demonstrate minimal receptor protein detected in cellular assay. Assays A-E were conducted in triplicate and with  $n = 8$  replicates. Data points displayed with mean  $\pm$  SEM. Statistical analysis: 1-way ANOVA (A-E);  $P$ -value limits:  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ .



**FIGURE S3. Supporting data for Figure 5.** A) Lung tissue qPCR for Heat shock and proteostasis intermediates (HSP40/DNAJB1, p62/SQSTM1) and vesicular trafficking intermediates (LAMP2, CERT1) in nonsmokers ( $n = 7$ ), smokers ( $n = 11$ ) COPD I-III ( $n = 17$ ), COPD IV ( $n = 20$ ) and severe asthma ( $n = 6$ ) lung tissue specimens represented as fold-change by  $\Delta\Delta Ct$  method and normalized to GAPDH. B) Pearson's correlation for IL33 expression with HSC70/HSPA8, HSP90 $\alpha$ /HSP90AA1 and nSMase2/SMPD3.



**FIGURE S4. Supporting data for Figure 6.** A) HSP90 $\alpha$  protein levels measured in lung tissue and bronchial wash fluid from representative specimens including nonsmokers ( $n = 7$ ), smokers ( $n = 10$ ) COPD I-III ( $n = 17$ ), COPD IV ( $n = 20$ ) and severe asthma ( $n = 6$ ). B) Soluble ST2 and RAGE protein levels measured by ELISA in SPIROMICS BAL specimens for GOLD Stage COPD 0 ( $n = 91$ ), COPD I ( $n = 28$ ), COPD II ( $n = 29$ ), COPD III ( $n = 4$ ) sample groups. C) Tetraspanin (CD63, CD81 and CD9) positive vesicles were verified using Exoview R100 analysis platform following manufacturer protocols. A representative image of CD9 and negative control capture spots (MlgG, lower subpanel) are represented. Particle counts are averaged from 3 different CD9 capture spots. Data

points displayed as mean  $\pm$  SEM. Statistical analysis: 1-way ANOVA (A, C); *P*-value limits: \**P* < 0.05.

**Table S1.** Demographic and clinical data for patient specimens in Washington University cohort: Lung transplant donor specimens without COPD (nonsmoker), rejected donors without COPD (smoker) or mild-moderate (GOLD Stage I-III) COPD<sup>#</sup> and lung transplant recipients with severe (GOLD Stage IV) COPD. <sup>#</sup>Rejected donor lungs grouped as COPD I-III separately from smokers based on the following criteria: 1) presence of emphysema on chest CT, 2) hyperinflation/air trapping on gross pathological examination and 3) positive tobacco history >10 pk-yr. \*Severe asthma clinical history with cause of death status asthmaticus.

| Characteristics           | Nonsmoker  | Smoker     | COPD I-III <sup>#</sup> | COPD IV      | Asthma <sup>*</sup> |
|---------------------------|------------|------------|-------------------------|--------------|---------------------|
| Number per group          | 7          | 11         | 17                      | 20           | 8                   |
| Mean age (range)          | 33 (12-78) | 41 (21-67) | 56 (21-71)              | 61 (49-75)   | 35 (24-44)          |
| Male:Female:Unknown       | 4:0:3      | 3:2:6      | 3:1:10                  | 7:13         | 2:2:2               |
| FVC (L)                   | Unknown    | Unknown    | Unknown                 | 1.9655       | Unknown             |
| FEV1 (L)                  | Unknown    | Unknown    | Unknown                 | 0.5235       | Unknown             |
| FEV <sub>1</sub> /FVC (%) | Unknown    | Unknown    | Unknown                 | 24.95        | Unknown             |
| Pack-years (range)        | N/A        | Unknown    | 40.8 (30-60)            | 42.7 (10-96) | Unknown             |

**Table S2.** Clinical characteristics of COPD patients based on GOLD Stage obtained from SPIROMICS cohort (3).

| Characteristics           | COPD 0       | COPD I        | COPD II         | COPD III     |
|---------------------------|--------------|---------------|-----------------|--------------|
| Number per group          | 91           | 28            | 29              | 4            |
| Mean age (range)          | 56 (40-74)   | 64 (46-75)    | 63 (52-74)      | 54 (41-68)   |
| Male:Female               | 40:51        | 16:12         | 18:11           | 3:1          |
| FVC (L)                   | ND           | ND            | ND              | ND           |
| FEV1 (L)                  | 4            | 2.27          | 1.53            | 1.84         |
| FEV <sub>1</sub> /FVC (%) | ND           | ND            | ND              | ND           |
| Pack-years (range)        | 30.9 (0-112) | 51.9 (20-118) | 58.4 (25.5-108) | 58.9 (25-90) |

**Table S3:** List of reagents and materials associated with Methods.

| Reagent                                              | Supplier            | Reference/<br>Identifier |
|------------------------------------------------------|---------------------|--------------------------|
| <b>Antibodies and Proteins</b>                       |                     |                          |
| Human IL-33 rabbit polyclonal (1:50 IF)              | Sigma Aldrich       | HPA024426                |
| Human IL-33 goat monoclonal (1:1000 WB)              | R&D systems         | MAB36253                 |
| Human IL-33 mouse monoclonal (Nessy-1) (1:1000 IF)   | Enzo                | ALX-804-840              |
| Human HSP70 antibody (6B3) (1:5000 WB, 1:100 IF)     | Cell Signaling      | 4873S                    |
| Human HSP90 antibody (1:100 IF)                      | Proteintech         | 60318-1-1g               |
| Soluble human RAGE-Fc                                | R&D systems         | 1145-RG                  |
| Soluble human ST2                                    | R&D systems         | 11272-ST                 |
| Anti-human Fc                                        | BD                  | 32935S                   |
| Anti-rat-HRP                                         | Santa Cruz          | sc-2032                  |
| Anti-rabbit vectafluor 594 kit                       | Vector labs         | DK-1594                  |
| Anti-rat-488                                         | Invitrogen          | A21208                   |
| IF = immunofluorescence, WB = western blot           |                     |                          |
| <b>Commercial lentiviruses</b>                       |                     |                          |
| Scrambled shRNA                                      | Santa Cruz          | sc-108080-V              |
| HSC70 shRNA                                          | Santa Cruz          | sc-62655-V               |
| HSP70 shRNA                                          | Santa Cruz          | sc-29352-V               |
| <b>Biological Samples</b>                            |                     |                          |
| SPIROMICS BAL                                        | NHLBI               | N/A                      |
| Control and COPD lung tissue                         | BJH, UNMC           | N/A                      |
| Control and COPD Bronchial wash fluid                | BJH                 | N/A                      |
| <b>Chemicals, Peptides, and Recombinant Proteins</b> |                     |                          |
| GW4869                                               | Sigma Millipore     | D1692                    |
| Phosphatidylserine                                   | Avanti Polar lipids | 840032P                  |
| Phosphatidylcholine                                  | Sigma Aldrich       | 63556                    |
| Pifithrin                                            | Sigma Aldrich       | P0122                    |
| Gefitinib                                            | Sigma Aldrich       | SML1657                  |
| Bafilomycin A                                        | Sigma Millipore     | B1793-10UG               |
| Rapamycin                                            | Sigma Aldrich       | R8781                    |
| Geranylgeranylacetone                                | Sigma Aldrich       | G5048                    |
| Ivermectin                                           | MedChemExpress      | MK-933                   |
| Importazole                                          | Calbiochem          | 401105                   |
| <b>Commercial Assays</b>                             |                     |                          |
| IL-33 ELISA DuoSet                                   | R&D Systems         | DY3625                   |
| HSP70 ELISA DuoSet                                   | R&D Systems         | DY1663                   |

|                                                                                                                         |                                        |            |
|-------------------------------------------------------------------------------------------------------------------------|----------------------------------------|------------|
| HSP90 ELISA                                                                                                             | Proteintech                            | KE00054    |
| ST2                                                                                                                     | R&D Systems                            | DY523B     |
| RAGE                                                                                                                    | R&D Systems                            | DY1145     |
|                                                                                                                         |                                        |            |
| Cell Lines                                                                                                              |                                        |            |
| HBE-1                                                                                                                   | Marisco Lung Institute                 | HBE1 C1p29 |
| HEK-Blue IL-33 Cells                                                                                                    | Invivogen                              | hkb-hil33  |
| A549 Dual Cells                                                                                                         | Invivogen                              | a549d-nfis |
| U937                                                                                                                    | ATCC                                   | CRL-1593.2 |
|                                                                                                                         |                                        |            |
| qPCR assays                                                                                                             | IDT PrimeTime® predesigned qPCR assays |            |
| HSPA1A                                                                                                                  | 421177696                              |            |
| HSPA8                                                                                                                   | 421177700                              |            |
| IL33                                                                                                                    | 441084120                              |            |
| STIP1                                                                                                                   | 454622465                              |            |
| STUB1                                                                                                                   | 454622457                              |            |
| HSP90AA1                                                                                                                | 416230057                              |            |
| SMPD3                                                                                                                   | 218154457                              |            |
| MUC5AC                                                                                                                  | 166039047                              |            |
| DNAJB1                                                                                                                  | 424729991                              |            |
| CERT1                                                                                                                   | 414584019                              |            |
| LAMP2                                                                                                                   | 206830890                              |            |
| SQSTM1                                                                                                                  | 454622461                              |            |
| CCT5                                                                                                                    | 508892978                              |            |
| CCT7                                                                                                                    | 508892982                              |            |
|                                                                                                                         |                                        |            |
| Constructs                                                                                                              |                                        |            |
| human Flag-IL-33 <sup>Δ34</sup> -His<br>C208S<br>C228S<br>C232S<br>C259S<br>C208/259S<br>C228/232S<br>C208/228/232/259S | pCDH – puro, neo                       |            |
| human IL-33 <sup>Δ34</sup> -birA-6His                                                                                   | pET21                                  |            |
| human IL-33 <sup>Δ34</sup> -MiniTurbo-V5                                                                                | pCDH - neo                             |            |
| Human HSP70-His                                                                                                         | pCDH - neo                             |            |
| Human HSP70 (K573Q)-His                                                                                                 | pCDH - neo                             |            |
| Human HSP70 (K589Q)-His                                                                                                 | pCDH - neo                             |            |

## SUPPLEMENTARY REFERENCES

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2. Greven JA, and Brett TJ. Production of Eukaryotic Glycoproteins for Structural and Functional Studies Using Expi293F Cells. *Curr Protoc*. 2022;2(8):e512.
3. Couper D, LaVange LM, Han M, Barr RG, Bleecker E, Hoffman EA, et al. Design of the Subpopulations and Intermediate Outcomes in COPD Study (SPIROMICS). *Thorax*. 2014;69(5):492.