Methods

Cell lines: Cell line HEK293A was purchased from ThermoFisher scientific Inc. (Whaltham, MA) and was cultured in high glucose DMEM without pyruvate, supplemented with 10% fetal bovine serum (FBS), L-glutamine (8mM) and penicilin/streptomicin (100U/ml) (Gibco, ThermoFisher Scientific Inc. Whaltham, MA).

Mice: KRN T cell receptor–transgenic mice were a gift from Drs. D. Mathis (Harvard Medical School, Boston, MA) and C. Benoist (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). HK2F/F mice were obtained from Dr. Hay(1) . These mice have the HK2 locus flanked by LoxP sites in exon 4 and exon 11. Col1a1Cre mice was obtained from Dr. Kisseleva(2). As described in Kisseleva's paper, collagen-α1 (I) promotor/enhancer we inserted into pGL3 (R2.1) using KpnI and Bgl II restriction enzymes. The construct was then cut with KpnI and SalI and inserted into fertilized C57BL/6J xCBA F1 hybrid embryos and implanted in pseudo-pregnant Swiss Webster Foster mothers. HK2F/F mice were crossed with Col1a1Cre mice through several generations until obtaining Col1a1-Cre ($HK2^{Col1}$) or negative littermates. All mice used in these experiments were bred on the C57BL/6 background and were 8–12 weeks old. All protocols involving animals received prior approval from the Institutional Review Boards and followed the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (National Research Council).

Reagents: Lipopolysaccharide (LPS) and 2-deoxy-D-glucose (2-DG) (Sigma Aldrich, San Luis, MO). Platelet-derived growth factor BB (PDGF) and tumor necrosis factor alpha (TNF) were obtained from R&D Systems Inc. (Minneapolis, MN). 7AAC was purchased from AdooQ Bioscience (Irvine, CA).

FLS silencing experiments: HuHK1 and huHK2 silencing in FLS was performed *in vitro* using the Magnetofection SilenceMag technology (OZbiosciences, San Diego, CA) according to manufacturer instructions. 10nM of siRNA was used, either ON-TARGET pool^{TM} siRNA control (Dharmacon, Lafayette, CO), siRNAHK1 (Santa Cruz Biotechnology), or ON-TARGET^{TM} siRNAHK2 plus SMART pool (Dharmacon, Lafayette, CO). After 16 hrs. of incubation, media was changed to complete media. Experiments were performed 72 hrs. post-transfection except specified otherwise.

Adenovirus (ad) experiments: Ad-mHK2 and ad-GFP were kindly provided by Dr. Miyamoto (Pharmacology Department, UCSD) and were developed as previously described(3). pDONR223-huHK1 and pDONR223-huHK2 were a gift from William Hahn & David Root (Addgene plasmid # 23730 and #23854). HuHK1 and huHK2 sequences coded in pDONR223-huHK1 and huHK2 were cloned into pAd/CMV/V5/DEST plasmid through the LR clonase II from Gateway technology systems (ThermoFisher Scientific Sci., Whaltham, MA). Both plasmids were transfected into HEK293A cell line for adenovirus production. Supernatants were collected, and freeze-thawed three times for cell disruption and 0.22um filtered for cell debris elimination. Titration of adenoviral vectors was assessed by cell lysis assay with HEK293A cells to obtain the multiplicity of infection (MOI) quantification. Overexpression experiments were performed by infecting FLS with 340 MOI. Experiments were performed 72 hrs. post- infection except specified otherwise.

Viability: RA FLS after 72 hours of silencing (siRNAsc, siRNAHK1, siRNAHK2) were fixed with 4% paraformaldehyde (PFA) and stained with 0.05% crystal violet (Sigma Aldrich, San Luis, MO) and images were captured with a Keyence Microscope at 20x. Cell density quantification was performed with Image J software**.**

Proliferation test: RA FLS cells were plated in a 12 well plated at 150.000 cell density. After 72 hours infection with ad-GFP, ad-HK1 or ad-HK2, cells were incubated with 10 uM EdU during 2 hours. Edu detection was performed with Click-iT ® Plus Edu Imaging Test (Life technologies, Thermo Fisher Scientific Inc., Whaltham, MA) following manufacturer's protocol. EdU positive cells were counted in one thousand cells Hoechst® positive cells per condition. Images were taken in the microscope facility (UCSD School of Medicine Microscopy Core) with Keyence Fluorescence Microscope.

Invasion test: One million FLS were trypsinized with 0.05% trypsine (Gibco, ThermoFisher Scientific Inc., Whaltham, MA) and resuspended in 40 μl of 1% FBS DMEM and 40 μl of Matrigel (356231, BD Biosciences, San Diego, CA). Then 4μl drops of the mixture were plated in a 6 well plate. After 5 minutes of incubation at 37°C, media was added in the presence or absence of PDGF (10ng/ml). The assay was stopped 24 hrs. later, by adding 4% paraformaldehyde (PFA). Cells were stained with 0.05% crystal violet (Sigma Aldrich, San Luis, MO) and images were captured with a 20X objective inverted microscrope. For quantification, 4 images were taken per condition and invasive area was measured by Image J software.

Migration test: FLS were seeded onto 6-well plates and allowed to come to confluence. A double cross scratch wound was done in each well with a sterile pipette tip. Cells were subsequently exposed to DMEM 1%FBS in presence or absence of PDGF (10ng/ml) for 24 hrs. FLS were fixed with buffered formalin 10%. Violet crystal was used at a concentration of 0.05% in distilled water for 30 minutes. After staining, cells were washed with water for 15 minutes and FLS migration across the wound margins was assessed, photographed and measured by ImageJ, the average of relative length between margins in 4 different fields at 24 hrs. was plotted as the difference between the percentage of migration control.

Immunoblotting (IB): FLS were disrupted in lysis buffer. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane through the semi-dry system (Invitrogen). After blocking for one hour with 5% milk, blots were incubated overnight at 4°C with the following antibodies: mouse anti-human HK2 at 1:500, mouse anti-human HK1 at 1:500 and mouse anti-human tubulin at 1:1000 (Santa Cruz Co, Santa Clara, CA). Horseradish peroxidase-conjugated anti-mouse IgG (Cell Signaling Technology, Danvers, MA) was used as secondary antibody at 1:2000 dilution. Membranes were developed using a chemiluminescence system (Clarity Western ECL, BIORAD, Hercules, CA). Images were captured by ChemiDoc^{TM} XRS+ System (BIORAD, Hercules, CA). Quantification was performed using ImageLab or ImageJ software.

Real-time quantitative PCR (qPCR): Total RNA was extracted from FLS with Trizol (Invitrogen). RNA was quantified and assessed for purity and concentration using a NanoDrop spectrophotometer (ND-1000). 1 ug of total RNA was used for cDNA synthesis using the Superscript VILO cDNA synthesis kit (ThermoFisher Scientific Inc., Whaltham, MA). qPCR was performed with SYBR Green PCR Master Mix Kit (Applied Biosystems, ThermoFisher Scientific Inc., Whaltham, MA). The relative amounts of transcripts were compared to those of 18s and normalized to untreated samples by the $^{\Delta\Delta}$Ct method. Primers are available upon request.

Lactate determination: Lactate secretion was determined by Lactate Fluorimetric assay kit (Biovision, San Francisco, CA) following the manufacturer's protocol.

Serum transfer and arthritis scoring in mice: Sera from adult, arthritic K/BxN mice were pooled for use in serum transfer. Recipient mice were injected intraperitoneally with 150 μl of the K/BxN mouse serum. Clinical arthritis scores were evaluated in the recipient mice after serum transfer, as described previously(4). On day 12 post–serum injection, the mice were euthanized and their paws were analyzed for histopathologic changes.

Adenovirus mice injection: A total of 1 x 10^8 viral particles in 8 μl were injected in the knees of control C57BL/6 mice. Adenovirus-GFP was injected in the right knee while adenovirus-HK2 was injected in the left knee. Fourteen days after the injection, mice were euthanized and knees were collected for histological studies and quantified as below (n=13).

Histology analysis: Joints were fixed in 10% formalin, decalcified in 10% EDTA for 2-3 weeks and paraffin embedded. Paraffin embedded human synovium and mice joints sections were stained with hematoxylin and eosin (H&E) and safranin O(4). A blinded semiquantitative scoring system was used to assess synovial inflammation, synovial hypertrophy, bone erosion and cartilage damage (0-5 scale) as previously described (4).

Immunohistochemistry (IHC): Paraffin sections prepared from arthritic mouse joints or human synovium were deparaffinized and hydrated for the IHC protocol. Antigen retrieval was performed with citrate buffer at 95 °C for 30 min and peroxidase activity was blocked with 3 % H_2O_2. After blocking the unspecific antibody binding sites with 2% BSA + 1% FBS in phosphate buffer saline (PBS), slides were incubated overnight at 4°C with the following antibodies: mouse anti-human HK1 (1:100, Santa Cruz Co, Santa Clara, CA), mouse anti-human HK2 (1:100, Santa Cruz Co, Santa Clara, CA), goat anti-mouse HK2 (1:100, Santa Cruz Co, Santa Clara, CA), rabbit anti-human vimentin (1:100, Cell Signaling Technology, Danvers, MA), rabbit anti-human alpha-smooth muscle actin (1:50, a-sma; Abcam, Cambridge, UK), goat anti-mouse metalloprotease 3 (MMP3) (1:100, Santa Cruz Co, Santa Clara, CA), mouse anti-ki67 (1;100, GeneTex, Irvine, CA), rat anti-human podoplanin (PDPN) (1:100, ebioscience™, ThermoFisher Scientific Inc., Whaltham, MA), rabbit anti-iNOS (1:100, Abcam, Cambridge, UK) and rat anti-F4/80 (1:100, ThermoFisher Scientific Inc.). Washing steps were performed with PBS and incubated with HRP or phosphatase alkaline-linked secondary antibody Impress system (Vector Labs, Burlingame,

CA). The signal was developed using diaminobenzidine (brown color) for single IHC, or adding niquel (black color) (Vector Labs, Burlingame, CA) and Immpact™ Vector® Red (Vector Labs, Burlingame, CA) for double-IHC. Sections were counterstained with hematoxylin. For PDPN staining, antigen retrieval was performed by incubating paraffin embedded formalin fixed slides in trypsin-EDTA for 15 minutes at 37 degrees. Slides were then incubated with hamster anti-mouse PDPN antibody (1:100; eBioscience) for 30 minutes at room temperature followed by incubation with goat anti hamster biotin (1:100; eBioscience) for 30 mins. Slides were then incubated with streptavidin HRP for 30 mins followed by DAB (Vector Labs, Burlingame, CA). Images were taken in the microscope facility (UCSD School of Medicine Microscopy Core) by Hamamatsu Nanozoomer Slide Scanning System (Hamamatsu Photonics, Japan).

Immunofluorescence: Paraffin sections prepared from arthritic mouse joints were deparaffinized as described and directly blocked with 3% BSA in 1x PBS. Goat anti-mouse HK2 (Santa Cruz Co, Santa Clara, CA) at 1:100, rabbit anti-vimentin (CST, Danvers, MA) at 1:100 were then incubated overnight at 4°C. Washing steps were performed with PBS and incubated with donkey anti-goat alexa-fluor 488 (1:200) and donkey anti-rabbit alexa-fluor 568 (1:200) at room temperature for 1 hour. Slides were then incubated with DAPI ((4',6-diamidino-2-phenylindole) for 20 minutes, washed and mounted. Images were taken in the microscope facility (UCSD School of Medicine Microscopy Core) by Hamamatsu Nanozoomer Slide Scanning System (Hamamatsu Photonics, Japan).

Confocal experiments: $2x10^4$ cells/well of RA FLS were plated on 18 mm coverslips placed in 6-well plates (n=3) and were infected with Ad-GFP, Ad-hHK1 or Ad-hHK2 24 hrs later as detailed in methods. 48 h post infection, cells were washed with PBS and fixed with 4% formaldehyde for 15 min at room temperature (RT). Cells were then permeabilized with 0.01% Triton X-100 and

blocked in 2%BSA for 60 min at RT. Rabbit anti-Tom20 (1:100 dilution, Santa Cruz) and mouse anti-HK-1 (1:100 dilution, Santa XCruz) or mouse anti-HK-2 (1:100 dilution, Santa Cruz) were incubated for 2 hours at RT. Secondary fluorescent antibodies were added for 1 hr. DAPI was used for nuclear counterstaining. Samples were imaged through a SP5 confocal microscope (Leica Microsystems, Buffalo Grove, IL, USA) 24 hrs. after mounting.

Preparation of single cells from the joints of arthritic mice: One hind leg was cleaned of all tissue and the upper and lower leg bones separated, while ensuring the surface of the knee joint remained intact. The exposed joints were digested in 1.5 ml of RPMI containing 2% FCS, 2.5 mg/ml collagenase D (Roche) and 20 μg/ml DNAase (Sigma-Aldrich) for 45 mins at 37°C with constant agitation. Digests were then filtered through 100 μm cell strainer and cells kept on ice. Bones were further digested for 30 min at 37°C in 1.5 ml RPMI containing 2% FCS, 2.5 mg/ml collagenase/dispase (Roche) and 20 μg/ml DNase (Sigma- Aldrich) with constant agitation. Combined cells were spun down, red cells lysed for 5 mins in ACK lysis buffer (Gibco) and cell counted and stained for flow cytometry. Cells were washed, filtered through a 70μm cell strainer, incubated with anti-live dead stain (zombie yellow, Bioledgend, 1:200 diluted in PBS) for 15 minutes. Cells were washed and incubated with anti-CD16/CD32 blocking antibody (1:200; ebioscience) for 10 min at room temperature, followed by staining with antibody cocktail at 4 °C. The following antibodies were used for membrane staining: PerCP-Cy5.5-labelled anti-CD45 (1:400; ebioscience), PE Cy7 labelled anti-CD31 (1:400; ebioscience) and PE labelled anti-Pdpn (1:800; ebioscience). Data was acquired using a BD LSR Fortessa X20. After staining the cell suspension with antibodies, the cell suspension was sorted into Pdpn+ and Pdpn- cells on a MoFlow Astrios flow cell sorter (Beckman coulter) with 100 μm nozzle at 20 psi. For the analysis with low input RNA-seq, the cells were sorted into 2% FBS HBS+ buffer spun down, and lysed

with extraction buffer (PicoPure™ RNA Isolation Kit, Thermo-fisher).

RNA-seq library preparation and sequencing: RNA was extracted from cells using the PicoPure™ RNA Isolation Kit (Thermo-fisher) according to manufacturer instructions. Library preparation was performed using the Nextera XT Library preparation kit (Illumina FC-131-1024) and performed following the Fludigm C1 adapted protocol on p28 SMARTer-C1-mRNA-Sequencing-Protocol (Fludigm document PN 100-5950 B1) from 'Tagmentation' onwards. 250 pg of cDNA was used per sample for library prep. Sequencing libraries were prepared using the Smart-Seq2 protocol. Libraries were pooled and sequenced with the Illumina HiSeq 2500 to a depth of 8-14M reads per library. RNA-seq data was analyzed starting with log2 transformed TPM expression values and differential gene expression performed. For RNAseq an n=6 mice was used for flow sorting with the pooled synovial cells from digested ankle and foot joints from both hind limbs processed for library preparation and sequencing.

Statistical analysis: Statistical analysis was performed with Prism software (version 5; GraphPad). Results are expressed as the mean±SEM. Normality of the variables was assessed using the Kolmogorov-Smirnov and D'Agostino-Pearson normality tests. For comparison between 2 groups, Student's 2-tailed *t*-tests or nonparametric Mann-Whitney tests were applied, depending on the normality of the distribution of the variables. For comparison between paired samples, paired *t*-tests or nonparametric Wilcoxon's matched pairs tests were applied. We compared 3 or more groups with analysis of variance, either Dunnett's post hoc test or the Bonferroni test was chosen, depending on the homogeneity of variances. Results were considered significant if the 2-sided *P* value was less than 0.05.

SUPPLEMENTARY FIGURES

Supplementary Figure 1: HK1 expression in synovial OA and RA tissues. Representative IHC images of HK1 staining of two different RA and OA synovium samples. B) Immunoblots (IB) of 5 different OA and RA FLS cell lines of the indicated proteins, along with IB quantification (mean ± SEM: OA: 0.64 ± 0.24; RA: 1.97 ± 0.47. *$p<0.05$).

Supplementary Figure 2: RA FLS phenotype after HKs deletion. A) Graphs representing relative mRNA levels of HK1 and HK2 after 48 hrs. or 72 hrs. of transfection with siRNAsc (control), siRNAHK1 and siRNAHK2. Three RA cell lines per condition. *$p<0.05$; ** $p<0.001$. B) IB of 3 RA FLS cell lines of the indicated proteins after 48 or 72 hrs. of transfection with siRNAsc, siRNAHK1 or siRNAHK2. C) Representative pictures of RA FLS cell line, 72 hours after transfection with siRNAsc, siRNAHK1 and siRNAHK2 (Image cell density measured by Image J: Values are the mean ± SEM. * =$p<0.05$). n=3 RA FLS. D) qPCR analysis of the indicated genes in RA FLS after siRNA-sc, siRNA-HK1 or siRNAHK2 transfection.

Supplementary Figure 3: RA FLS phenotype after HKs overexpression. A) Relative mRNA levels of three RA FLS cell lines after infection with adenovirus-GFP (ad-GFP), adenovirus-HK1 (ad-HK1) and adenovirus-HK2 (ad-HK2) during 48 hrs. or 72 hrs. *$p<0.05$; ** $p<0.001$. B) IB of 3 RA FLS cell lines of the indicated proteins after 72hrs. of adenovirus (ad)-HK1 (upper image) or ad-HK2 (bottom image) infection. C) Edu detection was conducted as described in methods in 3 RA FLS after ad-GFP, ad-HK1 and ad-HK2 infection. Results are average of 3 different RA FLS lines. Values are shown as mean ± SEM. * =$p<0.05$

Supplementary Figure 4: Intracellular distribution of HKs after HKs overexpression. A) Intracellular distribution of HKs (in green) and mitochondria (Tom20; in red) in FLS at baseline (left panels) and after Ad- infection (right panels) examined by confocal microscopy. Figure shows

also overlapping images (yellow, co-localization of HKs with Tom20). Representative images of three different RA FLS.

Supplementary Figure 5: Invasion in OA FLS after HK2 overexpression. Two 2 OA FLS were infected with either Ad-GFP or Ad-HK2 as detailed in methods. Representative images of invasion area are shown. Quantification, as detailed in methods of area of invasion after PDGF stimulation for 24 hrs are (right): mean± SEM: ad-GFP+PDGF: 10.4±0.78 vs ad-HK2+PDGF: 8.79±1.34; $p>0.05$; and (left): mean± SEM: ad-GFP+PDGF: 43.06±7.8 vs ad-HK2+PDGF: 28.38±2.70; $p>0.05$.

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