Online Data Supplement

Syndecan-1 Attenuates Lung Injury During Influenza Infection By Potentiating c-Met Signaling to Suppress Epithelial Apoptosis

Rena Brauer[1,2], Lingyin Ge[1], Saundra Y. Schlesinger[2], Timothy P. Birkland[1,2], Ying Huang[1], Tanyalak Parimon[1], Vivian Lee[2], Bonnie L. McKinney, John K. McGuire[4], William C. Parks[1,2], Peter Chen[1,2]

[1]Women's Guild Lung Institute, Cedars-Sinai Medical Center; Los Angeles, CA. USA

[2]Department of Medicine, Division of Pulmonary and Critical Care Medicine, University of Washington; Seattle, WA. USA

[3]Department of Pathology, University of Washington; Seattle, WA. USA

[4]Department of Pediatrics, University of Washington; Seattle, WA. USA

Supplemental Methods

Influenza Model Treatment Experiments

In some experiments, mice were treated with daily intraperitoneal injections of zVAD-FMK (10 mg/kg), a pan-caspase inhibitor (Bachem, Torrence, CA) or 10% DMSO (the carrier control). Mice were injected between 9 – 11 am daily starting on day 1 post-infection. Mice were also treated with murine recombinant HGF (Hepatocyte Growth Factor, carrier-free; eBioscience, San Diego, CA) by daily intranasal instillations of 0.2 mg/kg HGF or PBS control. Additionally, some mice were treated daily with the c-Met inhibitor, SGX-523 (25 mg/kg oral gavage; Selleckchem, Houston, TX) or methylcellulose control.

Syndecan-1 was cloned into an AAV expression plasmid, and AAV1 vectors were generated by the University of Pennsylvania (http://www.med.upenn.edu/gtp/vectorcore/). Mice were intranasally inoculated with 10^{11} genome copies of AAV1 virus in 30 µl sterile PBS (1). After waiting 21 days for optimal transduction of the transgene in the lung epithelium, mice were intranasally infected with 500 PFU PR8 influenza virus.

Cell culture treatment

BEAS-2b cells were infected with PR8 MOI 1.0 for indicated time points, and ALI cultures were infected with PR8 MOI 0.1 for 24h or MOI 1.0 for time course experiments. As indicated, some experiments included treatment of 50 µM zVAD in the conditioned medium for the duration of the experiment. When conditions were treated with either 50 ng/ml human or mouse recombinant HGF (PeproTech, Rocky Hill, NJ) or the c-Met inhibitor, SGX-523 (500 nM; Selleckchem, Houston, TX), cells were first pretreated for 1.5h prior to the addition of PR8 and left on for the course of infection.

Immunoblotting

Cells were lysed in ice-cold RIPA buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN). Frozen lung tissue was placed in 1 ml ice-cold RIPA buffer with the inhibitors and homogenized twice for 30 sec using a bead homogenizer (Omni Bead Ruptor Homogenizer, Omni International, Kennesaw, GA). Western blotting was performed with antibodies against AKT, phospho-AKT, c-Met, or phospho-c-Met (all 1:1000; Cell Signaling, Danvers, MA). Dot blot for syndecan-1 in bronchoalveolar lavage (BAL) fluid was performed using a rat anti-mouse syndecan-1 antibody (clone 281-2, 1:2000, BD Bioscience) as previously described (2). The membranes were then developed by chemiluminescence using SuperSignal West Femto Substrate (Pierce, Rockford, IL).

Lung and Bronchoalveolar Lavage (BAL) Fluid Analysis

Euthanized mice had BAL fluid collected by 3 consecutive lavages with 1 mL of sterile PBS. Inflammatory cells were counted and pelleted from the BAL fluid, and then used to create cytospins with subsequent Diff-Quik (Siemens, Newark, DE) staining for determining the neutrophil and macrophage percentages. Murine specific ELISAs were used to quantify levels of IgM (Bethyl Laboratories, Montgomery, TX), and total protein content was determined by BCA assay (Thermo Scientific/Pierce, Rockford, IL). Lungs were also used for either RNA, protein collection, or fixed with 10% formalin for histologic evaluation. Representative pictures were taken using a 10x/0.30, UPlanFI objective on an Olympus BX51 microscope.

Caspase-3 Assays

For detection of apoptosis, a fluorogenic activated caspase-3 assay was used following the manufacturer's protocol (Biovision, Milpitas, CA). Briefly, cells were infected with PR8 virus as described above. After 24 hours, cells were lysed in cold cell lysis buffer. Fluorogenic peptide was added, and fluorescence was read at 15-minute intervals for 4h. The slope of the

increasing signal was determined and used as a numerical representation of the caspase-3 activity.

To immunostain for apoptotic cells in lung tissue, immunohistochemistry was performed for cleaved caspase-3 (SignalStain Apoptosis; Cell Signaling, Danvers, MA). Representative pictures were taken using a 10x/0.30, UPlanFI objective on an Olympus BX51 microscope.

Viral quantification

We isolated total RNA from whole lung homogenate using the Qiagen RNeasy kit, and performed qRT-PCR for matrix protein 2 (M2) of influenza A virus subtype H1N1 using the following primers: 5' primer: CAT CCT GTT GTA TAT GAG GCC CAT; 3' primer: GGA CTC CAG CGT AGA CGC TT

We also used a plaque assay to quantify viral titers. In brief, MDCK cells ($1x10^6$ cells/well) were seeded into 6-well plates and infected the next day with ten-fold dilutions (10^{-1} to 10^{-6}) of virus containing samples (e.g., lung homogenate) for 1h at 37°C. Cells were overlayed with 0.6% agar in DMEM and incubated for 48h at 37°C before fixing and staining with crystal violet solution (0.25% in 20% ethanol) to identify viral plaques.

Supplemental Figures

Figure E1. Expression of chemokine, viral response genes, and influenza viral load in lung homogenates of *Sdc1*$^{-/-}$ mice following influenza infection. Gene expression of pro-inflammatory chemokines (**A**) *Ccl3*, (**B**) *Ccl2*, (**C**) *Cxcl1* and viral response genes (**D**) *Pkr* and (**E**) *Infa2* in total lung tissue over the time-course of influenza infection (250 PFU) as measured by qRT-PCR (n = 7 to 10; **p*<0.05, ****p*<0.001). **(F)** Influenza virus was quantified in mouse lung tissue by qRT-PCR to detect influenza virus RNA. Data are shown as relative expression ($2^{-\Delta Ct}$) ± SEM (n = 4 to 9 per time point). There was no statistical difference between genotypes by Two-Way ANOVA. Influenza viral plaque assay of **(G)** BAL fluid and **(H)** lung homogenates from influenza infected mice (day 6; 500 PFU; n = 4). **(I)** IL-1β and **(J)** IL-18 levels were measured in lung homogenates of mice after influenza infection (day 6; 500 PFU) by ELISA. All data was normalized to total protein concentration. All data shown as mean ± SEM.

Figure E2. AAV1 vectors transduce the lung epithelium *in vivo* and *in vitro*. (**A**) Immunohistochemical staining with an anti-GFP antibody (ab290, rabbit polyclonal) in WT mice transduced with AAV1-GFP or control (no AAV instilled). (**B**) Immunofluorescence for syndecan-1 (clone 281.2) in *Sdc1*$^{-/-}$ mice transduced with AAV1-Sdc1 or AAV1-GFP. Syndecan-1 expression can be seen primarily in airway epithelial cells and less so in alveolar type II cells, which is consistent with the distribution seen in WT conditions. The asterisk marks the airway lumens. (**C**) AAV vectors carrying the GFP transgene was added to ALI cultures. Membranes were fixed and mounted for immunofluorescent images 2 weeks after transduction and showed stable expression in the majority of cells. Scalebar = 50 μm. Control ALI cultures (no AAV infection) do not have any appreciable fluorescent signal when images were taken at the same exposure settings.

Figure E3. Syndecan-1 expression by the lung epithelial attenuates apoptosis during influenza infection. WT and *Sdc1*$^{-/-}$ mice were infected with influenza (500 PFU, Day 6) and lungs were processed for TUNEL staining (VitroView TUNEL Apoptosis/DAB Kit; GeneCopoeia). For each mouse lung, TUNEL+ epithelial cells were counted in ten random **(A)** airways (0.43 ± 0.25 vs. 8.03 ± 1.63) and **(B)** high power fields of alveolar cells (4.97 ± 1.08 vs. 25.90 ± 3.92). n = 3; **p*<0.0001.

Figure E4. Subcutaneous administration of zVAD suppresses apoptosis in the lungs of *Sdc1*$^{-/-}$ mice during influenza infection. Caspase-3 activity was measured in lung homogenates from WT and *Sdc1*$^{-/-}$ mice treated with DMSO (0.30 ± 0.05 vs. 0.64 ± 0.18) or zVAD (0.26 ± 0.15 vs. 0.24 ± 0.07) 6 days after influenza infection (500 PFU). **p*<0.05. All data shown as mean ± SEM.

Figure E5. Suppressing apoptosis reduces lung inflammation in *Sdc1*$^{-/-}$ mice during influenza infection. WT and *Sdc1*$^{-/-}$ mice treated with zVAD during influenza infection (500 PFU). Lung homogenates were analyzed by ELISA for pro-inflammatory cytokine, IL-6 (Mouse IL-6 ELISA; eBioscience, San Diego, CA) and for the chemokines, CXCL1 (KC) and CXCL2 (MIP-2α) (Mouse CXCL1/KC and Mouse CXCL2 DuoSet; R&D Systems, Minneapolis, MN) according to the manufacturer's protocols. n = 8 to 10; **p*<0.05, ***p*<0.01, ****p*<0.001; 6 days post-infection. All data shown as mean ± SEM.

Figure E6. Expression of activated AKT in the lung epithelium attenutes apoptosis and improves outcomes in mice infected with influenza. (**A**) ALI cultures were transduced with AAV1 carrying either the transgene for GFP or active (myristolated) AKT. Subsequently, ALI

cultures were infected with PR8 for 24 hours and processed for caspase-3 activity. (**B – C**) AAV1 vectors were intranasally instilled into WT and *Sdc1*$^{-/-}$ mice to transduce either GFP or active AKT in the lung epithelium, and subsequently infected intranasally with 500 PFU of PR8 virus. (**B**) Body weight loss (n = 4 – 5; **p*<0.01, ***p*<0.001 WT + GFP vs. *Sdc1*$^{-/-}$ + GFP, $^{\#}p$<0.05, $^{\#\#}p$<0.0001 *Sdc1*$^{-/-}$+ GFP vs. *Sdc1*$^{-/-}$ + Sdc1) and (**C**) total cell count (normalized to WT control). GFP: 1.0 ± 0.14 vs. 1.44 ± 0.14; AKT: 1.07 ± 0.06 vs. 1.14 ± 0.14. n = 4 – 5. **p* < 0.05. All data shown as mean ± SEM.

Figure E7. Syndecan-1 augments c-Met and AKT signaling in bronchial epithelial cells stimulated with HGF. Western blot for p-AKT, AKT, p-c-Met, c-Met, and β-actin in WT and *Sdc1*$^{-/-}$ ALI cultures stimulated with HGF. Total AKT and c-Met levels were identical in both genotypes similar to β-actin levels and are appropriate controls for p-AKT and p-c-MET immunoblots.

Figure E8. Apoptosis of WT ALI cultures after influenza infection is modulated by c-Met signaling. Caspase-3 activity was measured in WT and *Sdc1*$^{-/-}$ ALI cultures infected with PR8 virus with or without treatment with HGF or a c-Met inhibitor (SGX-523, Selleckchem #S1112); n = 3. All data shown as mean ± SEM.

Figure E9. HGF treatment of WT mice improved weight loss and survival after influenza infection. Mice were infected with PR8 and concomitantly treated with intranasal HGF. n = 10 for PBS; n = 15 for HGF. **p*<0.05, ***p*<0.01, ****p*<0.005.

Figure E10. Caspase-3 activity in WT mice after influenza infection. Mice were infected with PR8 and concomitantly treated with either **A)** a c-Met inhibitor by oral gavage (133.7 ± 14.92 vs.

248.5 ± 33.04; n = 4 – 5) or **B)** intranasal HGF (155.2 ± 11.79 vs. 82.82 ± 6.02; n = 9 – 12). Lungs were processed 6 days after infection for caspase-3 activity. *$p < 0.05$. All data shown as mean ± SEM.

Supplemental Figure E1

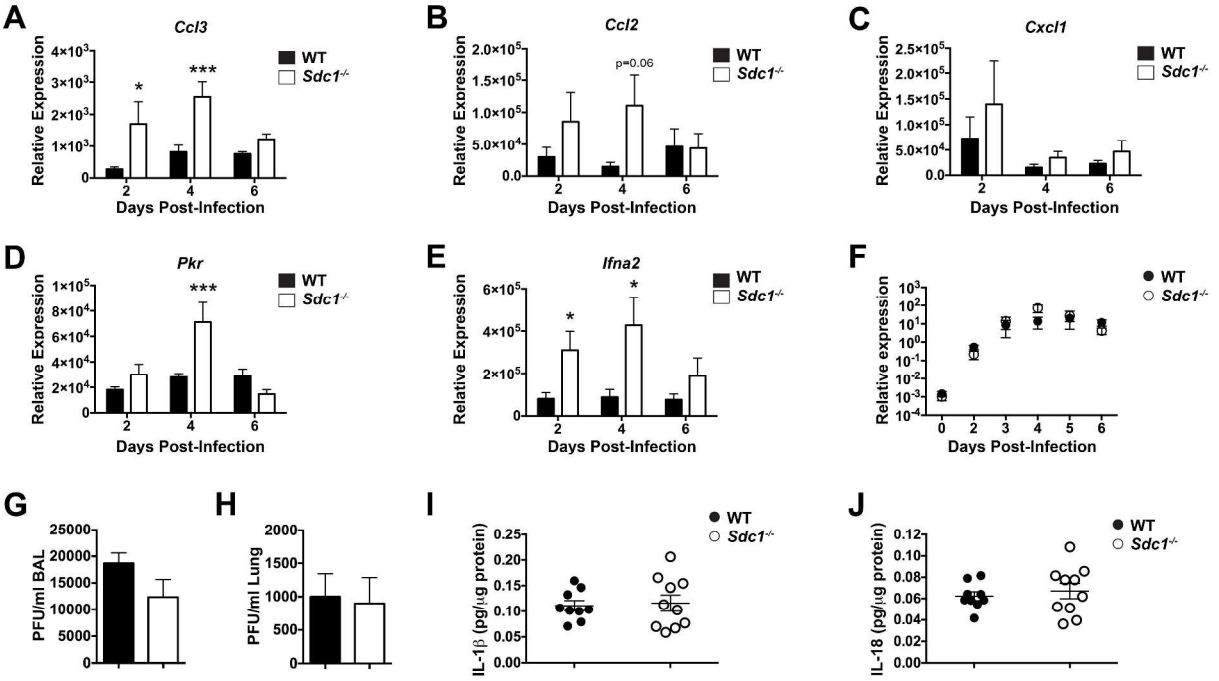

Figure E1. Expression of chemokine, viral response genes, and influenza viral load in lung homogenates of *Sdc1*$^{-/-}$ mice following influenza infection. Gene expression of pro-inflammatory chemokines (**A**) *Ccl3*, (**B**) *Ccl2*, (**C**) *Cxcl1* and viral response genes (**D**) *Pkr* and (**E**) *Infa2* in total lung tissue over the time-course of influenza infection (250 PFU) as measured by qRT-PCR (n = 7 to 10; **p*<0.05, ****p*<0.001). **(F)** Influenza virus was quantified in mouse lung tissue by qRT-PCR to detect influenza virus RNA. Data are shown as relative expression ($2^{-\Delta Ct}$) ± SEM (n = 4 to 9 per time point). There was no statistical difference between genotypes by Two-Way ANOVA. Influenza viral plaque assay of **(G)** BAL fluid and **(H)** lung homogenates from influenza infected mice (day 6; 500 PFU; n = 4). **(I)** IL-1β and **(J)** IL-18 levels were measured in lung homogenates of mice after influenza infection (day 6; 500 PFU) by ELISA. All data was normalized to total protein concentration. All data shown as mean ± SEM.

Supplemental Figure E2

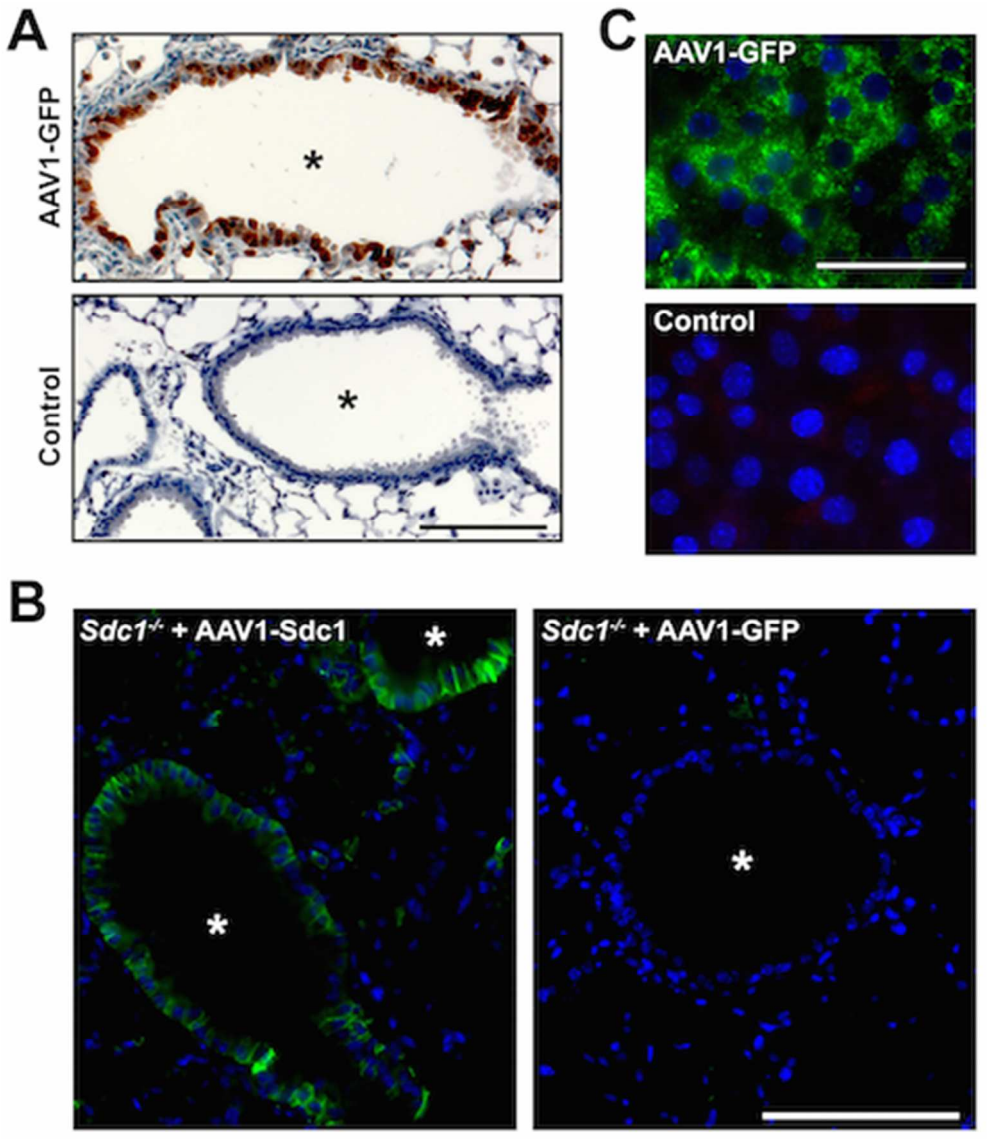

Figure E2. AAV1 vectors transduce the lung epithelium *in vivo* and *in vitro*. (**A**) Immunohistochemical staining with an anti-GFP antibody (ab290, rabbit polyclonal) in WT mice transduced with AAV1-GFP or control (no AAV instilled). (**B**) Immunofluorescence for syndecan-1 (clone 281.2) in *Sdc1*$^{-/-}$ mice transduced with AAV1-Sdc1 or AAV1-GFP. Syndecan-1 expression can be seen primarily in airway epithelial cells and less so in alveolar type II cells, which is consistent with the distribution seen in WT conditions. The asterisk marks the airway lumens. (**C**) AAV vectors carrying the GFP transgene was added to ALI cultures. Membranes were fixed and mounted for immunofluorescent images 2 weeks after transduction and showed stable expression in the majority of cells. Scalebar = 50 μm. Control ALI cultures (no AAV infection) do not have any appreciable fluorescent signal when images were taken at the same exposure settings.

Supplemental Figure E3

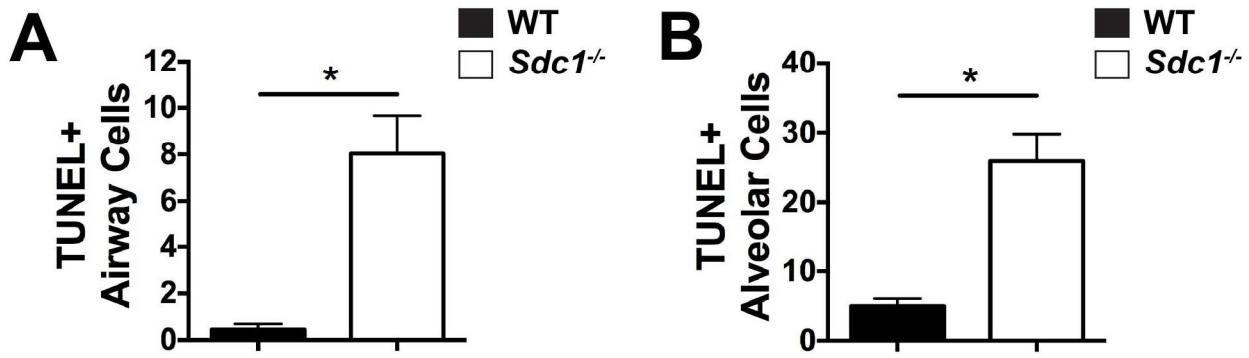

Figure E3. Syndecan-1 expression by the lung epithelial attenuates apoptosis during influenza infection. WT and *Sdc1*$^{-/-}$ mice were infected with influenza (500 PFU, Day 6) and lungs were processed for TUNEL staining (VitroView TUNEL Apoptosis/DAB Kit; GeneCopoeia). For each mouse lung, TUNEL+ epithelial cells were counted in ten random **(A)** airways (0.43 ± 0.25 vs. 8.03 ± 1.63) and **(B)** high power fields of alveolar cells (4.97 ± 1.08 vs. 25.90 ± 3.92). n = 3; **p*<0.0001.

Supplemental Figure E4

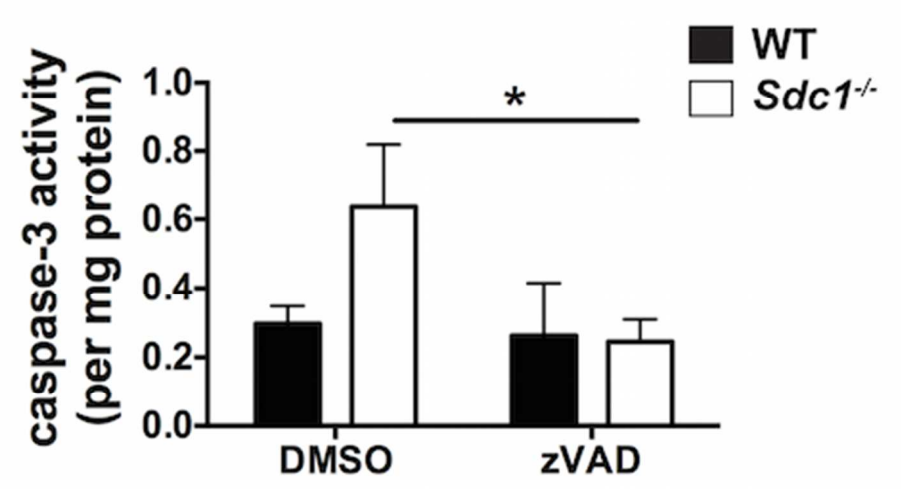

Figure E4. Subcutaneous administration of zVAD suppresses apoptosis in the lungs of *Sdc1*$^{-/-}$ mice during influenza infection. Caspase-3 activity was measured in lung homogenates from WT and *Sdc1*$^{-/-}$ mice treated with DMSO (0.30 ± 0.05 vs. 0.64 ± 0.18) or zVAD (0.26 ± 0.15 vs. 0.24 ± 0.07) 6 days after influenza infection (500 PFU). *p<0.05. All data shown as mean ± SEM.

Supplemental Figure E5

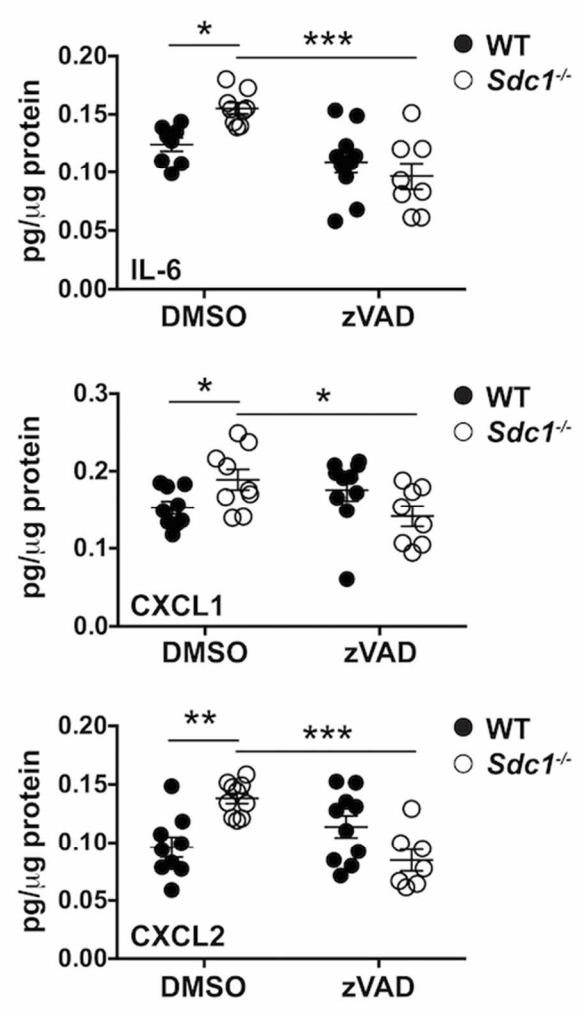

Figure E5. Suppressing apoptosis reduces lung inflammation in *Sdc1*$^{-/-}$ mice during influenza infection. WT and *Sdc1*$^{-/-}$ mice treated with zVAD during influenza infection (500 PFU). Lung homogenates were analyzed by ELISA for pro-inflammatory cytokine, IL-6 (Mouse IL-6 ELISA; eBioscience, San Diego, CA) and for the chemokines, CXCL1 (KC) and CXCL2 (MIP-2α) (Mouse CXCL1/KC and Mouse CXCL2 DuoSet; R&D Systems, Minneapolis, MN) according to the manufacturer's protocols. n = 8 to 10; *p<0.05, **p<0.01, ***p<0.001; 6 days post-infection. All data shown as mean ± SEM.

Supplemental Figure E6

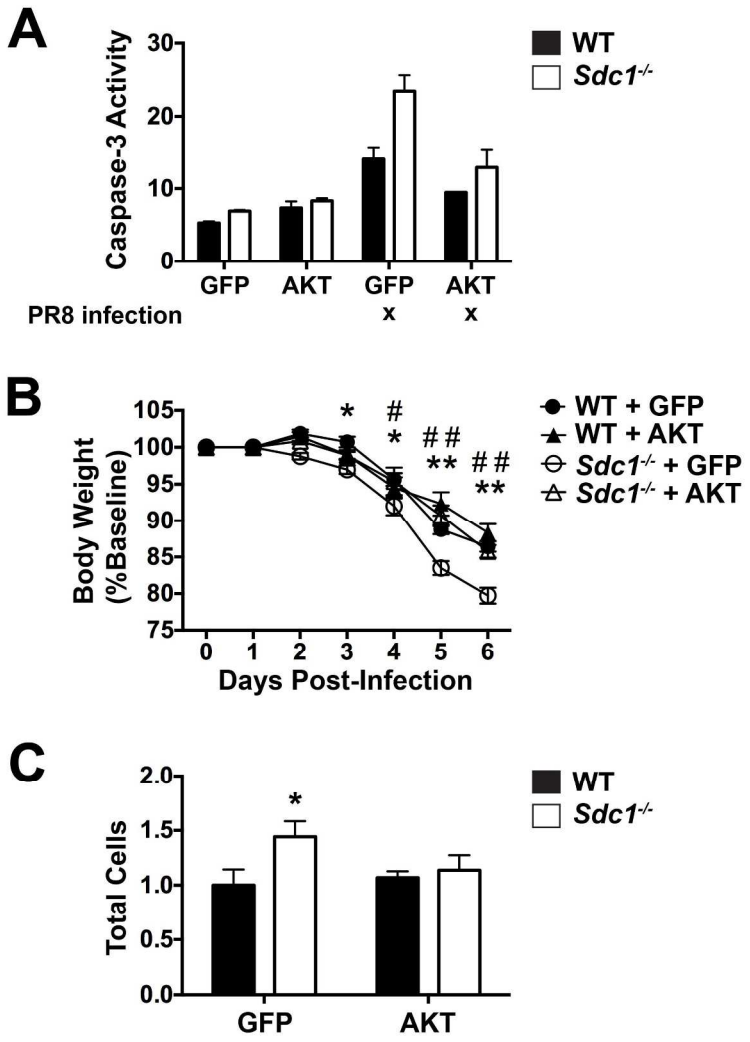

Figure E6. Expression of activated AKT in the lung epithelium attenutes apoptosis and improves outcomes in mice infected with influenza. (**A**) ALI cultures were transduced with AAV1 carrying either the transgene for GFP or active (myristolated) AKT. Subsequently, ALI cultures were infected with PR8 for 24 hours and processed for caspase-3 activity. (**B – C**) AAV1 vectors were intranasally instilled into WT and *Sdc1*$^{-/-}$ mice to transduce either GFP or active AKT in the lung epithelium, and subsequently infected intranasally with 500 PFU of PR8 virus. (**B**) Body weight loss (n = 4 – 5; $^{*}p<0.01$, $^{**}p<0.001$ WT + GFP vs. *Sdc1*$^{-/-}$ + GFP, $^{\#}p<0.05$, $^{\#\#}p<0.0001$ *Sdc1*$^{-/-}$+ GFP vs. *Sdc1*$^{-/-}$ + Sdc1) and (**C**) total cell count (normalized to WT control). GFP: 1.0 ± 0.14 vs. 1.44 ± 0.14; AKT: 1.07 ± 0.06 vs. 1.14 ± 0.14. n = 4 – 5. $^{*}p < 0.05$. All data shown as mean ± SEM.

Supplemental Figure E7

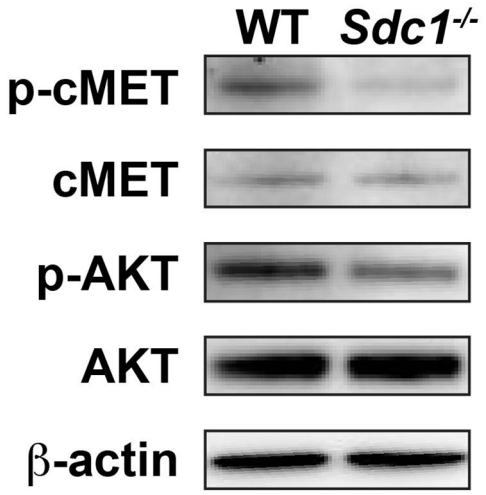

Figure E7. Syndecan-1 augments c-Met and AKT signaling in bronchial epithelial cells stimulated with HGF. Western blot for p-AKT, AKT, p-c-Met, c-Met, and β-actin in WT and *Sdc1*$^{-/-}$ ALI cultures stimulated with HGF. Total AKT and c-Met levels were identical in both genotypes similar to β-actin levels and are appropriate controls for p-AKT and p-c-MET immunoblots.

Supplemental Figure E8

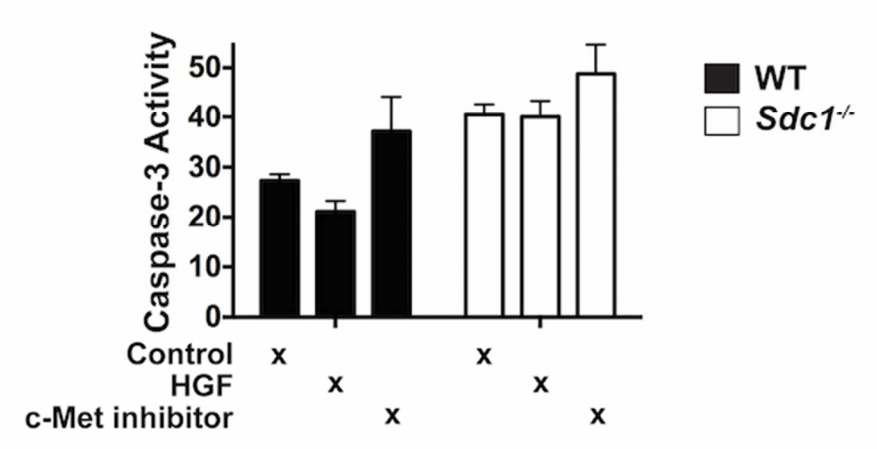

Figure E8. Apoptosis of WT ALI cultures after influenza infection is modulated by c-Met signaling. Caspase-3 activity was measured in WT and *Sdc1*$^{-/-}$ ALI cultures infected with PR8 virus with or without treatment with HGF or a c-Met inhibitor (SGX-523, Selleckchem #S1112); n = 3. All data shown as mean ± SEM.

Supplemental Figure E9

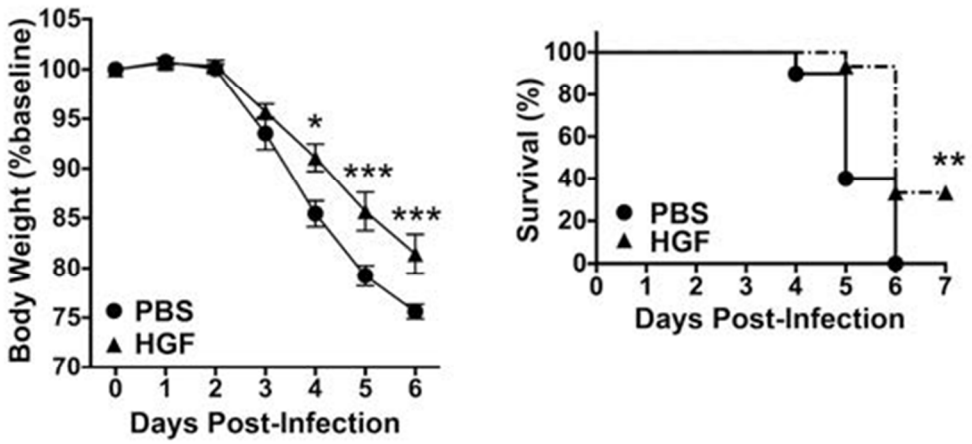

Figure E9. HGF treatment of WT mice improved weight loss and survival after influenza infection. Mice were infected with PR8 and concomitantly treated with intranasal HGF. n = 10 for PBS; n = 15 for HGF. *p<0.05, **p<0.01, ***p<0.005. All data shown as mean ± SEM.

Supplemental Figure E10

Figure E10. Caspase-3 activity in WT mice after influenza infection. Mice were infected with PR8 and concomitantly treated with either **A)** a c-Met inhibitor by oral gavage (133.7 ± 14.92 vs. 248.5 ± 33.04; n = 4 – 5) or **B)** intranasal HGF (155.2 ± 11.79 vs. 82.82 ± 6.02; n = 9 – 12). Lungs were processed 6 days after infection for caspase-3 activity. *$p < 0.05$. All data shown as mean ± SEM.

References

1. Limberis MP, Vandenberghe LH, Zhang L, Pickles RJ, Wilson JM. Transduction efficiencies of novel AAV vectors in mouse airway epithelium in vivo and human ciliated airway epithelium in vitro. *Mol Ther* 2009;17:294–301.
2. Li Q, Park PW, Wilson CL, Parks WC. Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. *Cell* 2002;111:635–646.