Supplemental Material.

Methods.

The authors declare that all supporting data are available within the article (and its online supplementary files), or can be made available from the corresponding author upon reasonable request. Materials included in this study will be made available upon request to the corresponding author.

Cell Culture, Treatments, and Transfections.

Cells. As published by us[23], Briefly, the aortic root was excised immediately and aseptically from hearts and rinsed with cold sterile DPBS to remove all blood components to limit VEC death and bacterial contamination. Valve leaflets were isolated carefully not to include any annular tissue, and placed in 15ml conical tube filled with 12ml cold DPBS and rinsed to remove debris. After removal of the endothelial cell layer using collagenase, leaflets were placed into 15ml centrifuge tube with 10ml collagenase solution per valve (3 leaflets) and incubated for 12-18hrs at 37°C. The digested tissue was then centrifuged for 5 minutes at 1000rpm and supernatant was aspirated. Cells were resuspended in complete media and cultured in a T75 flask. Complete media (CM) is defined as DMEM media (containing L-glutamine) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). pAVICs were utilized from passage number 2 to 6 for all the experiments reported. Human VICs (hVICs) were isolated from 2 non-diseased (healthy) and 2 calcified aortic valve donors (a kind gift from Dr. Robert Hinton). Healthy patients were aged 37 (sex-female, cause of death-cerebrovascular accident) and 26 (sex-male, cause of death-anoxia) years old, while the diseased cell lines were obtained

from 15 (sex-male, disease profile-BAV with severe aortic stenosis and aortic insufficiency) and 23 (sex-female, disease profile-BAV with aortic stenosis) year old patients. hVICs were maintained in MEM media with L-glutamine supplemented with 10% FBS and 1% P/S.

Calcification Stimuli. To stimulate calcification using a stiff matrix, pAVICs were cultured on glass cover slips in complete or low serum (2%) media and monitored for different stages of calcification (Stages I-III). Calcification was observed over a period of 10-14 days in culture. Treated cells were harvested when cells in the control calcified in a given experimental setup. Alternatively, pAVICs or hVICs were cultured for 4-5 days in osteogenic media (OM) (DMEM media with L-glutamine supplemented with 10% FBS and 1% P/S) supplemented with Dexamethasone (100nM), Ascorbic Acid (50 μM), and β-glycerophosphate (10 mM). For phosphate-induced calcification, sodium phosphate dibasic salt (Sigma #S9390) (pH7.4) was prepared at a stock concentration of 300mM and working concentrations of 1, 2.5, and 6mM were added and cells were treated for ~4-5 days.

KPT -330 treatment. KPT-330 was purchased from Apex Bio (B1464) and diluted in DMSO to a concentration of 50 mg/ml. KPT-330 treatment (100nM unless otherwise specified) and cell harvesting were performed at time points indicated the respective figure legends.

For prevention experiments using glass, KPT-330 was added at day 5 (Stage I) and treatment was continued by replenishing the media and drug every 2 days until cells in Stage I with vehicle (DMSO) treatment reached Stage III (calcific nodules). For experiments using OM and inorganic phosphate as calcification stimuli, KPT-330 was added a day after plating (day 1) cells along

with the OM or inorganic phosphate and replenished every 2 days until cells in OM calcified (~5 days).

For attenuation experiments using glass, KPT-330 was added at Stage II and for treatment experiments using glass, KPT-330 was added at Stage III and treated for ~72 hours. For hVICs, 20nM K PT-330 was added the day after plating the cells and cells harvested after 7-10 days.

For experiments involving siRNA transfections, KPT-330 was added to cells along with CM or OM, after transfecting with siRNA.

For experiments utilizing a proteasome degradation pathway inhibitor, pAVICs were treated as above and MG132 (10 μM; ab141003 (Abcam)) was added 3 hours prior to protein harvest.

Wnt Inhibition. XAV-939 was purchased from Selleckchem (S11890), and diluted in 1xPBS for use at a concentration of 10μM. pAVICs were treated with XAV-939 in OM for 24 hours after plating and harvested when calcific nodules were observed in vehicle controls.

C/EBPβ overexpression. To overexpress C/EBPβ in pAVICs, human recombinant C/EBPβ protein (rC/EBPβ) was added at a concentration of 400ng/μl (Abnova, H0000-1051-P01). Alternatively, empty pcDNA3 control and pcDNA3-C/EBPβ plasmid (pC/EBPβ) were purchased from Addgene (plasmid #49198; http://n2t.net/addgene;49198; RRID:Addgene 49198)) and 500ng was transfected into pAVICs using Lipofectamine3000 (explained in detail under "Dual-Luciferase Reporter Assay System") and cells were harvested 24 hours later.

XPO1 knockdown. In order to knock down *XPO1,* control (siGLO RISC-Free Control siRNA, 5 nmol; D-001600-01-05) and *XPO1* siRNA sequences were purchased from Dharmacon (ON-

TARGET plus Human XPO1 (7514) siRNA - SMARTpool, 5 nmol; L-003030-00-0005) and Dharmafect transfection reagent was utilized following the manufacturer's instructions.

Histological Staining.

Tissues. 4% paraformaldehyde (PFA)-fixed paraffin tissue sections (7μm thick) were deparaffinized using xylene and ethyl alcohol (100%, 95%, 75%, 50%, 25%) and subjected to boiling for 10 minutes in antigen unmasking solution (Vector Laboratories) for antigenic retrieval. Following 1hour in blocking solution, primary antibody (β-Catenin, ab16051 (Abcam), 1:200) was applied overnight at 4°C. Alternatively, slides were incubated with block only and serve as a no primary antibody control. Following primary antibody incubation, the appropriate secondary antibody was applied (Alexa Fluor 488 or 586; Invitrogen; 1:400). Tissue sections were then mounted in Vectashield anti-fade medium with DAPI (Vector Laboratories) to detect cell nuclei. Images were visualized using an Olympus BX51 microscope and captured using an Olympus DP71 camera and CellSens software. Image contrast and brightness were edited using Adobe Photoshop CC. To quantitate β-Catenin immunoreactivity, n=3 *Klotho*$^{-/-}$ and *wild-type* genotypes from 3 independent litters were used for analysis. The annular region of the aortic valve was selected based on anatomical features, and Image J was used to obtain raw integrated density. This was normalized to the annular area selected and the background fluorescence in each of the images to obtain the corrected total cell fluorescence (CTCF).

Cells. . Media was removed from treated pAVICs and cells were rinsed in 1xPBS, followed by fixation in 4% PFA solution for 20 minutes at room temperature. Cells were rinsed again in 1xPBS and blocked for one hour at room temperature. Primary antibody (or block only for no

primary antibody controls) was applied overnight at 4°C. The following primary antibodies were utilized for the study: Sclerostin, ab85799 (Abcam), 1:50; Runx2, sc-10758 (Santa Cruz), 1:200; Osteomodulin/Osteoadherin, sc-271102 (Santa Cruz), 1:100; Osteopontin, sc-10593 (Santa Cruz), 1:100; Cadherin-11/OB-Cadherin, #71-7600 (Thermo Scientific), 1:400; pHH3, #06-570 (Millipore), 1:300; C/EBPβ, ab53138 (Abcam), 1:50. Following primary antibody incubation, the appropriate secondary antibody was applied (Invitrogen). Cells were then mounted in Vectashield anti-fade medium with DAPI (Vector Laboratories) to detect cell nuclei. Images were visualized using an Olympus BX51 microscope and captured using an Olympus DP71 camera and CellSens software. Image contrast and brightness were edited using Adobe Photoshop CC.

pHH3 positive cells were counted using nine different field of views from 3 independent biological replicates and normalized to number of cell nuclei (DAPI). To quantitate C/EBPβ sub-cellular localization, empty pcDNA3 control and pcDNA3-C/EBPβ plasmid transfected cells were fixed in 4% PFA. After staining and imaging, three separate images from each of the three independent biological replicates were analyzed for sub-cellular localization of C/EBPβ. Number of cells with cytoplasmic, nuclear, and both (nuclear and cytoplasmic) localization were counted and prism was subject to statistical analysis as indicated.

Alizarin Red Staining. Following treatments, cells and murine tissue were fixed in 4% PFA for 20 minutes at room temperature, then rinsed in 1xPBS and water. Tissue sections (7μm thick) were deparaffinized using xylene and ethyl alcohol (100%, 95%, 75%, 50%, 25%). Fixed tissue sections and cells were then stained with 2% Alizarin Red stain in water (pH-4.2) for 10 minutes and washed in water, mounted using xylene-based mounting media and imaged. For

quantification, the number of Alizarin Red positive nodules were calculated and averaged for independent biological replicates. Alternatively, the area (arbitrary units shown) of Alizarin Red reactivity per magnified field/montage was calculated using Image J analysis, and averaged for independent biological replicates. Images were visualized using an Olympus BX51 microscope and captured using an Olympus DP71 camera and CellSens software. Image contrast and brightness were edited using Adobe Photoshop CC.

Alkaline Phosphatase (ALP) Staining. Staining was carried out according to the manufacturer's instructions as per the Abcam kit (ab242286). Briefly, cells were fixed using the fixing solution for 2 minutes at room temperature after a wash in PBST (phosphate buffered saline with Tween20). This was followed by another PBST wash and adding the ALP stain for 15-30 minutes protected from light. Cells were then washed in PBS and imaged using EVOS M700 (Invitrogen by Thermo Fisher Scientific, Inc). The area of ALP reactivity (arbitrary units shown) per magnified field/montage was calculated using Image J analysis, and averaged for independent biological replicates.

Caspase-Glo 3/7 Assay.

Caspase activity assay was performed as per the manufacturer's instructions (Promega, G8090). Briefly, 250,000 pAVICs were grown and treated as described in 6 well plates. After appropriate treatments/time, cells were trypsinized and pelleted. Equal number of cells from each sample were counted and added to each well of a white opaque 96 well plate-a total volume of 100µl and an equal volume of Caspase-glo 3/7 reagent was added to each well. The plate was incubated

in the dark for an hour and luminescence was read. All values were normalized to media only control and averaged.

MTT Assay.

The MTT assay was performed as per the manufacturer's instructions (Promega G400). Briefly, 250,000 pAVICs were grown and treated in 6 well plates. After appropriate treatments/time, cells were trypsinized and pelleted. Equal number of cells from each sample were counted and added to each well of a clear 96 well plate-a total volume of 100µl. Fifteen µl of dye solution was added to each well. Metabolically active cells convert the MTT tetrazolium component in the dye into a colored formazan product. The plate was incubated at 37°C for 1-4 hours, following which 100µl of solubilization/stop solution was added to each well to solubilize the formazan product. Absorbance was read at 570nm. All values were normalized to media only control and averaged.

Dual-Luciferase Reporter Assay System.

pAVICs were grown in 6 well plates in osteogenic media with/without KPT (see above). After 5-6 days in culture (calcific nodules seen in osteogenic media wells), cells were trypsinized and re-plated in 12 well plates at a density of 125,000 cells/wells. Twenty-four hours post plating, cells were transfected with 500ng of TOP/FOP Flash (Firefly) plasmids (M50 Super 8X TOP Flash plasmid; 12456 and M51 Super 8X FOP Flash plasmid; 12457) which were a gift from Randall Moon and 50ng of empty-Renilla plasmid (for transfection efficiency) using the Lipofectamine3000 transfection reagent kit from Invitrogen (L3000015) in serum free media. Twenty-four hours post transfection, cells were recovered in osteogenic media with/without KPT

and LiCl (10 mM) was added to all the wells in order to induce the Wnt/β-Catenin signaling response. Twenty-four hours post treatment (LiCl + KPT), Dual-Luciferase Reporter Assay was performed according to the manufacturer's instructions (Promega #E1910).

For AXIN1 luciferase reporter assay, pAVICs were plated in 12-well plates and transfected with 600 ng of AXIN-1 reporter plasmid (Genecopoeia;CS-HPRM44949-PL01-01-100). Secondary transfections were performed using 600 ng empty pcDNA3 control or pcDNA3-C/EBPβ plasmid (pC/EBPβ) (purchased from Addgene (plasmid #49198; http://n2t.net/addgene;49198; RRID:Addgene 49198)) using Lipofectamine3000. Twenty-four hours post transfection, cells were treated with DMSO OR KPT-330 and analyzed 24 hours post treatment. Dual-Luciferase Reporter Assay was performed according to the manufacturer's instructions (Promega #E2920). Luminescence Firefly values were normalized to Renilla and quantified using Prism.

Western Blotting.

Nuclear/Cytoplasmic Lysate Extraction. Nuclear and cytoplasmic fractions were extracted based on the manufacture's protocol provided with the NE-PER Nuclear and Cytoplasmic Extraction Kit from Thermo Scientific (#78833). Briefly, cells were rinsed in PBS, trypsinized and pelleted via centrifugation. Cell pellets were washed in PBS substituted with Complete EDTA-free protease inhibitor cocktail (Sigma-Aldrich, 11836170001) in order to inactivate the proteases. Ice-cold CER- 1 buffer was added to the cell pellet. Cells were vigorously vortexed for 15 seconds to dissolve the cell pellet and incubated on ice for 10 minutes. Ice-cold CER-2 buffer was added to the samples and they were incubated on ice for a minute after vortexing for 5 seconds. Samples were spun down for 5 minutes at ~16,000rcf. The supernatants (cytoplasmic

fraction) were transferred to a fresh tube and stored. Ice-cold NER buffer was added to the pellets and incubated on ice for 40 minutes. Samples were vortexed every 10 minutes for 15 seconds. They were then centrifuged and supernatants (nuclear fraction) was stored for analysis.

Protein Expression Analysis. Following treatments, protein from cells was harvested using 1X Cell Lysis Buffer (Cell Signaling, #9803S). Lysates were centrifuged for 10 minutes at 12,000 rpm at 4°C to remove cell debris. Supernatants were collected and BCA assay was utilized to determine protein concentration to normalize samples. Samples were run on PAGE gel, transferred to a nitrocellulose membrane using the dry transfer apparatus from Invitrogen. Membrane was blocked for an hour before applying primary antibodies. The following primary antibodies were utilized for the study: GAPDH, #2118S (Cell Signaling), 1:2000; Osteomodulin/Osteoadherin, sc-271102 (Santa Cruz), 1:1000; active β-catenin, #05-665 (Millipore), 1:500; β-catenin, #8480S (Cell Signaling), 1:500; C/EBPβ, sc-7962 (Santa Cruz), 1:500; AXIN1, #2087 (Cell Signaling), 1:500; XPO1/CRM1, sc-74454 (Santa Cruz),1:500; Lamin A/C, #4777S (Cell Signaling), 1:1000; β-tubulin, #2128S (Cell Signaling), 1:1000. Densitometric quantification on western blots was performed using Image J.

Animal Studies.

All mice for this study were housed in a USDA-certified, AALAC-accredited facility within Nationwide Children's Hospital Research Institute and Medical College of Wisconsin. The vivarium was monitored daily by the investigator and animal care technicians. All animal protocols for the proposed studies have been approved by the Institutional Animal Care and Use Committee (IACUC #AR11-00026, AUA0006769), and general animal training has been

provided to all individuals involved in this study. Experimental mice were euthanized using CO_2 anoxia, as recommended by the Panel of Euthanasia of the American Veterinary Medical Association, and great effort was made to ensure the mice do not suffer any unnecessary pain or discomfort.

Klotho heterozygous mice (B6;129S5-Kltm1Lex/Mmucd) were obtained from Mutant Mouse Resource and Research Centers supported by NIH (MMRCC) and bred to obtain *Klotho* wild-types, heterozygotes, and nulls. *Klotho* nulls and wild-types were blindly (prior to genotyping) and randomly assigned into experimental groups and injected intraperitoneally with Vehicle (Pluronic F-68 and PVP K29) or KPT-330 (dissolved in Pluronic F-68 and PVP K29) at a dosage of 30 mg/kg. Injections began at 3-4 weeks of age and lasted until week 7-8 (a total of 6-7 injections/mouse). Mice were then euthanized, and hearts were harvested for processing and analysis. For histological analysis, hearts were dissected and fixed in 4% PFA, paraffin-embedded and sectioned (7μm). Sex was considered as a biological variable, however differences were not observed and therefore, data are represented as both males and females.

The following wild-type (WT) and mutant (M) forward (FOR)/reverse (REV) primers were utilized for genotyping the *Klotho* mice. WT-FOR 5'-3': GATGGGGTCGACGTCA; WT-REV 5'-3': TAAAGGAGGAAAGCCATTGTC; M-FOR 5'-3'; GCAGCGCATCGCCTTCTATC; M-REV 5'-3': ATGCTCCAGACATTCTCAGC. The WT and M band were detected at 186 bp and 455 bp, respectively.

RNA-Seq Analysis.

pAVICs were plated as previously described and harvested either 5 days after plating (Day 5 or Stage I), ~8 days after plating (Day 8 or Stage II) (when pre-calcification was observed) or

DMSO/KPT added when nodules observed and harvested ~72 hours post KPT-330 treatment (Stage III/Stage III+KPT). Total RNA was extracted using the RNeasy Mini Kit from Qiagen (#74104).

Differential expression analysis. Gene-specific reads were calculated in the form of RPKM (Reads Per Kilobase of gene per Million fragments mapped). The RPKM of 10 reads was defined as the Detection Threshold, which was calculated from the formula: (10 reads / 2kb) / (total mapped reads/1M). Values that were smaller than detection threshold were replaced with the average value of detection threshold across all samples. The Reliable Quantification Threshold (RQT) was defined as an RPKM equivalent to 50 reads and was calculated from the formula: (50 reads / 2kb) / (total mapped reads/1M). Only the 13,934 porcine genes showing counts above RQT in one or more samples were analyzed for differential expression analysis. A 1-way ANOVA was performed on the log2 RPKM data for the 13,934 detectable porcine genes to examine the effect of group (Stage I, Stage II, Stage III+DMSO and Stage III+KPT). Tukey tests were performed as a post-hoc test to determine the effect of treatment within each group (e.g. Stage I vs Stage II). Likewise, fold changes were calculated for the same comparisons by taking the ratio of the mean values from both groups in the comparison. For example, when comparing Stage I vs. Stage II a fold change of 2.5 would mean that the gene expressed at Stage I was 2.5 times higher than at Stage II, or expression was higher in Stage I than Stage II. This logic was applied for all stage comparisons. ANOVA and Tukey P values were adjusted to control the False Discovery Rate according to the method of Benjamini and Hochberg (1995) J Roy Stat Soc B. 57:289-300. All statistical analysis was performed using R version 3.2.2 statistical computing software. Differential gene expression between stages was

visualized utilizing volcano plots, which were generated with the R ggplot 2 package version 2.2.1.

Functional Annotation. To examine the differential gene expression between different stages, we used an FDR cut off of 0.05 for each comparison. This threshold identified the 1944 differentially expressed genes between Stage I and II, 2262 differentially expression genes between Stage II and III, and 3652 differentially expressed genes between Stage III and KPT treated cells. Functional annotation was then performed on each of these sets of differentially expressed genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8. Gene Ontology (GO) Direct terms were utilized, which provide GO mappings directly annotated by the source database and have been filtered to remove broad parent terms. Enriched GO biological processes were then analyzed using the R package GOplot version 1.0.2, including multiple testing statistics (Benjamanini, Bonferroni). Bubble plots were generated using the GoBubble function to visualize the top 15 GO biological processes. The bubble plots represent each GO term as a circle, and these size of each circle correlates to the number of genes within the term and is plotted as –log (FDR) vs. z-score. The z-score is a crude measurement and predicts if a term will be up or downregulated. It is calculated by taking the number of upregulated genes and subtracting the number of downregulated genes and then dividing this number by the square root of the number of genes in each pathway. The top 12 GO biological processes were also visualized as a circle plot through the GoCircle function. The circle plots are able to show overall gene expression changes associated with each GO term, visualized by the red (upregulated genes) and blue (downregulated genes) dots. The circle plot also highlights the terms association to the z-score, visualized by color of the inner circles, where red is associated with terms that are

predicted to have an overall increase, while blue is associated with an overall decrease. Chord plots where also generated, using the GoChord function, to highlight important GO terms and their association with statistically significant gene expression changes in genes associated with valve development and disease.

We also aimed to identify and functionally annotate the genes expressed explicitly during specific stages. We assessed detectable genes with an average RPKM above the reliable quantification threshold, that have ANOVA P values by group < 0.05 and a fold change > 2 in any of the 6 pairwise comparisons among the four groups. A gene was considered detectable in a group if the gene's average RPKM value was above the average Reliable Quantification Threshold (RQT) value for that group. Venn Diagram was used to generate overlapping data sets and to generate Venn Diagrams. Genes in the different overlapping datasets were analyzed using DAVID version 6.8 and were utilized the GO Direct terminology for functional annotation. The raw data resulting from this RNA-seq study has been deposited in GeoDataSets.

RNA-cDNA-qPCR analysis.

For RNA-seq validation, total mRNA was isolated using standard Trizol-phenol extraction. In brief, 200µl of Trizol was added to cells with the addition of 10% v/v of chloroform. Following cold centrifugation, the top clear layer was precipitated with isopropanol overnight at $^{-}20^{o}$C, and the RNA pellet was washed in 70% ethanol following centrifugation the next day, before being resuspended in 20µl RNAase free water. Following, 2µg mRNA was transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, #4387406) as per instructions. Confirmation of cDNA generation was determined by performing qPCR analysis to detect expression of sus scrofa *18s FOR 5'-CAACTCGACCGAGGGCACAA-3', REV 5'-*

GTCCCGACGTGACTGCTCG-3' as a housekeeping gene. For this, and all other qPCR analyses, the One-Step-Real-Time RT-PCR master mix system was utilized (ThermoFisher Scientific) as recommended by the manufacturer, using sus scrofa-specific primers in conjunction with Sybr green master mix (Tm 60^{o}C, 40 cycles) to detect expression of *Sost* (FOR 5'-3': TTTAAGTGCCTGCAACCCA-, REV 5'-3': GTCTGTGACTCAGTTTCCTCCC), *Osteomodulin* (FOR 5'-3': TCGCCTGGACCAAAATAAGC, REV 5'-3': GGGTCCACGTGTTCTTCTGA), *Osteoglycin* (FOR 5'-3': ACAATGCCCTGGAATCCGTG, REV 5'-3': GATGGGATTATCTTCCAAGCGT), and *Ostf1* (FOR 5'-3': ACAAAAAGAAACTTTGGGGCG, REV 5'-3': CTGGAGTTCTGGGTTCAAATGT), normalized to *Gapdh* (FOR 5'-3': TGAACCACCAACTGCTTAGC, REV 5'-3': GGCATGGACTGTGGTCATGAG.

Mass Spectrometry/Proteomic Analysis.

pAVICs were plated on glass coverslips and harvested either 5 days after plating (Day 5 or Stage I), or DMSO/KPT added on Stage I (prevention group) (Stage III/Stage III+KPT at Stage I) or when nodules observed and (rescue group) harvested ~72 hours post KPT-330 treatment (Stage III/Stage III+KPT at Stage III). Whole cell pellets were isolated via trypsinization, washed with PBS. Nuclear pellets were extracted at described above. Samples were sent to Duke Proteomic Core, Duke University, Durham, North Carolina for analysis.

Sample Preparation: Frozen samples were thawed to room temperature and 100μL of 0.5% RapiGest in 50mM ammonium bicarbonate (AmBic) was added to each. The samples were probe sonicated three times at power level 3 and each pulse was three seconds long. The samples were

cooled on ice between pulses. A Bradford assay (Thermo) was performed with 5µL of each sample diluted with AmBic to 0.25% RapiGest to minimize interference in the measurement.

Sample Normalization and Digestion: Following the Bradford assay, 20µg of each sample were normalized to an equivalent concentration (w/v) for the digestion using 0.5% RapiGest in AmBic was added to bring the total sample volume to 100µL. Cysteine reduction was performed with 10 mM DTT, at 80oC for 15 minutes with shaking at 750rpm. Alkylation was performed with 2mM iodoacetamide at room temperature in the dark for 30 minutes. Sequencing grade trypsin (Promega) at 100ng/µL in AmBic was added at a ratio of 1:50 (w/w) trypsin:protein. The samples digested at 37oC overnight with shaking at 750rpm. After digestion, each sample was acidified with 500fmol ADH1_YEAST to a final concentration of 1% trifluoroacetic acid (TFA), 2% acetonitrile (MeCN) in water. The samples were heated to 60^{o}C for two hours with shaking at 750 rpm and then cooled to 4oC for one hour. The samples were centrifuged at 15000 rpm for 5 minutes to pellet any undigested protein. 100µl of the resulting supernatant was pipetted into an autosampler vial for analysis by LC-MS/MS. A Study Pool QC (SPQC) was made by combining 3µL from each of the samples.

Quantitative Analysis of Whole Cell Lysate Proteins: Quantitative one-dimensional LC-MS/MS was performed once per sample using 2µL (~250 ng) of the protein digest. Samples were analyzed using a nanoAcquity UPLC system (Waters) coupled to a Q Exactive Plus Orbitrap high resolution accurate mass tandem mass spectrometer (Thermo) via a nanoelectrospray ionization source. The sample was first trapped on a Symmetry C18 300µm x 180mm trapping column (5µL/min at 99.9 0.1 v/v H2O/MeCN). Next, the analytical separation was performed using a 1.7 um Acquity HSS T3 C18 75µm x 250mm column (Waters) with a 90 minute gradient

of 5 to 40% MeCN in 0.1% formic acid at a flow rate of 400nL/min and a column temperature of 55oC. Data collection on the Q Exactive Plus mass spectrometer was performed in a data-dependent MS/MS manner, using a 70,000 resolution precursor ion (MS1) scan followed by MS/MS (MS2) of the top 10 most abundant ions at 17,500 resolution. MS1 was accomplished using an automatic gain control (AGC) target of 1e6 ions and mass accumulation of 60 msec. MS2 used AGC target of 5e4 ions, 60msec mass accumulation, 2.0m/z isolation window, 27V normalized collision energy, and 20s dynamic exclusion. The total analysis cycle time for each sample injection was approximately 2 hours, and the experiment totaled 21 injections (19 for quantitative analysis).

The SPQC was analyzed twice to condition the column and was analyzed four additional times (at the beginning and after every 5 samples). Following the analyses, the data was imported into Rosetta Elucidator v4.0 (Rosetta Biosoftware, Inc.), and all LC-MS files were aligned based on the accurate mass and retention time of detection ion ("features") using a PeakTeller algorithm (Elucidator). The relative peptide abundance was calculated based on area-under-the-curve (AUC) of aligned features across all runs. The dataset had 835,562 MS/MS spectra for sequencing by database searching. This MS/MS data was searched against a custom RefSeq NCBI database with *Sus scrofa* taxonomy (downloaded on 12/19/2016) with additional and standard and contaminant proteins, including yeast ADH1 and bovine serum albumin, as well as an equal number of reversed-sequence "decoys" for false discovery rate (FDR) determination. Amino acid modifications allowed in database searching included fixed carbamidomethyl on Cys (+57), oxidation on Met (+16), deamidation on Asn and Gln (+1). The data was searched with 5 ppm precursor, 0.02Da product ion tolerance, and tryptic enzyme specificity, allowing up to two

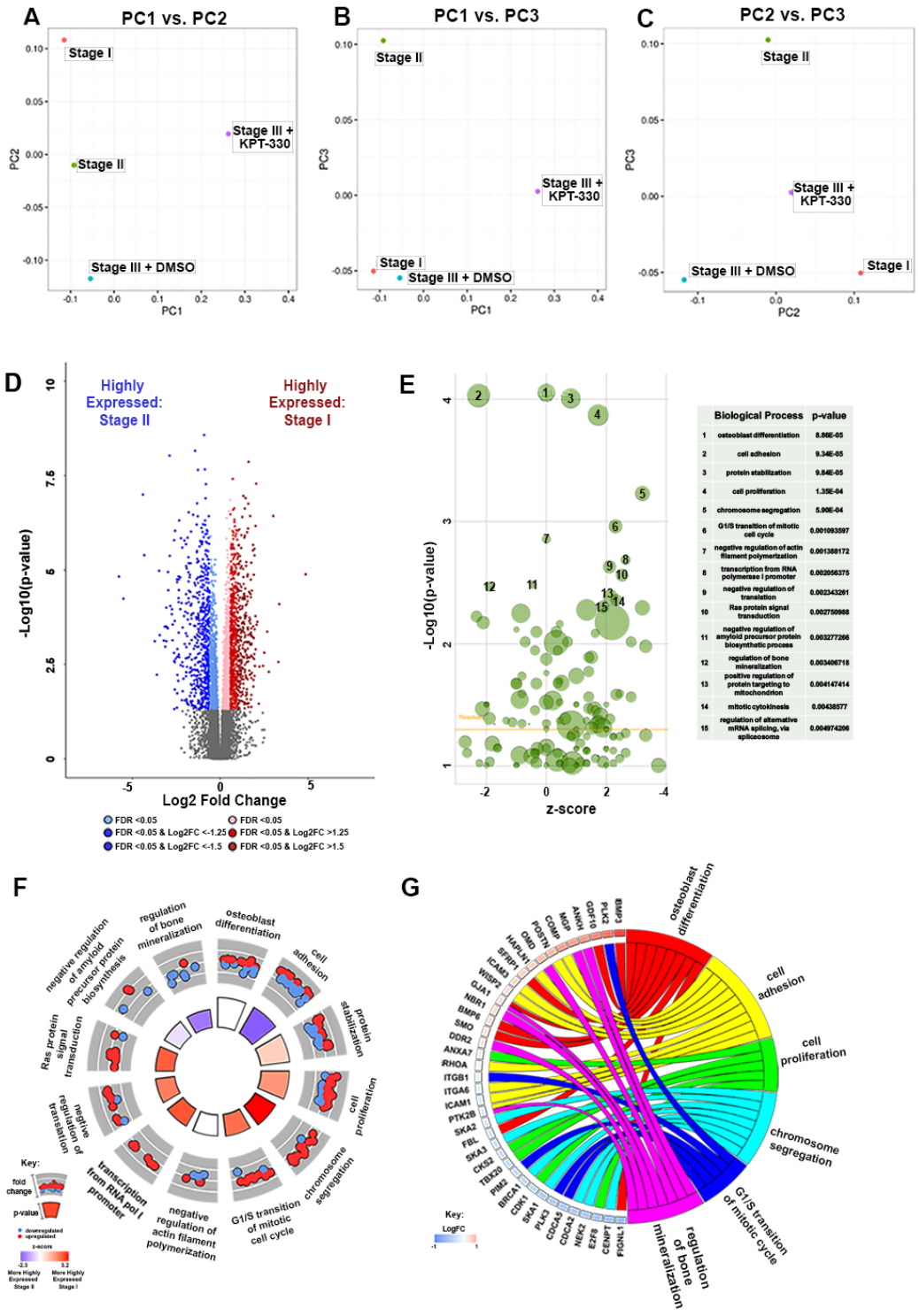
missed cleavages. The data was annotated at 1.2% peptide FDR using the PeptideTeller scoring algorithm.

During quantitative processing, the data was first curated to contain only high quality peptides with appropriate chromatographic peak shape. To obtain the most robust protein quantification results, peptides with %CV > 50% were removed from the dataset prior to summing to the protein level, to yield a 'robust' protein quantification dataset. The tool db2db on BiodbNet (https://biodbnet-abcc.ncifcrf.gov/db/db2dbRes.php) was used to convert the RefSeq protein identifier ("Primary Protein Name") in Table S5 to a Gene Symbol to improve ease of interpretation.

General Statistics.

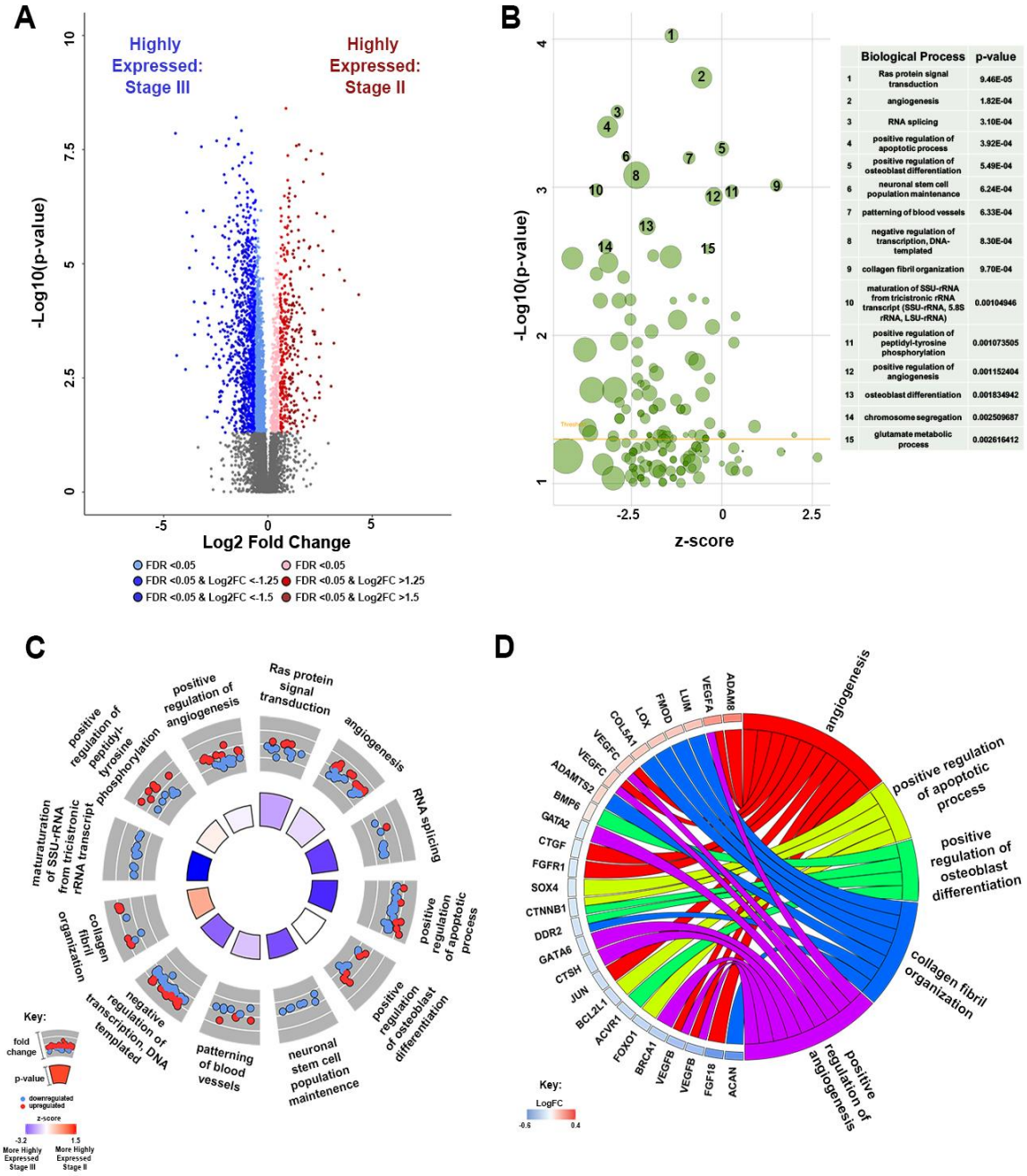
The priori power calculation was applied prior to the study to determine the sample size needed in order to detect some level of effect with inferential statistics. For datasets $n<6$, Bonferroni-corrected non-parametric Mann-Whitney testing was applied to determine significance between-group comparisons. For comparisons across more than two experimental datasets (Figures 1B, 1C and 1E), one-way ANOVA was performed followed by post-hoc pairwise testing (two-tailed), unless otherwise stated in the methods (RNA-seq and Mass Spec analysis). All statistical analysis and generation of graphs was performed using Prism GraphPad 9.0, or Excel as necessary to determine exact p-values.

Supplementary Figures.

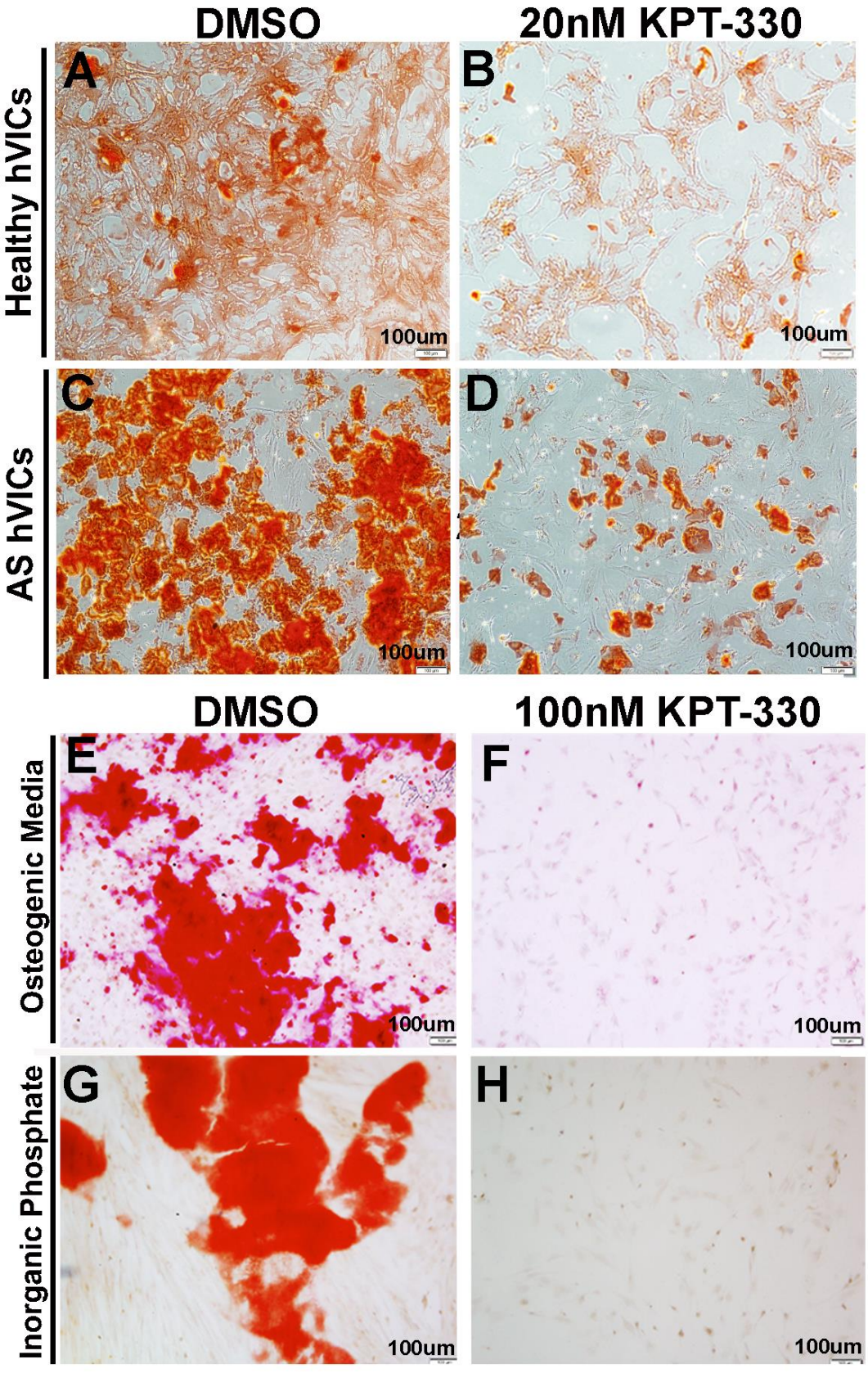


Supplementary Figure I. *(A-C)* PCA biplots to show principal component scores of samples (represented as dots). The principal components are created in order of the amount of variation

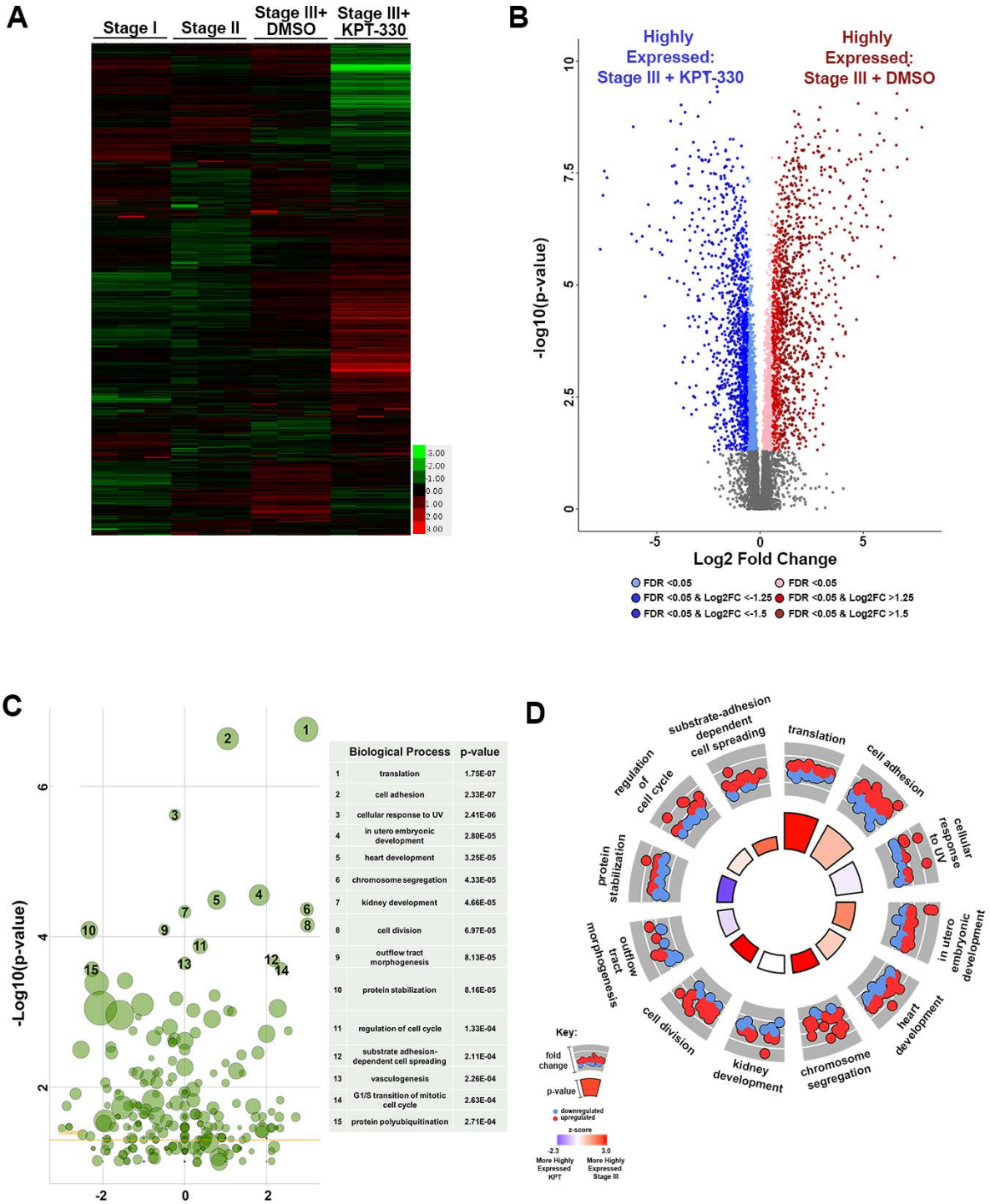
that they cover, with PC1 capturing the most variation, PC2, the second and PC3, the third. (*D*) Differential gene expression between Stage I and Stage II pAVICs, represented by a volcano plot. Dots represent average expression values $p<0.05$ and increased at Stage I (red), or Stage II (blue). (*E*) Enrichment of gene ontology biological processes between Stage I and Stage II pAVICs, represented as a bubble plot. Top 15 most significant pathways are labeled. (*F*) Circle plot highlighting the 12 most significant biological processes and associated gene annotations. The inner circle size represents the p-value, with the larger correlates to a more significant p-value. Additionally, the inner circle color reflects the z-score, which is a prediction of a bias in gene regulation, blue predicts an association with Stage II, while red predicts an association with Stage I. The scatterplots in the outer circle represent the differential gene expression, red dots represent upregulated genes, while blue dots represent downregulated genes. (*G*) Chord graph representing 39 statistically significant differentially expressed genes between Stage I and Stage II, highlighting the association of these genes to the corresponding biological processes. The color associated with the gene name represents the LogFC, where blue corresponds to downregulation and red corresponds to upregulation.


A
Highly Expressed: Stage III
Highly Expressed: Stage II
-Log10(p-value)
Log2 Fold Change
FDR <0.05
FDR <0.05
FDR <0.05 & Log2FC <-1.25
FDR <0.05 & Log2FC >1.25
FDR <0.05 & Log2FC <-1.5
FDR <0.05 & Log2FC >1.5
B
-Log10(p-value)
z-score
Biological Process
p-value
1 Ras protein signal transduction 9.46E-05
2 angiogenesis 1.82E-04
3 RNA splicing 3.10E-04
4 positive regulation of apoptotic process 3.92E-04
5 positive regulation of osteoblast differentiation 5.49E-04
6 neuronal stem cell population maintenance 6.24E-04
7 patterning of blood vessels 6.33E-04
8 negative regulation of transcription, DNA-templated 8.30E-04
9 collagen fibril organization 9.70E-04
10 maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA) 0.00104946
11 positive regulation of peptidyl-tyrosine phosphorylation 0.001073505
12 positive regulation of angiogenesis 0.001152404
13 osteoblast differentiation 0.001834942
14 chromosome segregation 0.002509687
15 glutamate metabolic process 0.002616412
C
Ras protein signal transduction
angiogenesis
RNA splicing
positive regulation of apoptotic process
positive regulation of osteoblast differentiation
neuronal stem cell population maintenence
patterning of blood vessels
negative regulation of transcription, DNA templated
collagen fibril organization
maturation of SSU-rRNA from tricistronic rRNA transcript
positive regulation of peptidyl-tyrosine phosphorylation
positive regulation of angiogenesis
Key:
fold change
p-value
downregulated
upregulated
z-score
More Highly Expressed Stage III
More Highly Expressed Stage II
D
angiogenesis
positive regulation of apoptotic process
positive regulation of osteoblast differentiation
collagen fibril organization
positive regulation of angiogenesis
ADAM8
VEGFA
LUM
FMOD
LOX
COL5A1
VEGFC
VEGFC
ADAMTS2
BMP6
GATA2
CTGF
FGFR1
SOX4
CTNNB1
DDR2
GATA6
CTSH
JUN
BCL2L1
ACVR1
FOXO1
BRCA1
VEGFB
VEGFB
FGF18
ACAN
Key:
LogFC

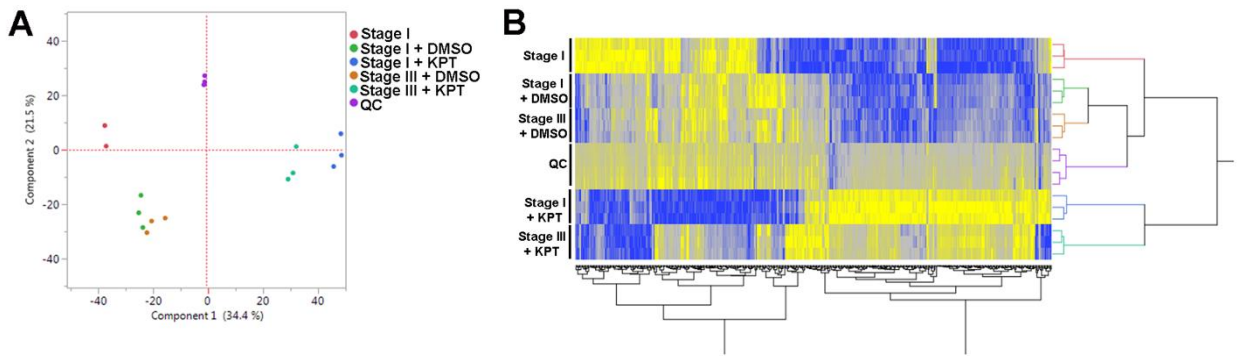
Supplementary Figure II. (*A*) Differential gene expression between Stage II and Stage III pAVICs, represented by a volcano plot. Dots represent average expression values $p<0.05$ and increased at Stage II (red), or Stage III (blue). (*B*) Enrichment of gene ontology biological processes between Stage II and Stage III pAVICs, represented as a bubble plot. Top 15 most significant pathways are labeled. (*C*) Circle plot highlighting the 12 most significant biological processes and associated gene annotations. The inner circle size represents the p-value, with the larger the size correlating to a more significant p-value. Additionally, the inner circle color reflects the z-score, which is a prediction of a bias in gene regulation, blue predicts an association with Stage III and red predicts an association with Stage II. The scatterplots in the outer circle represent the differential gene expression, red dots represent upregulated genes, while blue dots represent downregulated genes. (*D*) Chord graph representing 27 statistically significant differentially expressed genes between Stage II and Stage III, highlighting the association of these genes to the corresponding biological processes. The color associated with the gene name represents the LogFC, where blue corresponds to downregulation and red corresponds to upregulation.


DMSO
20nM KPT-330
Healthy hVICs
A
B
100um
100um
AS hVICs
C
D
100um
100um
DMSO
100nM KPT-330
Osteogenic Media
E
F
100um
100um
Inorganic Phosphate
G
H
100um
100um

Supplementary Figure III. (A-D) Alizarin red staining of healthy and aortic stenosis (AS) human aortic VICs treated with DMSO or 20nM KPT-330 in OM (n=2). **(*E*-*H*)** Alizarin red staining of pAVICs treated with DMSO or KPT-330 in OM (*E*, *F*) or 6mM inorganic phosphate (*G*, *H*) (n=3).


A
Stage I
Stage II
Stage III+ DMSO
Stage III+ KPT-330
B
Highly Expressed: Stage III + KPT-330
Highly Expressed: Stage III + DMSO
-log10(p-value)
Log2 Fold Change
FDR <0.05
FDR <0.05 & Log2FC <-1.25
FDR <0.05 & Log2FC <-1.5
FDR <0.05
FDR <0.05 & Log2FC >1.25
FDR <0.05 & Log2FC >1.5
C
-Log10(p-value)
z-score
Biological Process | p-value
1 translation 1.75E-07
2 cell adhesion 2.33E-07
3 cellular response to UV 2.41E-06
4 in utero embryonic development 2.80E-05
5 heart development 3.25E-05
6 chromosome segregation 4.33E-05
7 kidney development 4.66E-05
8 cell division 6.97E-05
9 outflow tract morphogenesis 8.13E-05
10 protein stabilization 8.16E-05
11 regulation of cell cycle 1.33E-04
12 substrate adhesion-dependent cell spreading 2.11E-04
13 vasculogenesis 2.26E-04
14 G1/S transition of mitotic cell cycle 2.63E-04
15 protein polyubiquitination 2.71E-04
D
translation
cell adhesion
cellular response to UV
in utero embryonic development
heart development
chromosome segregation
kidney development
cell division
outflow tract morphogenesis
protein stabilization
regulation of cell cycle
substrate-adhesion dependent cell spreading
Key:
fold change
p-value
downregulated
upregulated
z-score
More Highly Expressed KPT
More Highly Expressed Stage III

Supplementary Figure IV. (*A*) Heat map from RNA-Seq analysis demonstrating three replicates of Stage I, II, III+DMSO, and Stage III+KPT-330 (*B*) Differential gene expression between Stage III and KPT-330 treated pAVICs, represented by a volcano plot. Red dots represent average expression values with a p-value <0.05 and an increased expressed in Stage III, while blue dots represent average expression values that have a p-value <0.05 and have an increased expressed during KPT-330 treatment. (*C*) Enrichment of gene ontology biological processes between Stage III and KPT-330 treated pAVICs, represented as a bubble plot. Top 15 most significant pathways are labeled. (*D*) Circle plot highlighting the 12 most significant biological processes and associated gene annotations. The inner circle size represents the p-value, with the larger the size correlating to a more significant p-value. Additionally, the inner circle color reflects the z-score, which is a prediction of a bias in gene regulation, red predicts pathways associated with Stage III and blue predicts an association with KPT-330 treatment. The scatterplots in the outer circle represent the differential gene expression, red dots represent upregulated genes, while blue dots represent downregulated genes.



Supplementary Figure V. (*A*) Principal Component Analysis of whole cell proteomics data from at the protein level. No outliers were observed. (*B*) 2D Hierarchical clustering of 471 proteins putatively differentially expressed in the nuclear prep based on one–way ANOVA p<1e-5 across all samples.

Lay Sentence. This study is relevant to public health as it addresses the need to discover novel therapeutic targets to prevent, or treat calcific aortic valve disease.