

## **SUPPLEMENTAL MATERIALS AND METHODS:**

### **Immunohistochemistry, Immunofluorescence, and Hematoxylin and Eosin (H&E)**

#### **Stains**

*Immunohistochemistry*—Human control and IPAH lung sections were stained using a Ventana Benchmark XT automated immunostainer (Roche Diagnostics Corporation, Indianapolis, IN) combined with a Ventana OptiView DAB Detection kit. CC2 retrieval was performed for 32 minutes followed by Opti-View amplification (4 minutes). HQ Universal Linker and HRP Multimer dilution options were both employed. Primary antibody, mouse anti-O-GlcNAc (RL-2 antibody generous gift from Dr. Gerald Hart) was diluted 1:50 and incubated for 32 minutes with heat. Slides were counterstained with Hematoxylin II, dehydrated, cleared, and permanently mounted for viewing using the same microscope and capture software as the H&E staining.

*Immunofluorescence*—Paraffin embedded human lung tissue sections were fixed with the FLEX Signature Series reagents (Richard-Allan Scientific, Thermo Scientific, USA) and stained with a primary biotinylated WGA lectin (1:500) followed by retrieval with a streptavidin conjugated Alexa-fluor-533 antibody (1:500). Tissues were mounted with media containing DAPI overnight and sealed before images were taken. Snapshots of distinct regions were taken using a Leica DM 5500B equipped with a 20x (numerical aperture 0.4) objective. Images were taken using an attached QImaging Retiga-SRV camera and QCapture 7.0 software.

*Hematoxylin and eosin (H&E) stains*—Lung tissues taken from explanted lungs were fixed and embedded in paraffin and 4  $\mu\text{m}$  sections were prepared. The sections

were stained with H&E using standard procedures for proper lung orientation and morphological assessment. Snapshots of histology were taken using a Leica DM 5500B microscope equipped with a 20x (numerical aperture 0.4) objective. Images were generated using an attached Leica DFC 425C camera and the high-performance Leica LAS software.

### **Fluorophore Assisted Carbohydrate Electrophoresis (FACE) UDP-Sugar Analysis**

PASMCs were washed and collected in cold PBS scraped with a rubber policeman. PASMCs were centrifuged, pelleted and fixed with 75% cold ethanol. Lysates were generated by sonication in the 75% ethanol and kept on ice. An aliquot of lysate was taken and stored at -20 °C for DNA quantitation and normalization using the Quant-IT PicoGreen dsDNA kit (Life technologies, NY, USA). Cell debris was removed by centrifugation (16,000 × *g* for 10 min at 4°C). The supernatants were dried and reconstituted in 10 mM ammonium bicarbonate. The UDP-sugars were then isolated and purified using an Envi-Carb column (Sigma, St. Louis, MO, USA) procedure(1) with slight modifications. Briefly, reconstituted samples were applied to the column and washed with (i) sterile filtered water, followed by (ii) a 25% acetonitrile solution, and then (iii) a 50 mM triethylamine acetate buffer (pH 7). UDP-sugars were eluted with 2 ml of 25% acetonitrile in 50 mM triethylamine acetate buffer (pH 7) and speed-vacuumed to dryness. The UDP-sugars were prepared for AMAC conjugation by subjecting the dried sample to mild acid hydrolysis (50 mM HCl) at 100°C for 20 minutes to remove the nucleotide from the sugar monosaccharide. Acid hydrolyzed samples were then dried,

and monosaccharides were solubilized in acetic acid followed by derivatization with aminoacridine (AMAC) and sodium cyanoborohydride for FACE analysis(2). A monosaccharide standard was also run simultaneously to determine the mobility of GlcNAc-AMAC within the FACE gel. In addition, a known purified amount of UDP-GlcNAc was processed using this protocol and the percent yield was determined (data not shown). Images of the gel were captured using the G:BOX Chemi XR5 system and Gene Tools software v4.3.00. The densitometry of GlcNAc-AMAC within the FACE gel was determined using ImageJ(3) software and normalized to total cellular DNA.

### **siRNA Transfection and OGT Inhibitor Experiments**

PASMCs at 85-90% confluency were transfected with either a scrambled (Ambion Applied Biosystems, Inc.; cat # AM4611, NY, USA) or an antisense siRNA oligonucleotide against OGT (Ambion Applied Biosystems, Inc.; cat # 13301, NY, USA) at 60 nM using lipofectamine 2000 (Invitrogen, USA) and incubated in Opti-MEM (Cleveland Clinic Media Core) overnight. The following day, transfection medium was changed to the SmGM-2 medium and cells were grown for an additional 24 hours. At this point, confluent PASMCs were trypsinized and re-seeded in a six-well dish at a density of  $1.5 \times 10^5$  cells/mL. PASMCs were allowed to adhere, and siRNA transfection was repeated to enrich knock-down. At 48 hours post-transfection, cells were either taken for Western blot or BrdU incorporation and flow cytometric analysis. Cell images were taken using an Olympus CKX41 microscope containing a 10x (0.25 PHP)

objective with an attached SC30 camera at 2 hours and 48 hours post-enrichment transfection.

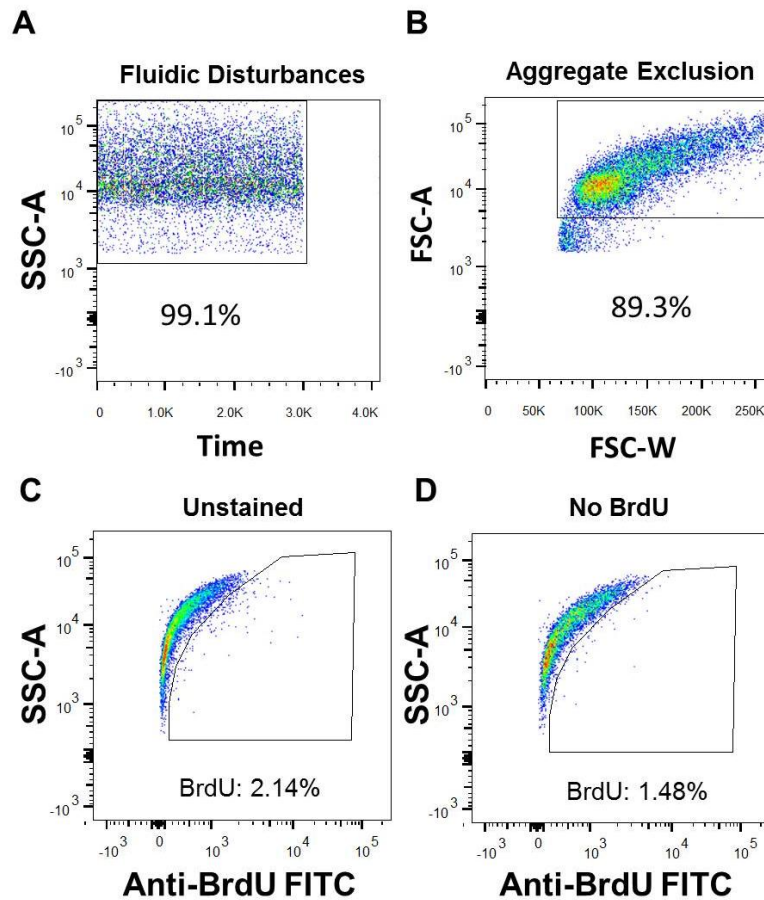
### **Cell Proliferation and Flow Cytometry Analysis**

Following a 4 hour serum starvation, PSMCs were pulsed for 2 hours with 10  $\mu$ M 5-bromo-2'-deoxyuridine (BrdU) (Sigma, St. Louis, MO, USA) followed by trypsinization to detach cells. Subsequently, the cell suspension was centrifuged for 10 minutes (RT) at 400 x g (all centrifugation steps used these conditions). Supernatants were aspirated, and cells were subjected to ice cold 70% ethanol to a final concentration of  $1 \times 10^6$  cells/100  $\mu$ L and stored in the -80°C freezer until all BrdU incorporation assays were finished. For preparation of flow cytometric staining, cells were thawed and washed in PBS containing 0.5% BSA, and then centrifuged and resuspended in 100  $\mu$ L denaturing solution (PBS containing 0.5% Tween-20, 0.5% BSA). Following 20 minutes of incubation at room temperature, cells were washed, centrifuged, and resuspended 0.1 M sodium borate (pH 8.5) to neutralize any residual acid. Upon extensive washing, an unconjugated mouse monoclonal antibody against BrdU (1:50, Ab-3 clone BRD.3, Thermo Scientific, Waltham, MA, USA) was added to the sample and incubated at room temperature for 20 minutes followed by washes and centrifugation to remove any unbound primary antibody. The secondary FITC-conjugated goat anti-mouse (1:50, BD Pharmingen, San Diego, CA, USA) was added to the cells, incubated at room temperature for 20 minutes, and subsequently washed and centrifuged to remove unbound secondary. As a biological control, cells in parallel

experiments were prepared with no BrdU incubation or analyzed with only secondary antibody (unstained) (Supplemental Figure 2). Data were generated by flow cytometry using the LSRFortessa cell analyzer (Becton Dickinson, USA) using the standard configuration. Cells were excited at 488 nm, and the BrdU linked green fluorescence (FITC) was detected through a 515/20 bandpass filter. Flowjo software (version 10) was used for analysis and the percentage of BrdU incorporated cells was determined. The gating strategy and the relevant controls for flow cytometric analysis are shown in Supplemental Figure 1.

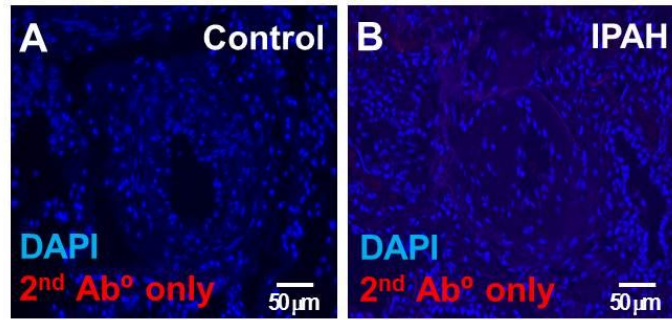
**SUPPLEMENTAL FIGURES:**

**Supplemental Figure 1**



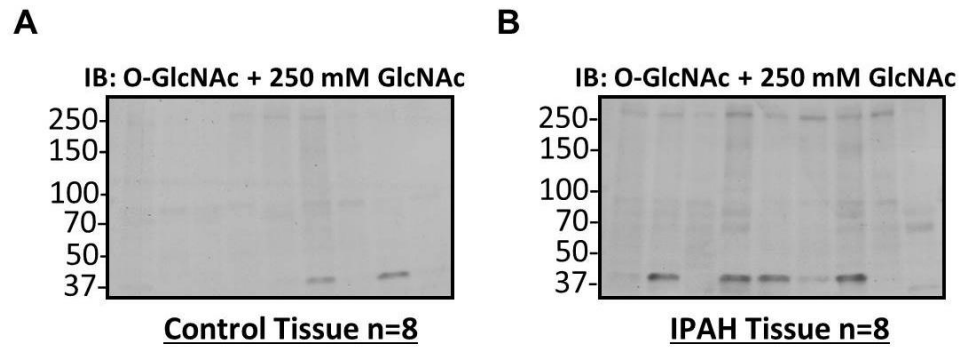
**Supplemental Figure 1. Flow cytometry control parameters.** (A) Time gating was performed on a Time/SSc plot to control for fluidic disturbances. (B) Aggregates were excluded using a FSC-W/FSC-A plot. Percent BrdU positive cells were determined on a pseudo-color plot as depicted in Figure 3. As a biological control, cells were treated with (C) only secondary (unstained) or (D) no BrdU in parallel experiments.

**Supplemental Figure 2**



**Supplemental Figure 2. Secondary control for Control and IPAH lung tissue staining of WGA (Figure 1 A and B).** Representative secondary controls for control (A) and IPAH (B) lung tissue.

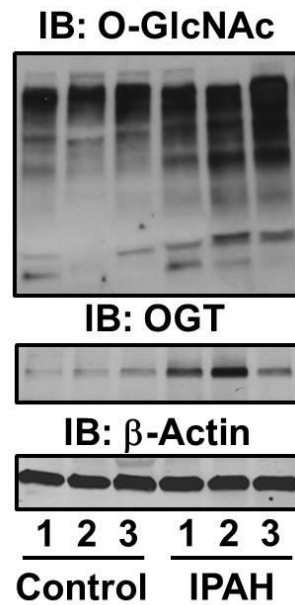
### Supplemental Figure 3



**Supplemental Figure 3. O-GlcNAc antibody (CTD 110.6) control for reactivity.** The O-GlcNAc primary antibody was subjected to a pre-incubation with 250 mM GlcNAc in 5% BSA (GlcNAc block) for an hour at room temperature. Transferred nitrocellulose membranes were then exposed to the GlcNAc block and continued to completion as described in the *Materials and Methods*.

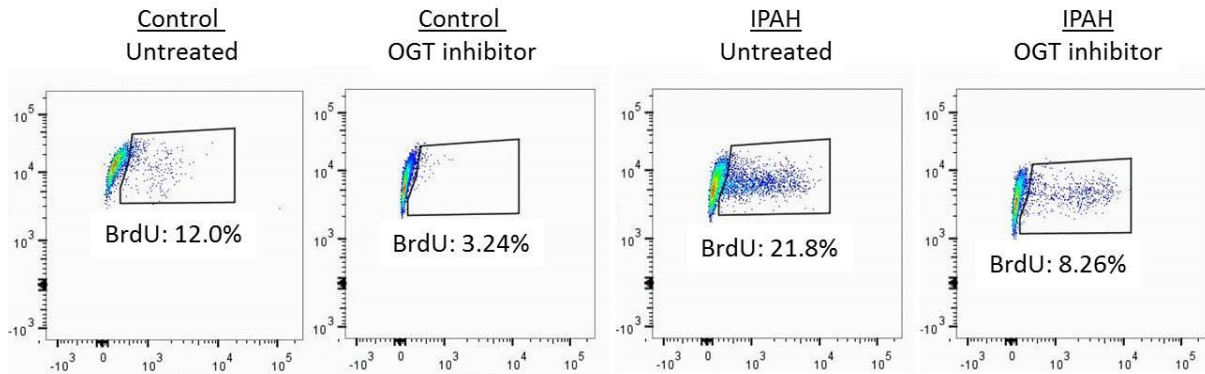


**Supplemental Figure 4**



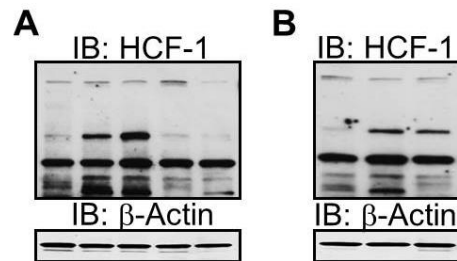
**Supplemental Figure 4. Global O-GlcNAc and OGT levels are increased in PAECs.** A Representative Western blot for the Global O-GlcNAc and OGT examined in multiple patient PAECs, control and IPAH (n=3). As a loading control,  $\beta$ -Actin was used.

## Supplemental Figure 5



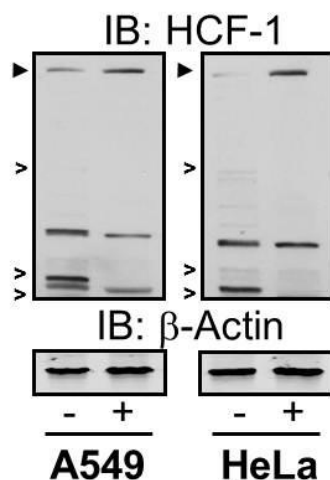
**Supplemental Figure 5. Chemical inhibition of OGT by Alloxan reduces cell proliferation in PAH PSMCs.** Control and IPAH PSMCs were plated and treated with and without alloxan monohydrate (5mM) for 24hrs, followed by BrdU incorporation for 2 hours. The cells were subsequently collected and subjected to flow cytometry analysis (see *Supplemental Materials and Methods*).

Supplemental Figure 6



**Supplemental Figure 6. A longer exposure of the Immunoblot for HCF-1 as denoted in Figure 4. (A) siRNA specific KD of OGT and (B) an inhibitor to OGT under the conditions specified.**

## Supplemental Figure 7



**Supplemental Figure 7. Elevated OGT levels leads to an increase in HCF-1 cleavage/activation in IPAH that is analogous to the cancer cell mechanism.** A549 and HeLa cells were treated in the presence (+) or absence (-) of OGT inhibitor (TT40) for 24 hours and HCF-1 was analyzed. Arrowheads represent the precursor HCF-1 and open arrows indicate HCF-1 specific cleavage products generated by OGT. A non-specific, but antibody reactive band is not marked in figure.

## REFERENCES:

1. Oikari S, Venalainen T, Tammi M. Borate-aided anion exchange high-performance liquid chromatography of uridine diphosphate-sugars in brain, heart, adipose and liver tissues. *Journal of chromatography A*. 2014;1323:82-6. doi: 10.1016/j.chroma.2013.11.004. PubMed PMID: 24309714.
2. Lauer ME, Mukhopadhyay D, Fulop C, de la Motte CA, Majors AK, Hascall VC. Primary murine airway smooth muscle cells exposed to poly(I,C) or tunicamycin synthesize a leukocyte-adhesive hyaluronan matrix. *J Biol Chem*. 2009;284(8):5299-312. doi: 10.1074/jbc.M807965200. PubMed PMID: 19088077; PubMed Central PMCID: PMC2643504.
3. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nature methods*. 2012;9(7):671-5. PubMed PMID: 22930834.