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## Supplementary information

# Targeting immune–fibroblast cell communication in heart failure

In the format provided by the authors and unedited

## Materials and Methods

### Human single nuclei isolation for snRNA-seq

Cardiac tissues from ischemic cores at the time of heart transplantation were flash frozen using liquid nitrogen. Identical regions from the apex of LV from explanted donors were used. Single nuclei suspensions were generated as previously described. In brief, flash frozen sections were minced with a razor blade, transferred to a Dounce Homogenizer containing 1 mL of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 0.1% NP-40 in nuclease-free water) on ice. Samples were homogenized using five strokes, an additional 1 mL of lysis buffer added, and incubated on ice for 15 min. Samples were then filtered with a 40 um filter, which was rinsed with 1 mL of lysis buffer. The mixture was then centrifuged at 500 *g* for 5 min 4 °C, resuspended in 1 mL nuclei wash buffer (2% BSA and 0.2 U $uL^{-1}$ RNase inhibitor (Thermo Fisher, cat. no. AM2694) in 1X PBS) and, filtered using a 20 um pluristrainer (Pluriselect, cat. No. SKU43-50020-03). Filtered solution as centrifuged using the above criteria and resuspended in 300 uL Nuclei Wash Buffer and transferred into a 5 mL tube for flow cytometry. Subsequently, 1 uL DRAQ5 (5 mM solution; Thermo Fisher, cat. no. 62251) was added, sample gently vortexed and allowed to incubate for 5 min prior to sorting. $DRAQ5^+$ nuclei were sorted into 300 uL Nuclei Wash Buffer using a BD FACS Melody (BD Biosciences) with a 100 uM nozzle. Sorted nuclei were then centrifuged using the above conditions and resuspended in Nuclei Wash Buffer for a final target concentration of 1,000 nuclei/uL – nuclei were counted on a hemocytometer. Based on the nuclei concentration, 10,000 target nuclei were loaded onto a Chip G for GEM generation using the Chromium Single Cell 5' Reagent v1.1 kit from 10X Genomics. Reverse transcription, barcoding, complementary DNA amplification and purification for library preparation were performed as per the Chromium 5' v1.1 protocol at the McDonnel Genome Institute. Sequencing was performed on a NovaSeq 6000 platform (Illumina) at a target read depth of 100,000 at the McDonnel Genome Institute.

### snRNA-seq reference map generation

Nuclei fastq files were aligned to the whole genome pre-mRNA reference generated from the GRCh38 transcriptome (with the intron flag included) using CellRanger v6.1 (10X Genomics). Nuclei were filtered to include those with 1000 < RNA UMI count < 10,000 and mitochondrial reads < 5%. After initial QC, scrublet was ran on each sample separately in Python with default parameters to score nuclei and nuclei with a doublet score > 0.2 were excluded from downstream analysis. We then leveraged supervised doublet removal as previously described on a per cell type basis before combining all objects. Briefly, clusters were annotated into major cell populations, each major cell type was subset, and re-normalized, PCA, UMAP embedding, clustering, and DE analysis performed. Sub-clusters which did not express the gene signature of the cell type or had overlapping genes across different cell types were removed. Post-contamination, all cell type objects were merged to construct a cleaned object. After QC and doublet removal downstream analysis was performed in Seurat v4. The cleaned object was normalized using SCTransform[1] with regressing out mitochondrial percent and RNA UMI counts. We then computed the principal components and used these to integrate all samples with harmony. Informed by the ElbowPlot, we used 80 components to construct the UMAP embedding, find nearest neighbors, and clustered the data at multiple resolutions. We then used the FindAllMarkers function in Seurat to perform differential expression testing and annotated clusters into distinct cell types based on canonical gene markers. To define cell states within each major cell type, we subset the major cell populations, re-normalized, re-clustered, re-computed the UMAP, and annotated cell states. Both the global object and each cell type object was saved and used for downstream analysis and plotting in Seurat. All differential gene expression used to identify cell types or cell states was performed using the normalized assay with the FindAllMarkers function and the Wilcoxon rank sum test with a min.pct = 0.1 and logfc.threshold = 0.25.

**Integration of spatial transcriptomics data**
Processed .h5ad objects were acquired for all spatial transcriptomic samples as previously published[2]. Data was re-normalized using SCTransform to keep analysis consistent with the human data processing. The snRNA-seq reference map was used to impute voxel annotations from selected spatial transcriptomic samples – mapping scores and gene signatures were also plotted to validate mapping. SPOTlight[3] was used for spot deconvolution and Pearson correlation coefficients calculated to identify cells which co-localize in space. To validate findings from isolated patients in a broader array of patients, the above analysis was performed on several samples and an integrated UMAP embedding of spatial spots was constructed for 28 samples using the same analysis pipeline as for snRNA-seq reference map construction.

**Gene Editing Design**
Analysis of genomic DNA sequence surrounding the target region, using the Benchling (www.benchling.com) guide RNA design tool, identified a gRNA sequence with a suitable target endonuclease site in *Fap* gene start site. Predicted off-target sites for the gRNA (crRNA2-AAGGTTAGTGCGTGCCGTCT) were identified using the specificity model developed and documented by Hsu and colleagues and embedded in the Benchling gRNA design tool. Design considerations for off-target editing were as follows. Higher off-target editing risk was considered when the "Score" is >2.0 with a canonical SpCas9 PAM (NGG), >50.0 with a chromosomally unlinked non-canonical PAM (NAG) or >20.0 with a linked non-canonical PAM yet in an annotated protein coding region. These "Score" thresholds were based on empirical targeted sequencing analysis of 20 different predicted off-target loci in 15 different mouse zygote gene editing experiments.

**Microinjection of gRNAs and plasmid donor DNA repair templates**
*Streptococcus pyogenes* Cas9 (SpCas9) V3 protein and gRNAs were purchased as part of the Alt-R CRISPR-Cas9 system using the crRNA:tracrRNA duplex format as the gRNA species (IDT, USA). Alt-R CRISPR-Cas9 crRNAs (Product# 1072532, IDT, USA) were synthesized using the gRNA sequences specified in the DESIGN section and hybridized with the Alt-R tracrRNA (Product# 1072534, IDT, USA) as per manufacturer's instructions. This gRNA duplex was used in all subsequent gene editing processes. The sequence of the double stranded DNA donor plasmid, that functions as the DNA repair template, was synthesized by Genscript. Features included asymmetric homology arm lengths of 1.6 kb and 2.0 kb, flanking the 2.2 kb insertion sequence. The final concentration of the gRNA, SpCas9 and donor components in the microinjection mixture was 50ng/ul, 100 ng/ul and 10 ng/ul, respectively. All animal work was approved by the Jackson Laboratory Animal Care and Use Committee and adhered to the standards of Guide for the Care and Use of Laboratory Animals set forth by the NIH. Microinjection was performed as described (PMID: 27210041) using C57BL/6J (Stock# 000664, The Jackson Laboratory, USA) donor female mice (3–4 weeks of age).

**Sequence characterization of Founder and N1 mice**
PCR primers flanking the gRNA target site, but outside the repair template homology arms, were used to amplify the region of interest from founder progeny. PCR amplicons were subjected to Sanger sequencing to identify founders with precise desired changes. Founders carrying desired mutations were bred with C57BL/6J mice (Stock #000664, The Jackson Laboratory, USA) to produce N1 progeny. N1 animals were confirmed by the same PCR-Sanger sequence analysis the founder population was subjected to. In contrast to founders, N1 animals are obligate heterozygotes, with one allele derived from an un-manipulated chromosome, enabling deconvolution of the two alleles in the mixed PCR amplicon. PCR primer sequences were as follows: Short Range PCR, Fap SR forward and Cre SR reverse; 5'-

AGAACGCCCCCAAAATCTGT-3' and 5'- ATTCAACTTGCACCATGCCG-3', left homology arm PCR, Fap LHA forward and Cre SR reverse; 5'-GCTGATGGCTTTCTCTCGGA-3' and 5'-ATTCAACTTGCACCATGCCG-3', and right homology arm PCR, ERT RHA forward and Fap RHA reverse; 5'-CGGGCTCTACTTCATCGCAT-3' and 5'-CAGGTGACATGCTGAGGTGT-3'.

**PET imaging chemical.**

FAPI-46 was provided by SOFIE under material transfer agreement. $^{68}$Gallium, $^{68}$Ga ($t_{1/2}$ = 68 min; $\beta^+$ =89%; $E_{max}$ = 1.92 MeV) was obtained from the GalliaPharm® $^{68}$Ge/$^{68}$Ga Generator and Modular-Lab system from Eckert & Ziegler Radiopharma GmbH. Ultra-pure 0.1 M HCl (5 mL) was used to elute $^{68}GaCl_3$. The eluate was passed through the Strata X-C cation exchange cartridge (Phenomenex®, 30 mg/1 mL). The trapped $^{68}GaCl_3$ was then released from the resin with 0.02 M HCl/98% acetone (0.8 mL). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and used as received. Water with a resistivity of 18.2 MΩ·cm was prepared by a Milli-Q® Integral 5 Water Purification System (Bedford, MA).

**$^{68}$Ga Radiolabeling of FAPI-46**

Radiolabeling of FAPI-46 was performed following our previous report[4]. $^{68}GaCl_3$ (962 MBq to 1073 MBq) in 0.02 M HCl/98% acetone (800 µL) was transferred to a microcentrifuge tube (1.5 mL) and heated for 15 min at 95 °C to evaporate the acetone. For the labeling of FAPI-46, 400 µL of 0.2 M ammonium acetate buffer (pH 5) containing 2.5 mg/mL gentisic acid was added before adding FAPI-46 at a ratio of 1 µg peptide per 7.4 MBq $^{68}GaCl_3$. The reaction mixture was incubated at 95 °C for 10 min in a thermomixer (without agitation). The radiolabeled compound was analyzed using radio-HPLC to ensure more than 95% radiochemical purity prior to animal studies.

**Characterization of $^{68}$Ga-FAPI-46.**

Reversed-phase high performance liquid chromatography (RP-HPLC) was performed on an Agilent 1200 Series with photodiode array detector and an ORTEC® scintillation photon counter. Both RP- and radio-HPLC analysis were performed using Phenomenex® Luna 5 µm C18(2) 100 Å, 150 × 4.6 MM with a mobile phase of A: water (0.1 % TFA) and B: acetonitrile (0.1 % TFA), using a gradient of 0–90 % acetonitrile over 10 min with a 2 mL/min flow rate.

**$^{68}$Ga-FAPi-46 PET/CT Imaging**.

At day 7 after AngII/PE induction or sham operation, AngII/PE mice were anesthetized with isoflurane and injected with ca. 5.55 MBq of $^{68}$Ga-FAPI-46 in 100 µL of saline via the tail vein. Small animal PET scans (45 to 60 min dynamic scan) were performed on Inveon PET/CT system (Siemens, Malvern, PA). The PET images were corrected for attenuation, scatter, normalization, and camera dead time and co-registered with microCT images. For PET scans performed with Inveon PET/CT system, the PET images were reconstructed with the maximum a posteriori (MAP) algorithm and analyzed by Inveon Research Workplace. The uptake was calculated as the percent injected dose per gram (%ID/g) of tissue in three-dimensional regions of interest (ROIs) without the correction for partial volume effect.

**Fibroblast flow cytometry and sorting for single cell RNA seq analysis**

After ice cold PBS perfusion, heart tissue from mice were minced with a razor blade on ice and transferred to a 15 mL conical tube containing 3 mL DMEM with 170 uL Collagenase IV (250 U/mL final concentration), 35 uL DNAse1 (60 U/mL), and 75 uL Hyaluronidase (60 U/mL) and incubated at 37 °C for 45 min with agitation. After 45 min, the digestion reaction was quenched with 6 mL of HBB buffer (2% FBS and 0.2% BSA in HBSS), filtered through 40 um filters into a 50 mL conical tube and transferred back into a 15 mL conical tube to obtain tighter pellets. Samples were then spun down at 4 °C, 1200 rpm for 5 min and the supernatant was discarded. Pellet resuspended in 1 mL ACK Lysis buffer (Gibco A10492-01) and incubated at room

temperature for 5 min followed by the addition of 9 mL DMEM and centrifugation (4 °C, 5 min, 1200 rpm). Supernatant was discarded and the pellet was resuspended in 2 mL FACS buffer (2% FBS and 2 mM EDTA in calcium/magnesium free PBS) and centrifugation was repeated in above conditions and supernatant aspirated. Samples were then incubated in Pdpn (BioLegend, 127423, clone 8.1.1), Cd140a (Biolegend, 135911, clone APA5), Cd31 (Biolegend, 102409, clone 390), Fap (Creative Biolabs, HPAB-0171-CN-PE), and Cd45 (Biolegend, 103132, clone 30-F11) with a 1:200 dilution for 30 min. A no Fap negative control was used which contained all antibodies except Fap. Solution was washed 3X with FACS buffer following same centrifugation as above and then resuspended in 500 uL of FACS buffer and 1 uL DAPI (BD Biosciences, 564907) and filtered into filter-top FACS tubes. Gating was performed as follows: Pdpn+, singlets, Cd45-/Cd31-, Pdpn+/PDgfra+, and Fap vs Pdgfra gate was used to look at Fap+ fibroblasts. The no Fap antibody negative control was used to draw the Fap gate. For the fibroblast scRNA-seq experiment, 4 isotype Ang II/PE and 4 anti- IL-1b mAb + Ang II/PE mice FAP+ and FAP- fibroblasts were sorted into 300 uL cell resuspension buffer (0.04% BSA in PBS). Collected cells were centrifuged as above, pooled across mice, and resuspended in collection buffer to a target concentration of 1,000 cells/uL. Cells were counted on a hemocytometer before proceeding with the 10x protocol. cDNA construction and library preparation were performed for 4 libraries (Isotype + Ang II/PE FAP+, Isotype + Ang II/PE FAP-, anti- IL-1b mAb + Ang II/PE FAP+, and anti- IL-1b mAb + Ang II/PE FAP-. cDNA synthesis, library construction, and sequencing were performed using the same protocol as the human CITE-seq data. Flow cytometry dtaa collection was performed on BD FACSChorus version 1.1 and software FACSDiva version 8. Flow cytometry data analysis was performed in FlowJo 10.8.2.

**Histology and Immunofluorescence**

Flash frozen LV samples were fixed for 24 hr at 4 °C in 10% neutral buffered formalin, washed in 1X PBS, and embedded in paraffin. Paraffin-embedded sections were cut at an 8 um thickness using a microtome. Slides were baked at 65 °C for 1 hr, deparaffinized with serial xylene washes, and rehydrated with ethanol. Then slides underwent methanol treatment (10% MeOH + 3% $H_2O_2$) for 20 min at RT followed by 3X TBS-T washes (5 min each). Antigen retrieval was performed using the AR6 buffer (Ankoya Biosciences, AR600250ML) for 15 min in the microwave and then cooled to room temperature. Tissue sections were marked with a hydrophobic pen and blocked in 10% BSA in TBS-T for 30 min at RT. Slides were then stained with the primary antibody diluted in 10% BSA in TBS-T overnight at 4 °C: FAP (Abcam, ab207178, 1:250) and POSTN (Abcam, ab215199, 1:250). Next, the primary antibody was detected using Opal Polymer HRP Ms + Rb (PerkinElmer Opal Multicolor IHC system). The PerkinElmer Opal Multicolor IHC system was utilized to visualize antibody staining per manufacturer protocol. Slides were imaged using a Zeiss Axioscan Z1 automated slide scanner. Image processing was performed using Zen Blue and Zen Black (Zeiss). For POSTN quantification, the whole heart was circled, and an MFI calculated in sham, isotype + Ang II/PE and anti-IL-1b mAb + Ang II/PE representative sections. For the $Il1r^{fl/fl}Dermo1cre^{+}$ mice 28-day Ang II/PE infusion, mice were sacrificed on day 28 and FFPE sections prepared as above. Trichrome staining was performed using Gomori's Trichrome Stain Kit (ThermoScientific, 87020) and subsequently percent fibrosis was quantified in serial cross-sections. For the CCR2 GFP mice, frozen sections were prepared using the following: hearts perfused with cold PBS, placed in 4% PFA overnight at 4 °C, washed with PBS 3X, infiltrated with 30% sucrose overnight at 4 °C, and embedded in OCT and stored in a -80 °C freezer. Prior to staining, 10 um sections were cut using a Leica Cryostat, washed with PBS, incubated with 0.25% Triton-X in PBS at RT for 5 min, blocked in 5% BSA/PBS for 1hr at RT, stained with primary antibody: anti-GFP (Abcam, ab13970) and IL-1b (R&D systems, AF-401-NA, 1:50) co-stained with an appropriate secondary antibody, and mounted with DAPI fluoroshield (R&D, F6057). Slides were then imaged on a Zeiss confocal microscopy and representative images included.

RNAscope and Immunofluorescence co-staining for in vivo lineage tracing study: RNAscope in situ hybridization (ISH) To detect RNA transcripts for POSTN in mouse vascular tissues, a commercially available kit (#323100, RNAscope Multiplex Fluorescent Reagent Kit v2, Advanced Cell Diagnostics) was used according to the manufacturer's instructions. Briefly, 5-µm-thick 4% paraformaldehyde-fixed mouse heart sections were air-dried for 1 hr at room temperature, and treated with hydrogen peroxide for 10 min to block endogenous peroxidase activity. After antigen retrieval by boiling in target antigen retrieval solution for 5 min at 95-100°C, slides were treated with protease III for 30 min at 40°C. Target probe (#418581, mouse POSTN) was hybridized for 2 hr at 40°C, followed by a series of signal amplification and washing steps. Hybridization signals were detected by TSA Plus Cyanine 5, and co-stained with ACTA-2 antibody (#F3777, Sigma Aldrich). Slides were counterstained with DAPI by using ProLong Gold antifade reagent (#P36962, Invitrogen). Images were analyzed in Zeiss Zen Blue version 3.2 and Zeiss Zen Black Version 2.0.

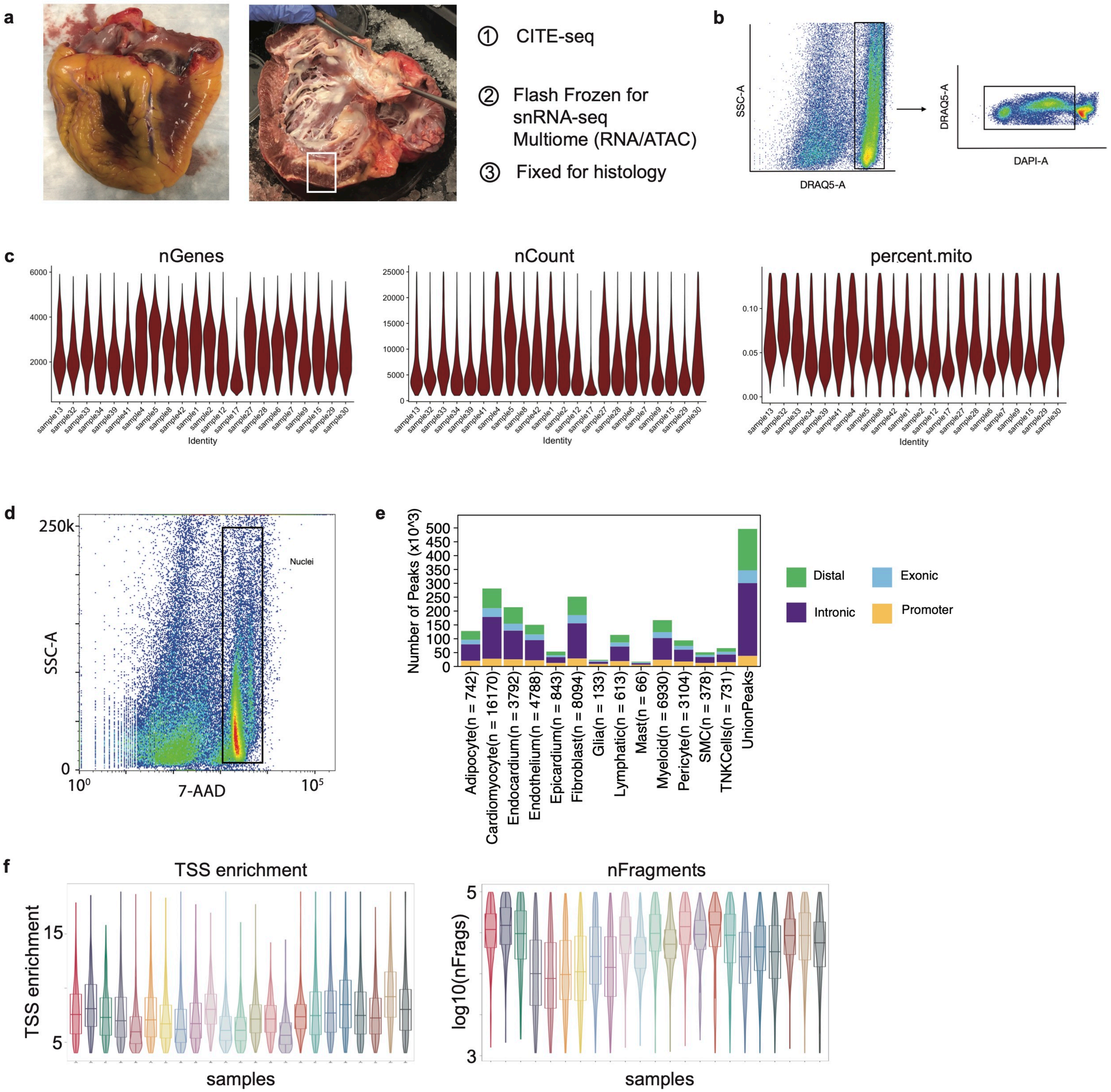
**RT- PCR**

RNA was extracted from flash frozen mouse apex using the Qiagen RNeasy Plus Mini Kit. RNA concentration was then measured using a nanodrop spectrophotometer and cDNA synthesis was performed using the HighCapacity RNA to cDNA synthesis kit (Applied Biosystems). Quantitative real time PCR reactions were prepared with sequence-specific primers with PowerUP™ Syber Green Master mix (Thermo Fisher Scientific) in a 20 uL volume. Real time PCR was performed using QuantStudio3 (Thermo Fisher Scientific). mRNA expression was normalized to 36B4. The primers were purchased from ADT (Postn: Fwd: 5'-GGTGCCCTAGAAAGGATCATGG-3'; Rev: 5'-CAGAGCACTGGAGGGTATTTAG-3'), (Il1r1: Fwd: 5'-AGGTGGAGGACTCAGGATATT-3'; Rev: 5'-CCAGGGTCATTCTCTAACACAG-3').

## References

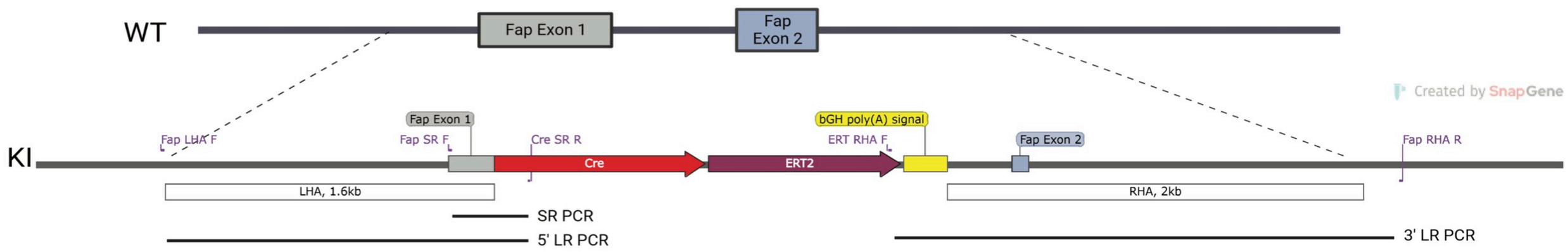

1. Hafemeister, C. & Satija, R. Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. *Genome Biol* **20**, 1–15 (2019).
2. Kuppe, C. *et al.* Spatial multi-omic map of human myocardial infarction. *Nature* (2022) doi:10.1038/s41586-022-05060-x.
3. Elosua-Bayes, M., Nieto, P., Mereu, E., Gut, I. & Heyn, H. SPOTlight: seeded NMF regression to deconvolute spatial transcriptomics spots with single-cell transcriptomes. *Nucleic Acids Res* **49**, e50–e50 (2021).
4. Strunk, M. *et al.* Toward Quantitative Multisite Preclinical Imaging Studies in Acute Myocardial Infarction: Evaluation of the Immune-Fibrosis Axis. *J Nucl Med* **65**, 287–293 (2024).

## Supplementary Data



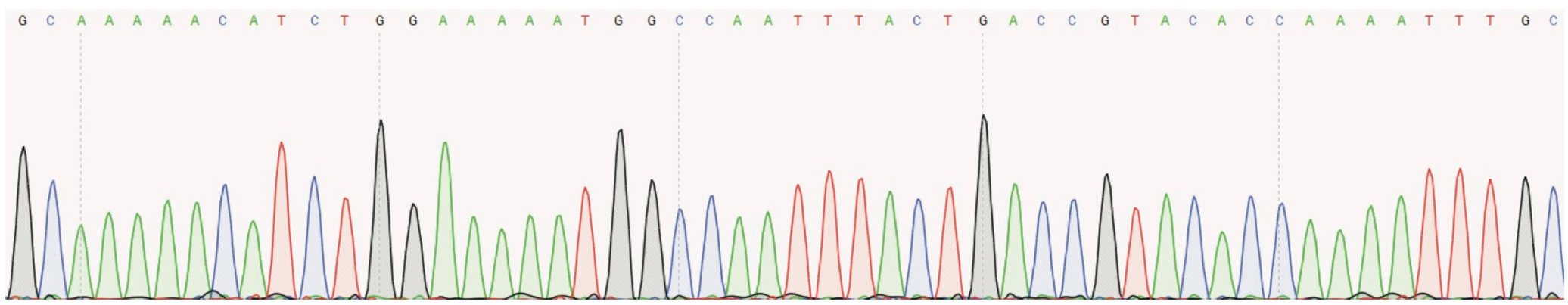

**Supplementary Figure 1**. (a) Explanted heart and dissected region boxed used for CITE-seq, snRNA-seq, histological analysis with (b) flow cytometry panel to sort live cells. (c) QC metrics post filtering. (d) Flow cytometry gating scheme to isolate nuclei using 7-AAD. (e) MACS2 peak calling summary from ArchR by cell type. (f) Multiome samples quality control metrics post QC and doublet removal box-violin plot split by sample; TSS enrichment (left) and log10 (nFrags) calculated in ArchR.

**a**



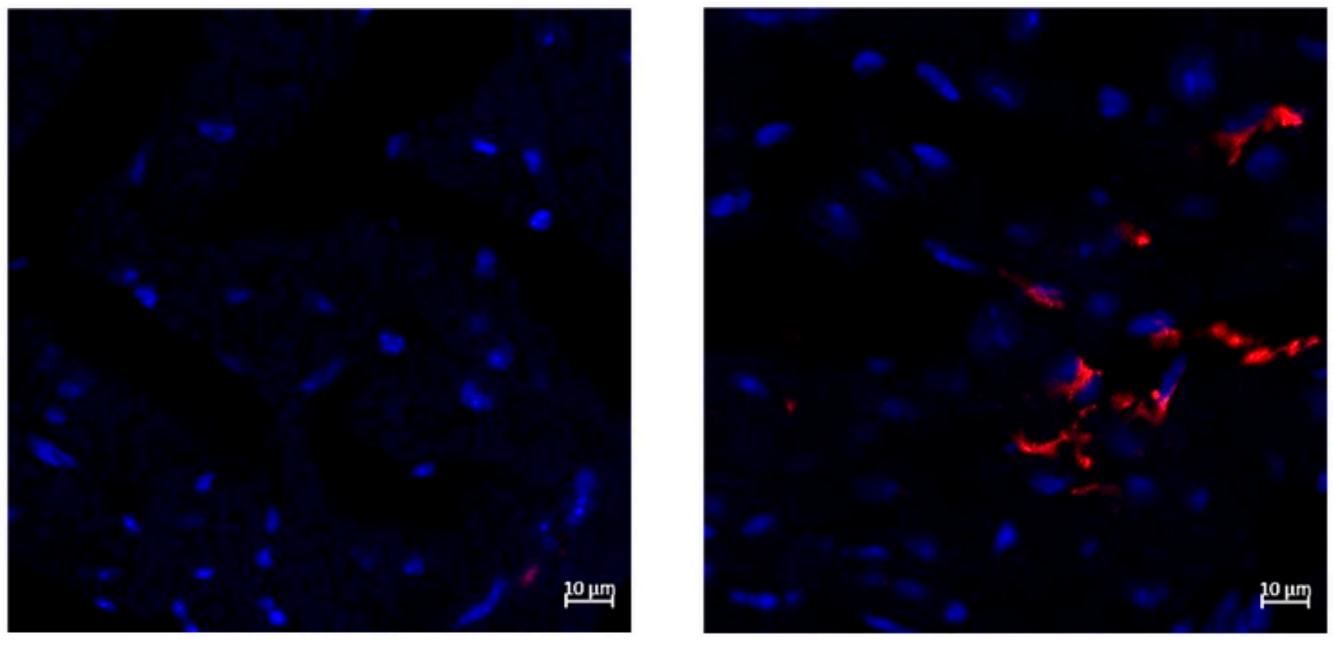

**b**


**c**

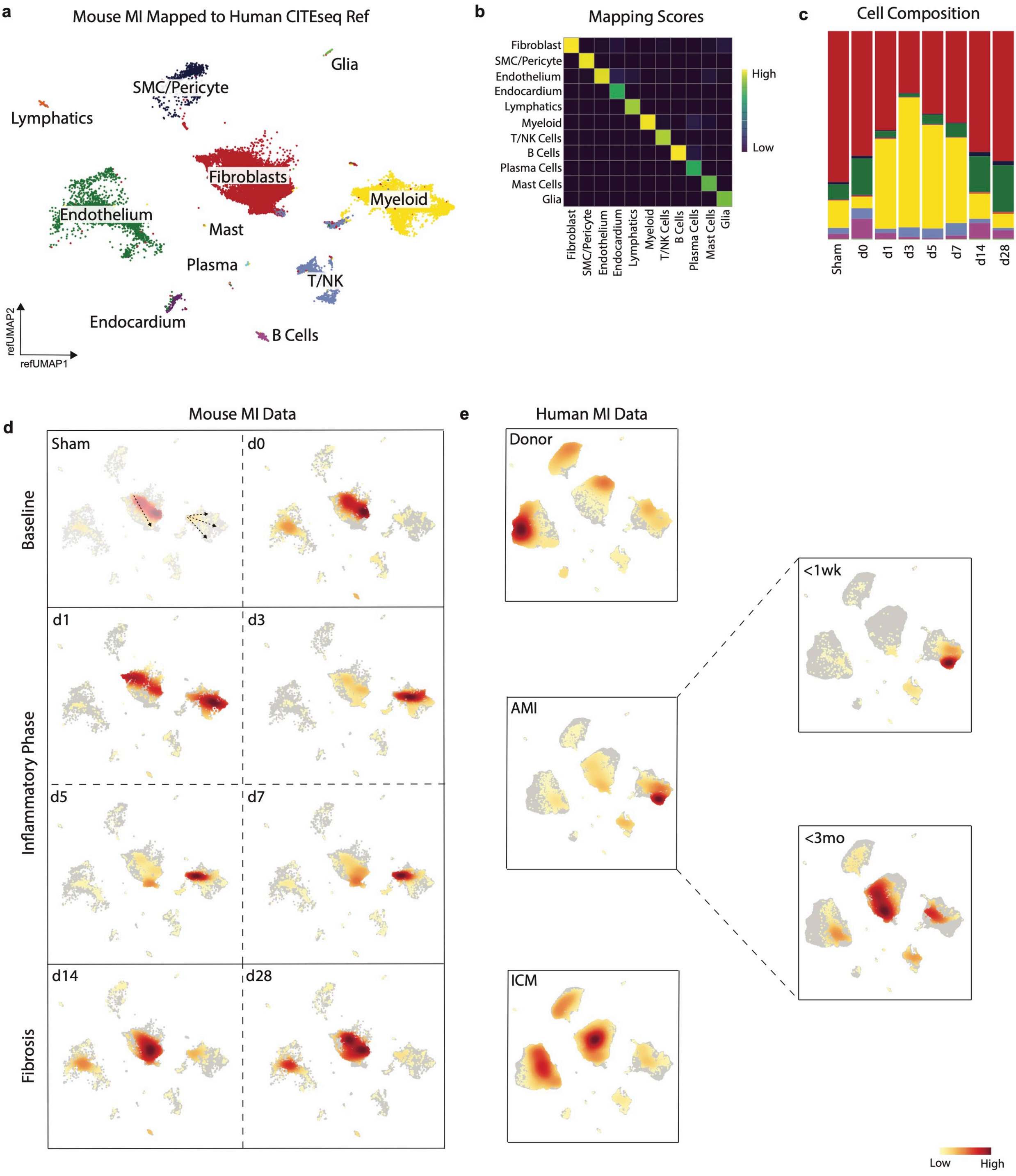



WT GCAAAAACATCTGGAAAAATGAAGGTTAGTGCGTGCCGTCTCGGGGACGACC
KI GCAAAAACATCTGGAAAAATGGCCAATTTACTGACCGTACACCAAAATTTGC
CRE M A N L L T V H Q N L

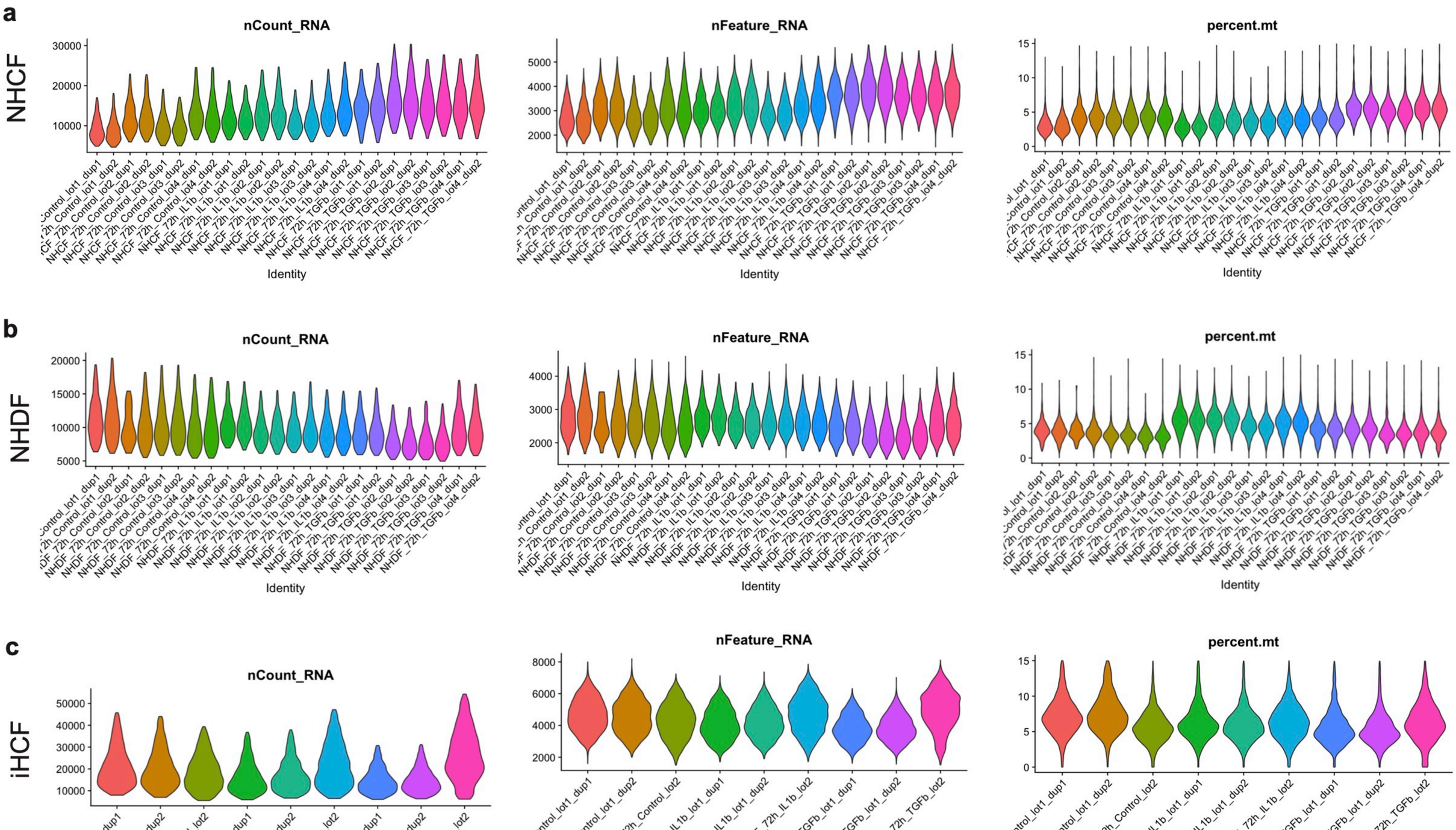
**d**



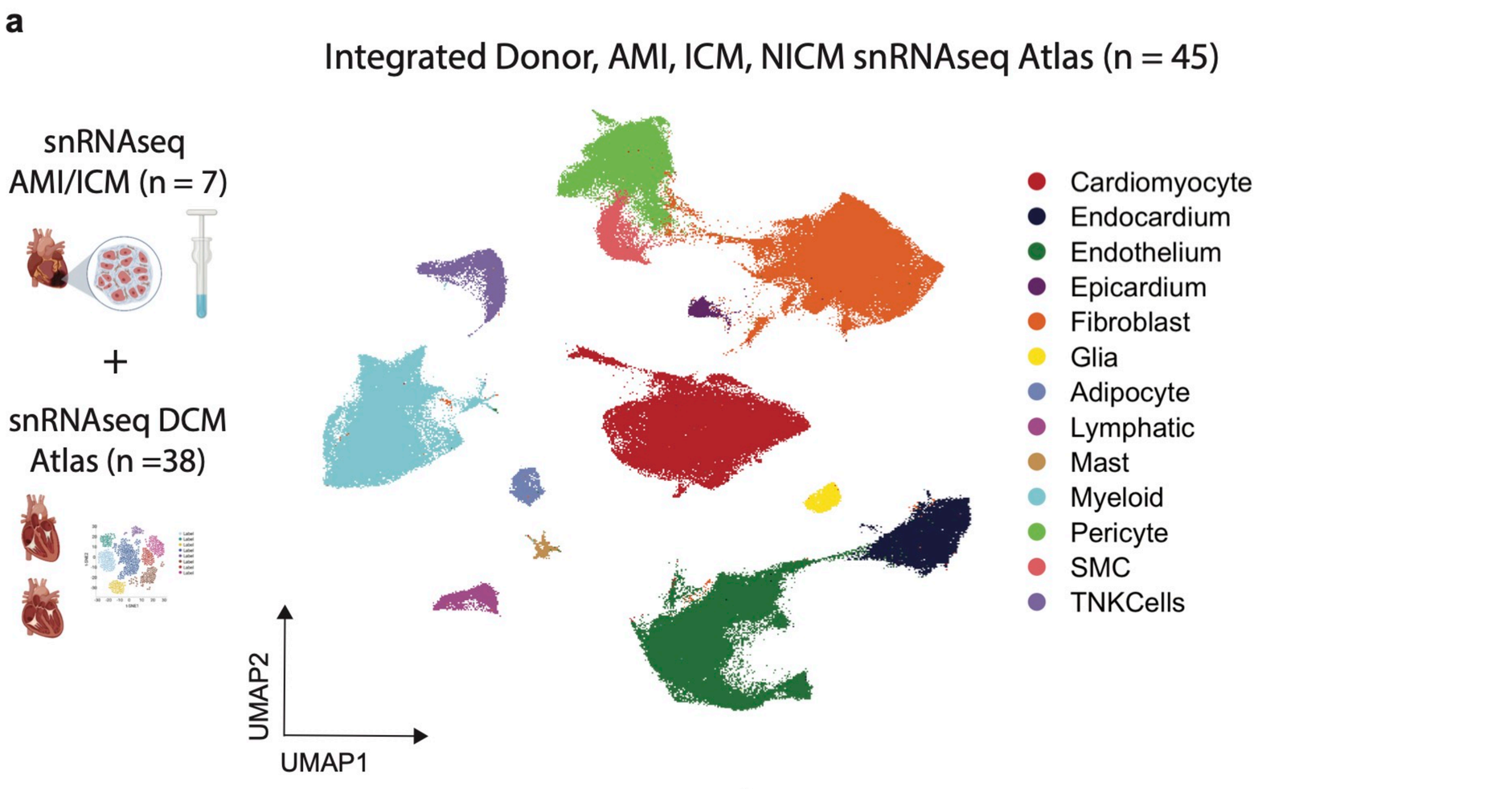

**Supplementary Figure 2.** (a) Schematic representing the homology directed repair to incorporate Cre recombinase and estrogen receptor 2 (ERT2) followed by a bovine growth hormone poly A (bGH poly (A) termination sequence, called KI into the start codon of the endogenous mouse Fap gene (WT). PCR primer sequence is shown in purple and amplicon lengths are as follows; 5' LR PCR- 1798 bp, SR PCR- 400 bp, 3' LR PCR- 2498 bp. (b) Genomic sequence of the FapCreERT2 mouse allele and genotyping primers. (c) Sanger sequencing showing the wild-type (WT) Fap gene and the knock-in (KI) sequence with the protein translation (CRE). (d) tdTomato immunofluorescence in Ang II/PE day 14 with and without TAM induction.



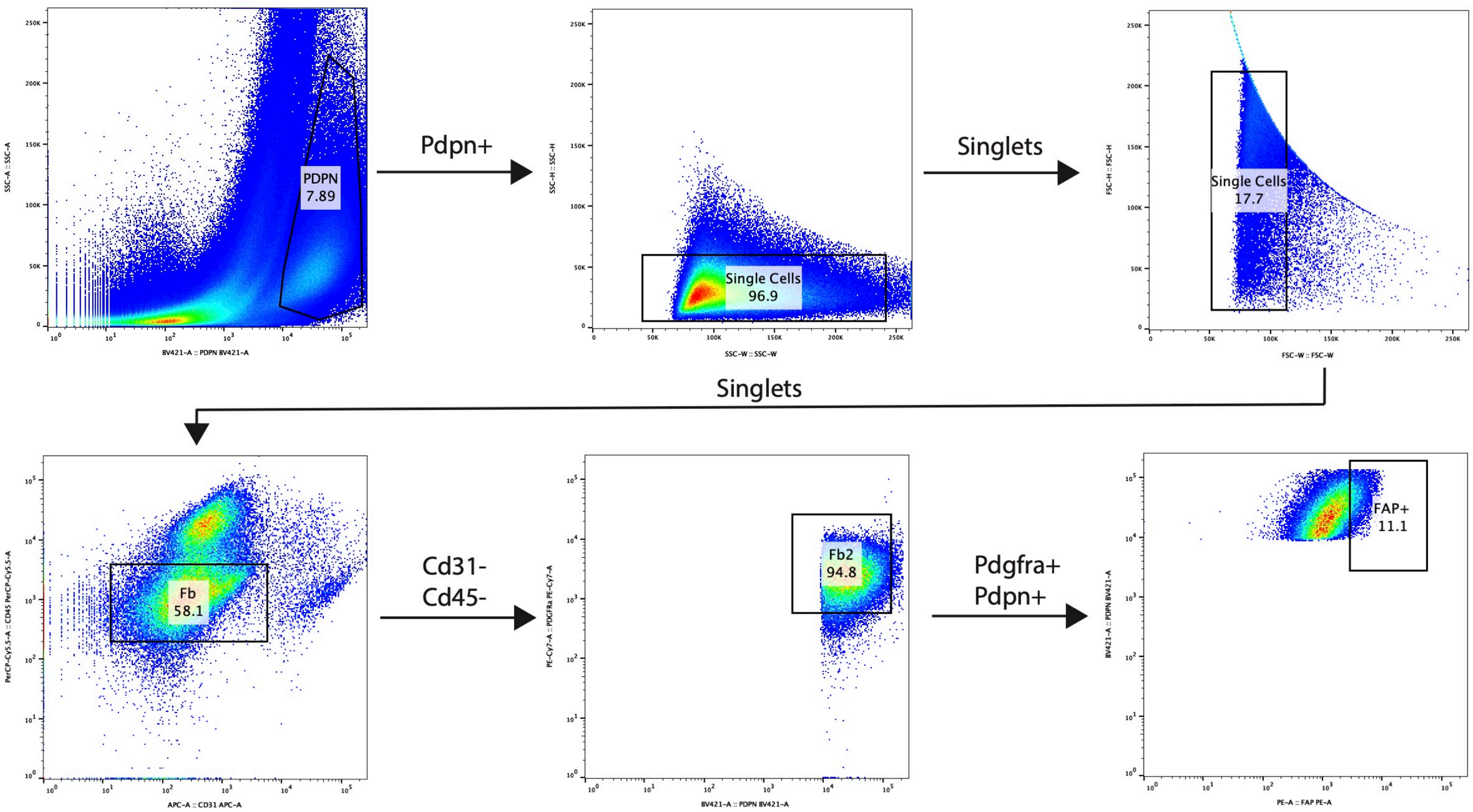

**Supplementary Figure 3.** (a) Mouse MI data mapped onto human CITE-seq global object. (b) Seurat reference mapping scores for different cell types. (c) Cell composition from imputed annotations split by time point. (d) Gaussian kernel density embedding plots in mouse MI data split by condition and biological phase and (e) human MI data grouped by MI category.




**Supplementary Figure 4.** (a) Quality control metrics post filtering grouped by each biological replicate for each experimental condition (vehicle, TGF-β, and IL-1β) in (a) NHCF, (b) NHDF, and (c) iHCF. (d) Integrated data from 3 cell lines in a UMAP embedding colored by biological replicate for each experimental condition.




**Supplementary Figure 5.** (a) UMAP embedding plot of integrated snRNA-seq atlas. (b) FAP RNA expression in example Visium spatial transcriptomics samples from donor, AMI, and ICM. (c) Gene signature for cardiomyocyte, perivascular cells (endothelium, VSMC, and pericyte), fibroblast, myeloid, and T/NK-cells in AMI section with H&E zoom in.




**Supplementary Figure 6.** (a) Flow cytometry gating scheme for fibroblasts. (b) FAP+ gate construction with a no antibody-stained negative control.