Fig. S1. Microfibrils are not deposited by HDF cultures when FN is depleted. (A) Immunofluorescence microscopy of HDF cultures (8 days), showing deposition of fibrillin-1 (Fibr-1; B/W, red) and FN (B/W, green), with nuclei stained with DAPI (blue). Images were taken using a 20× objective. Specific band-pass filter sets for DAPI, FITC, and Cy3 or Cy5 were used to prevent bleed-through. Control cultures (Con) showed partial colocalisation of fibrillin-1 and FN (yellow). FN knockdown (kd) HDF cultures had no detectable microfibrils. EXT1 knockdown (kd) HDFs had disordered microfibrils but no apparent FN changes. Scale bars = 100 μm. n = 2 (B) Fibrillin-1 deposition was quantified using ImageJ. The percentage area was calculated by applying an equal threshold value over all the images and measuring the area of the brightest pixels corresponding to fibrillin fibrils. For each cell type, at least 3 images were used and the graph shows the mean percentage area and SEM. Statistical significance for deviation from the control cell-line values was calculated using a 2-way ANOVA with a Bonferroni's multiple comparisons test using GraphPad Prism V6. Asterisk indicate P values where *** = $P \leq 0.001$. (C) Medium and cell layer extracts from HDF cells in control (Con) and knockdown (kd) experiments (perlecan, EXT1, PKCα, FN, syndecan-2 or syndecan-4) were separated on 3-8% Tris-acetate gels in reducing conditions, and analyzed by western blotting for fibrillin-1 (antibody HPA021057) or FN (antibody FN-3E2), or β-actin (mAbAC-74) as loading control for cell layer extracts. Molecular weight markers are indicated. Quantification of band intensity is shown as a percentage of control band intensity (where Con = 100%). Data shown are from a single representative experiment, with biological and technical repeats exhibiting the same trends (n = 3).

Fig. S2. Relative expression levels in ARPE-19A and B cells, podocytes, and human dermal fibroblasts (HDFs). (A) Real-time quantitative PCR (qPCR) analysis of gene expression of fibrillin-1 (Fibr-1), fibronectin (FN), EXT1, perlecan (Perl), PKCα and syndecans (Syn) 1-4 in (i) ARPE-19A, (ii) ARPE-19B, (iii) podocyte (all 7 days) and (iv) HDF cultures (4 days). (B) Real-time qPCR analysis of gene expression of E-cadherin (E-cad), N-cadherin (N-cad), PDGF receptor-β (PDGFRβ), smooth muscle α-actin (SMA), SNAI1, SNAI2 and TWIST 1 in (i) ARPE-19A, (ii) ARPE-19B, and (iii) podocyte cultures (all 7 days). For both (A) and (B), expression is reported relative to TATA box binding protein (TBP), where TBP expression is equal to 1. Data are represented as the mean ± s.e.m. See Table S1 for details of n values for ARPE-19A and B cultures; for podocytes, n = 3; for HDF in (A) (iii), n = 6-12, depending on gene.

Fig. S3. Real-time quantitative PCR analysis of expression levels following knockdown of EXT1, perlecan, or fibronectin, and syndecan-2, syndecan-4 or PKCα. (A-D) qPCR analysis of expression levels of fibrillin-1 (Fibr-1), fibronectin (FN), EXT1, Perlecan (Perl), PKCα (PKCα) and syndecans (Syn) 1-4, following knockdown of EXT1, perlecan, or FN in (A) ARPE-19A, (B) ARPE-19B cells (both 7 days) and (C) HDF cultures (4 days). (D) qPCR analysis of expression levels of fibrillin-1 (Fibr-1), fibronectin (FN), syndecans (Syn) 1-4, E-cadherin (E-cad), N-cadherin (N-cad), PDGF receptor-β (PDGFRβ), smooth muscle α-actin (SMA), SNAI1, SNAI2 and TWIST 1 following knockdown of FN in podocyte cultures (7 days). Samples were either normalised to a combination of GAPDH/TBP expression (A, B and D), or to TBP expression (C) prior to being reported as fold changes relative to lipofectamine-treated control cell expression of either ARPE-19A, ARPE-19B, HDF or podocyte accordingly (where Con = 1). All data are represented as the mean ± s.e.m. and analyzed by 2-way ANOVA, with *P<0.05; **P<0.01; ***P<0.001. See Table S1 for details of n values for ARPE-19A and B cultures; for HDF, n = 3-16, depending on gene and knockdown condition. (E-H) qPCR analysis of expression levels of fibrillin-1 (Fibr-1), fibronectin (FN), EXT1, Perlecan (Perl), PKCα (PKCα) and syndecans (Syn) 1-4, following knockdown of syndecan-2, syndecan-4 or PKCα in (E) ARPE-19A, (F) ARPE-19B cells (both 7 days) and (G) HDF cultures (4 days). Samples were either normalised to a combination of GAPDH/TBP expression (E and F), or to TBP expression (G) prior to being reported as fold changes relative to lipofectamine-treated control cell expression of either ARPE-19A, ARPE-19B, or HDF accordingly (where Con = 1). All data are represented as mean ± s.e.m. and analyzed by 2-way ANOVA, with *P<0.05; **P<0.01; ***P<0.001. See Table S1 for details of n values for ARPE-19A and B cultures; for HDF, n = 3-16, depending on gene and knockdown condition. (H) Cell lysate samples of ARPE-19A and B cells (control and PKCα knockdown) were separated on 4-12% Bis-Tris gels in reducing conditions, and analyzed by western blotting for PKCα or β-actin (mAbAC-74) as loading control for cell layer extracts. Molecular weight markers are indicated. Verification of PKCα kd was achieved via quantification of band intensity ("Expression"), shown as a percentage of control band intensity (where Con = 100%).

Fig. S4. Effects of adding cellular fibronectin on microfibril and FN deposition by ARPE-19B cultures. Immunofluorescence microscopy of ARPE-19B cells (control and FN kd, 12 days), showing deposition of fibrillin-1 (Fibr-1; B/W, red) and FN (B/W, green), with nuclei stained with DAPI (blue), in the presence or absence of cellular FN (cFN; 10 μg/ml) . Addition of cFN to FN kd ARPE-19B cells failed to rescue deposition of fibrillin-1. Images were taken using a 20× objective. Specific band-pass filter sets for DAPI, FITC and Cy3 or Cy5 were used to prevent bleed-through. Scale bars = 100 μm.

Fig. S5. Effects on microfibrils of knocking down perlecan, or syndecans-2 or -4, or PKCα, in ARPE-19A, ARPE-19B and HDF cultures. Immunofluorescence microscopy of (A) ARPE-19A cells and (B) ARPE-19B cells (both 7 days), showing deposition of perlecan (B/W, red) and FN (B/W, green), with nuclei stained with DAPI (blue). Images were using a 20× objective. Boxed areas are shown as zoomed images on right. Control cultures (Con) showed partial colocalisation of fibronectin and perlecan (yellow). FN knockdown (kd) ARPE-19A cultures exhibited prominent perlecan staining, but FN knockdown ARPE-19B cultures had no detectable perlecan. Perlecan knockdown in both ARPE-19A and B cultures had no effect on FN deposition, and loss of perlecan was shown. Scale bars for first three lanes = 100 μm; scale bar for perlecan zoom = 25 μm; for A and B n = 3. (C) Immunofluorescence microscopy of HDF cultures (8 days), showing deposition of fibrillin-1 (Fibr-1; B/W, red) and perlecan (B/W, green), with nuclei stained with DAPI (blue). Boxed areas are shown as zoomed images on right. Control cultures (Con) showed partial colocalisation of fibrillin-1 and perlecan (yellow). FN knockdown (kd) HDFs had no detectable perlecan or microfibrils. Perlecan knockdown HDFs had some microfibrils. (D) Immunofluorescence microscopy of HDF cultures (8 days), showing deposition of fibrillin-1 (Fibr-1; B/W, red) and FN (B/W, green), with nuclei stained with DAPI (blue). Boxed areas are shown as zoomed images on right. PKCα, syndecan-2 and syndecan-4 knockdown (kd) HDFs had few microfibrils but abundant FN. For both (A) and (B), images were taken using a 20× objective. Scale bars = 100 μm, n = 3. For all microscopy, specific band-pass filter sets for DAPI, FITC, and Cy3 or Cy5 were used to prevent bleed-through.

Fig. S6. Real-time quantitative PCR analysis of syndecan and E-cadherin expression levels following siRNA treatments. qPCR analysis of expression levels of (A) syndecans 1-4 and (B) E-cadherin, following knockdown of EXT1, FN, perlecan, PKCα, syndecan-2 or syndecan-4 in ARPE-19A cells (black bars) and ARPE-19B cells (white bars; both 7 days). Samples were normalised to a combination of GAPDH/TBP expression prior to being reported as fold changes relative to ARPE-19A lipofectamine-treated control cell expression (where ARPE-19A Con = 1). E-cadherin expression is lower in control ARPE-19B cultures compared to control ARPE-19A cultures (see also Fig. 2). In both ARPE-19A and B cells, knockdown of EXT1 results in an increase in E-cadherin expression. None of the other gene knockdowns tested changed E-cadherin expression in ARPE-19B cells, whereas knockdown of perlecan and PKCα had consequences for E-cadherin expression in ARPE-19A cells. (C) Real-time quantitative PCR (qPCR) analysis of gene expression of syndecans 1-4 in control (Con) and FN kd ARPE-19A and B cells. For (A), (B) and (C), the "Gene Study" functionality of CFX Manager was utilised. In order to use the "Gene Study" software throughout the experiment, datasets were generated for ARPE-19A and B cells (for (A) and (C), n = 3-5, and for (B) n = 3-9, depending on the knockdown condition). All data are represented as the mean ± s.e.m. and analyzed by 2-way ANOVA, with **P*<0.05; ***P*<0.01; ****P*<0.001.

Fig. S7. Effects of actomyosin inhibitors and EXT1 knockdown on microfibril and FN deposition by ARPE-19A and B cultures. (A) Immunofluorescence microscopy of ARPE-19A and ARPE-19B cells (both cultured for 7 days), showing deposition of fibrillin-1 (Fibr-1; B/W, red) and FN (B/W, green), with nuclei stained with DAPI (blue). Images were taken using a 20× objective. ARPE-19A and B cells were incubated for 7 days in the presence of the myosin II inhibitor blebbistatin (10 μM), or the Rho kinase inhibitor Y27632 (10 μM), or the RhoA activator lysophosphatidic acid (LPA) (20 μM), with DMSO control cultures (DMSO). Microfibrils and FN were greatly reduced in both ARPE-19A and B cells by blebbistatin or Y27632, with only faint fine microfibril arrays observed. Scale bars for first three lanes = 100 μm; scale bar for Fibr-1 zoom = 25 μm. (B) Immunofluorescence microscopy of (i) ARPE-19A and (ii) ARPE-19B cells (both cultured for 7 days), showing deposition of fibrillin-1 (Fibr-1; B/W, red) and FN (B/W, green) after EXT1 knockdown (kd), with nuclei stained with DAPI (blue). Images were taken using a 20× objective. Control cultures (Con) showed partial colocalisation of fibrillin-1 and FN (yellow). EXT1 kd in both ARPE-19A and B cultures ablated microfibrils, with only cellular fibrillin-1 staining detected, and dense punctuate pericellular FN. Scale bars = 100 μm. For A, n = 2, and B, n = 4. For all microscopy, specific band-pass filter sets for DAPI, FITC, and Cy 3 or Cy5 were used to prevent bleed-through. Boxed areas are shown as zoomed images on right.

Fig. S1

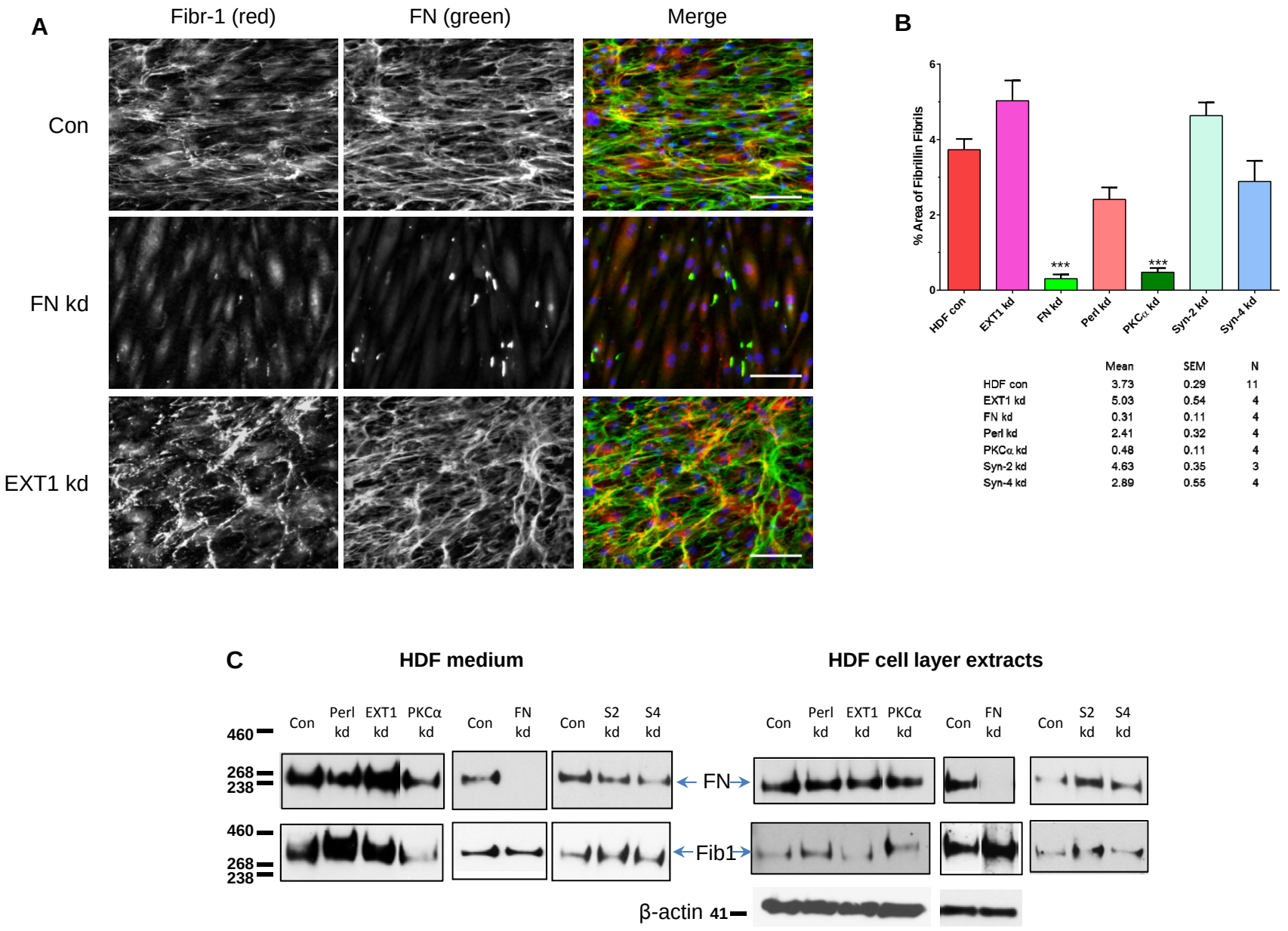
A
Fibr-1 (red)
FN (green)
Merge
Con
FN kd
EXT1 kd
B
% Area of Fibrillin Fibrils
6
4
2
0
***
***
HDF con
EXT1 kd
FN kd
Perl kd
PKCα kd
Syn-2 kd
Syn-4 kd
Mean SEM N
HDF con 3.73 0.29 11
EXT1 kd 5.03 0.54 4
FN kd 0.31 0.11 4
Perl kd 2.41 0.32 4
PKCα kd 0.48 0.11 4
Syn-2 kd 4.63 0.35 3
Syn-4 kd 2.89 0.55 4
C
HDF medium
HDF cell layer extracts
Con
Perl kd
EXT1 kd
PKCα kd
Con
FN kd
Con
S2 kd
S4 kd
460
268
238
FN
Fib1
β-actin 41

Fig. S2

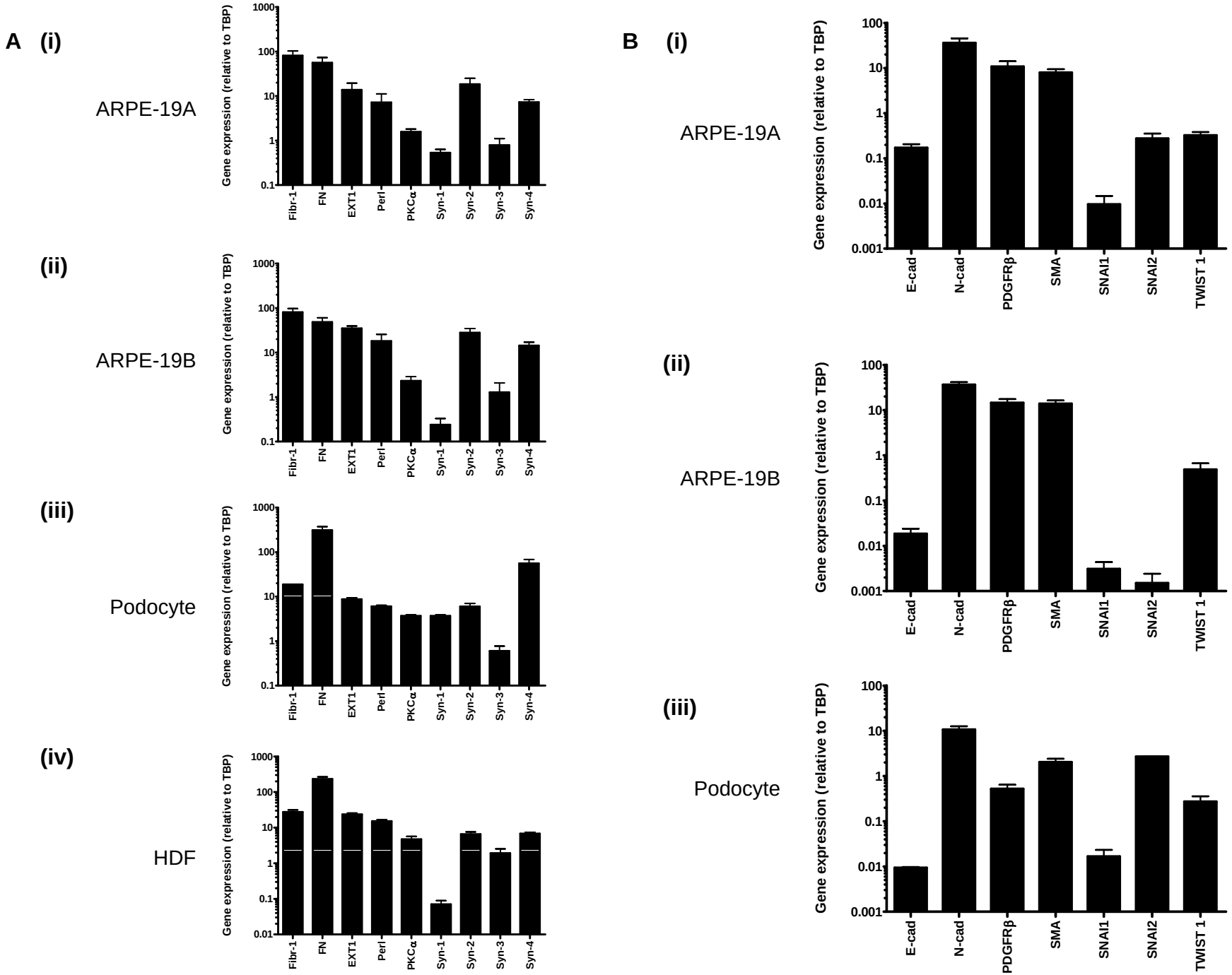
A
(i)
ARPE-19A
(ii)
ARPE-19B
(iii)
Podocyte
(iv)
HDF
Gene expression (relative to TBP)
Fibr-1
FN
EXT1
Perl
PKCα
Syn-1
Syn-2
Syn-3
Syn-4
B
(i)
ARPE-19A
(ii)
ARPE-19B
(iii)
Podocyte
Gene expression (relative to TBP)
E-cad
N-cad
PDGFRβ
SMA
SNAI1
SNAI2
TWIST 1

Fig. S3

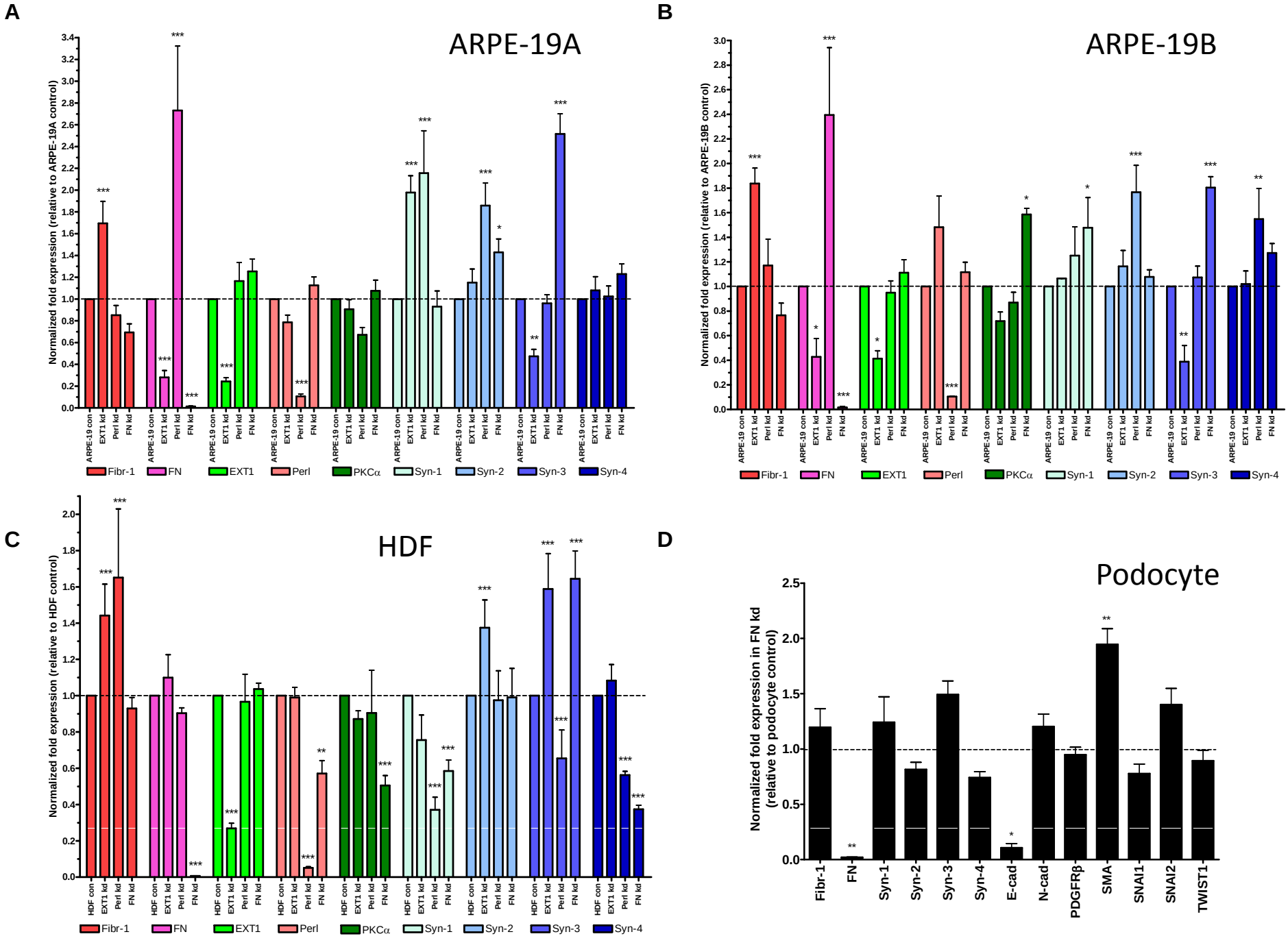
A
ARPE-19A
Normalized fold expression (relative to ARPE-19A control)
B
ARPE-19B
Normalized fold expression (relative to ARPE-19B control)
C
HDF
Normalized fold expression (relative to HDF control)
D
Podocyte
Normalized fold expression in FN kd (relative to podocyte control)
ARPE-19 con
HDF con
EXT1 kd
Perl kd
FN kd
Fibr-1
FN
EXT1
Perl
PKCα
Syn-1
Syn-2
Syn-3
Syn-4
Fibr-1
FN
Syn-1
Syn-2
Syn-3
Syn-4
E-cad
N-cad
PDGFRβ
SMA
SNAI1
SNAI2
TWIST1

Fig. S3

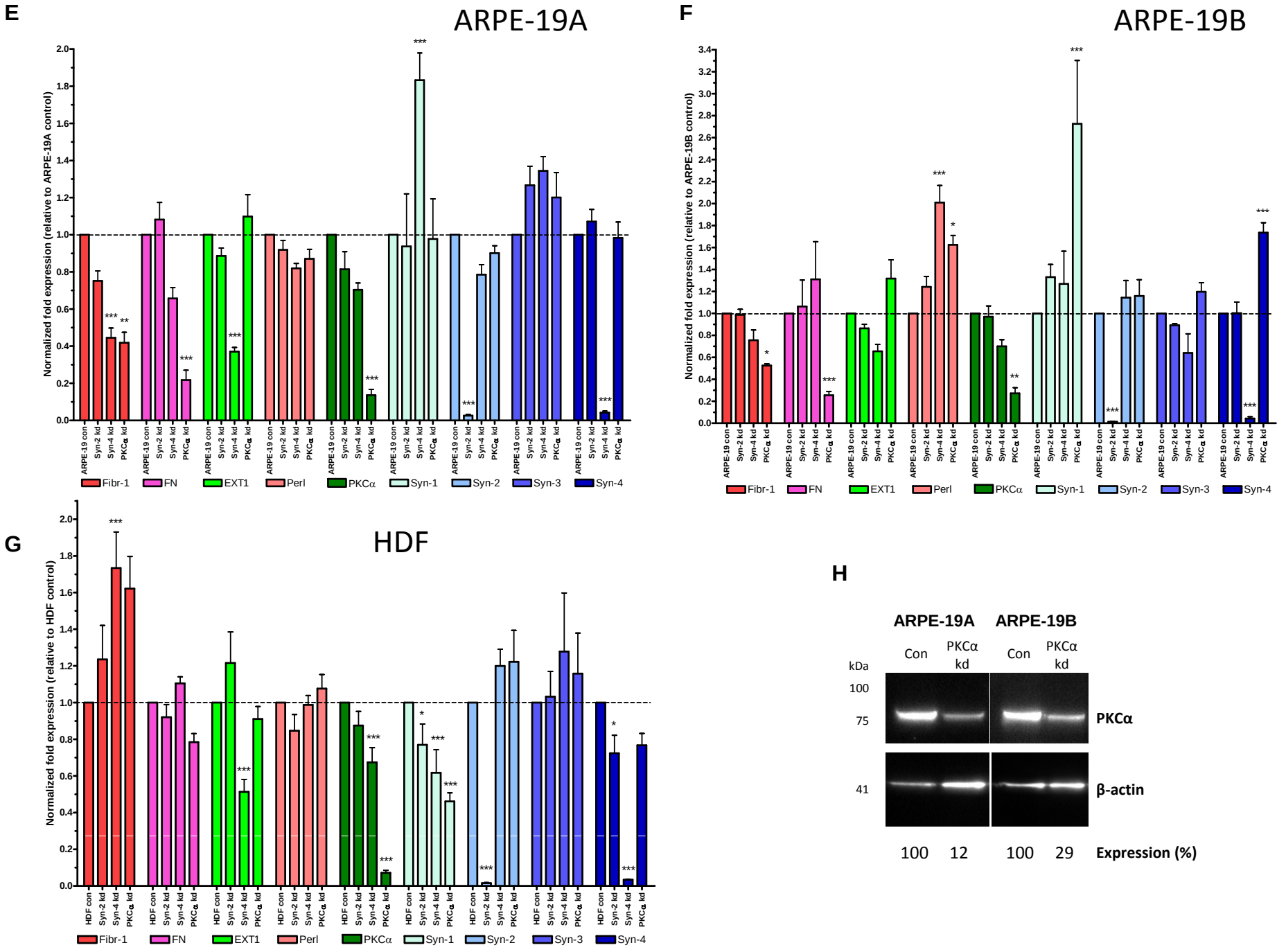
E
ARPE-19A
Normalized fold expression (relative to ARPE-19A control)
F
ARPE-19B
Normalized fold expression (relative to ARPE-19B control)
ARPE-19 con
Syn-2 kd
Syn-4 kd
PKCα kd
Fibr-1
FN
EXT1
Perl
PKCα
Syn-1
Syn-2
Syn-3
Syn-4
G
HDF
Normalized fold expression (relative to HDF control)
HDF con
H
ARPE-19A
ARPE-19B
Con
PKCα kd
kDa
100
75
41
PKCα
β-actin
100
12
100
29
Expression (%)

Fig. S4

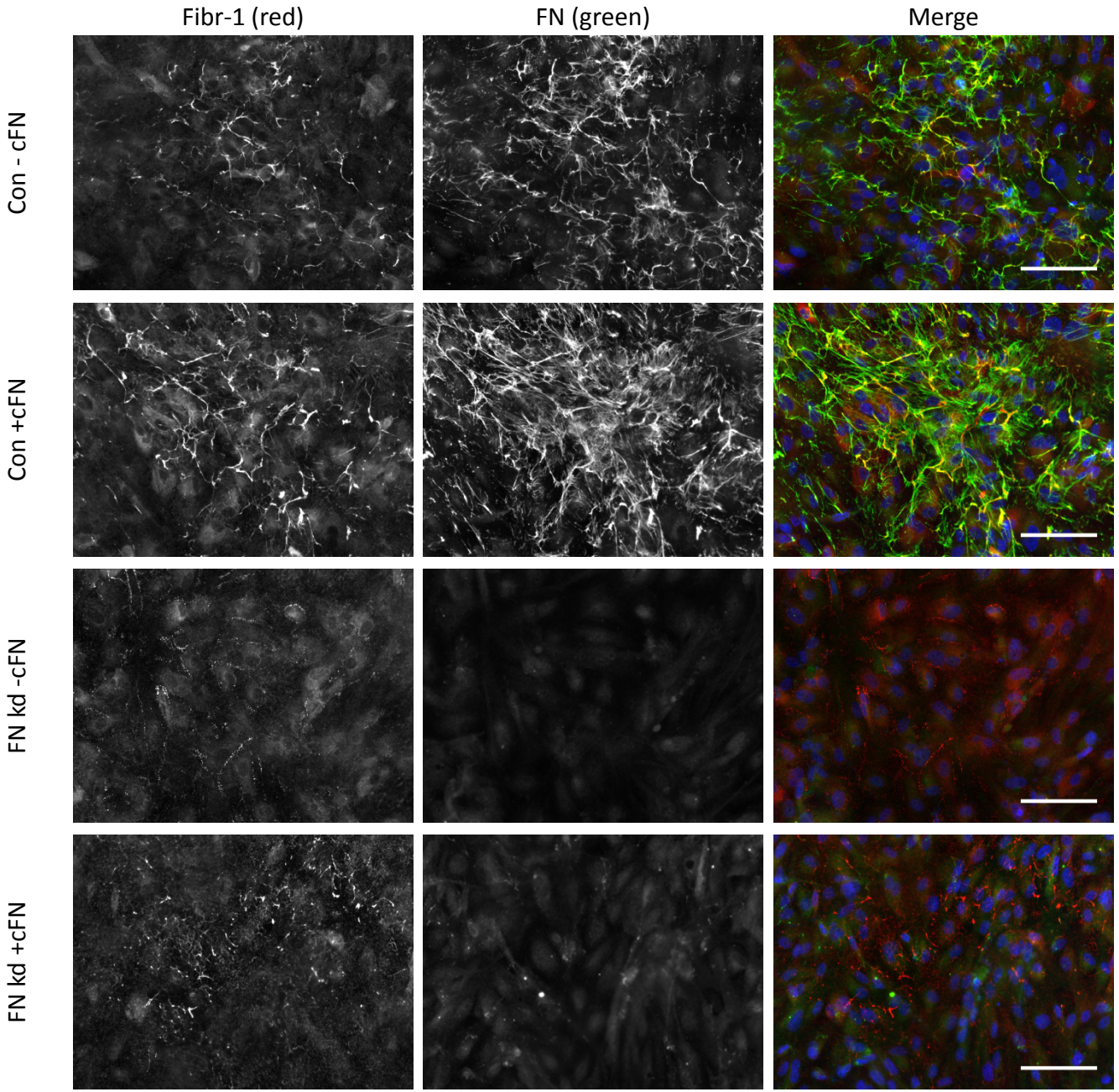

Fig. S5

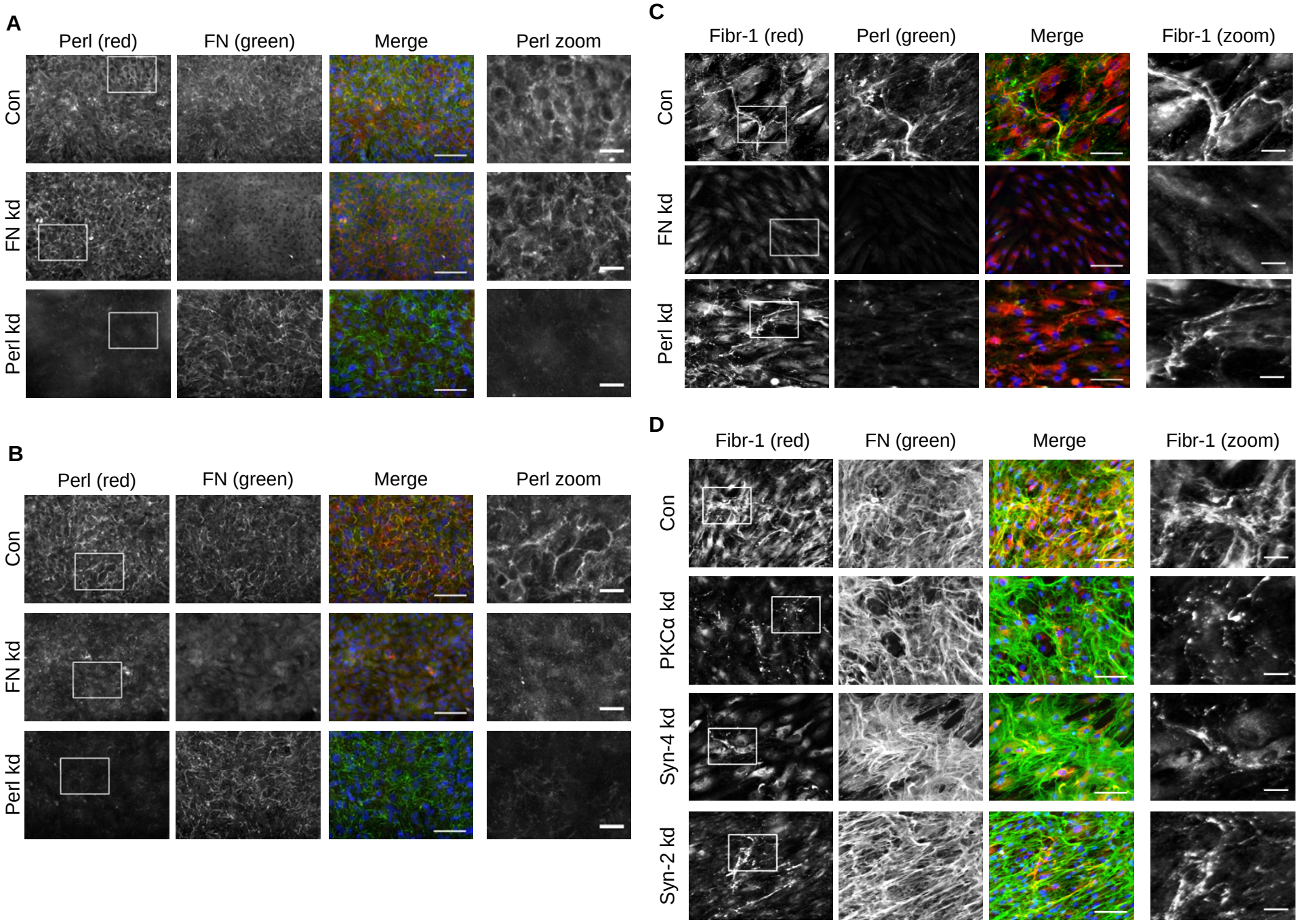
A
Perl (red)
FN (green)
Merge
Perl zoom
Con
FN kd
Perl kd
B
Perl (red)
FN (green)
Merge
Perl zoom
Con
FN kd
Perl kd
C
Fibr-1 (red)
Perl (green)
Merge
Fibr-1 (zoom)
Con
FN kd
Perl kd
D
Fibr-1 (red)
FN (green)
Merge
Fibr-1 (zoom)
Con
PKCα kd
Syn-4 kd
Syn-2 kd

Fig. S6

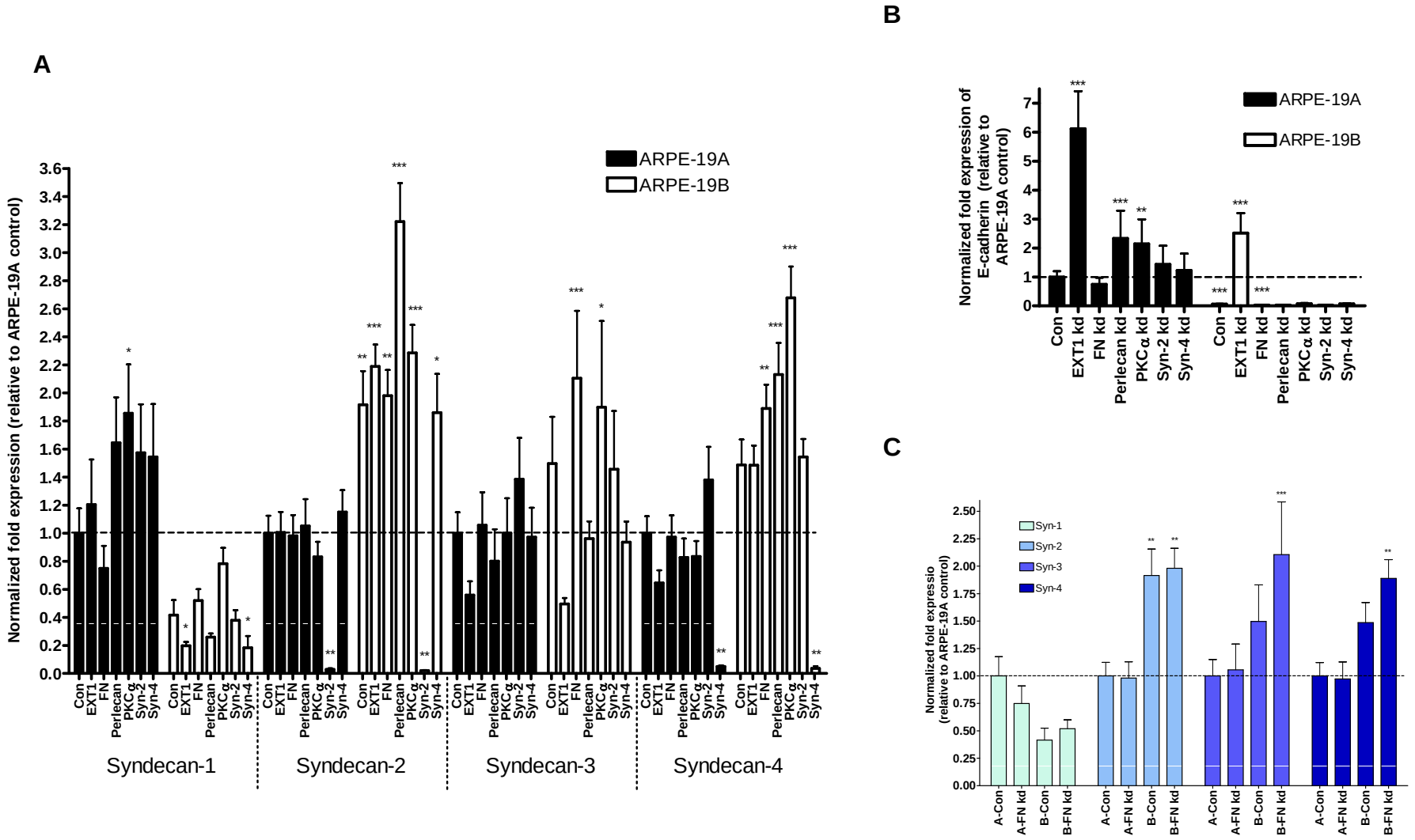
A
ARPE-19A
ARPE-19B
Normalized fold expression (relative to ARPE-19A control)
Con
EXT1
FN
Perlecan
PKCα
Syn-2
Syn-4
Syndecan-1
Syndecan-2
Syndecan-3
Syndecan-4
B
Normalized fold expression of E-cadherin (relative to ARPE-19A control)
ARPE-19A
ARPE-19B
Con
EXT1 kd
FN kd
Perlecan kd
PKCα kd
Syn-2 kd
Syn-4 kd
C
Normalized fold expressio (relative to ARPE-19A control)
Syn-1
Syn-2
Syn-3
Syn-4
A-Con
A-FN kd
B-Con
B-FN kd

Fig. S7

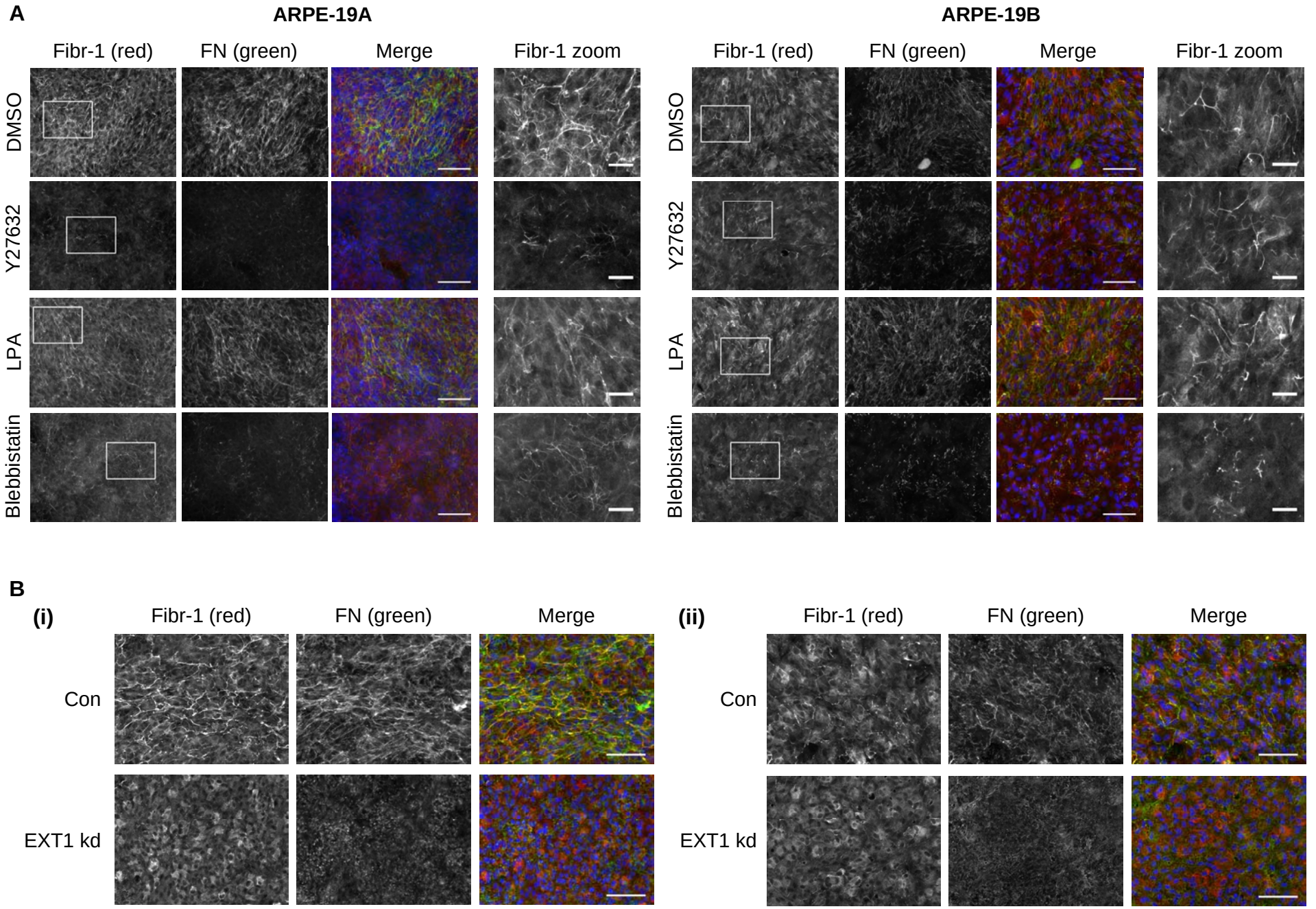
A
ARPE-19A
ARPE-19B
Fibr-1 (red)
FN (green)
Merge
Fibr-1 zoom
DMSO
Y27632
LPA
Blebbistatin
B
(i)
(ii)
Con
EXT1 kd

Table S1. Oligonucleotide primers used for qPCR analyses.

	Forward Primer (5′ – 3′)	**Reverse Primer (5′ – 3′)**
EXT1	GCTCTTGTCTCGCCCTTTTGT	TGGTGCAAGCCATTCCTACC
FN	CTGCGAGAGCAAACCTGAAG	TTTAGGACGCTCATAAGTGTCAC
TBP	TCGTGCCCGAAACGCCGAAT	CAGTGCCGTGGTTCGTGGCT
GAPDH	AAGGGCATCCTGGGCTAC	GTGGAGGAGTGGGTGTCG
PKCα	TGACGTGGAGTGCACCAT	GAGTGCAGCTGCGTCAAG
Fibrillin-1	GGGCATTTGCCAGAACAC	CGCTGAGGCATTCGTTTT
Syndecan-1	CCAGCCAAGCTGACCTTC	GAGGCTCCATCCTCAGCA
Syndecan-2	CCTGCTGTTGGTGTATCG	GCAGCACTGGATGGTTTG
Syndecan-3	GGAGCCTGACATCCCTGA	GGGGTCTGAGCCACCTCT
Syndecan-4	ACTGTGCAGGGCAGCAAC	AAGAGGATGCCCACGATG
Perlecan	TGCGCTGGACACATTCGTACCT	CCACTGCCCAGGTCGTCTCCT
E-cadherin	CATGAGCCACTGCACCTG	GCGATGGAGCGAAACTGT
N-cadherin	AATGACCCCACAGCTCCA	GAGCTCAAGGACCCAGCA
SMA	CATCACCAACTGGGACGA	GGTGGGATGCTCTTCAGG
PDGFRβ	CTCGGGGACCTACACCTG	ACGTAGCCGCTCTCAACC
SNAIL 1	TCCCATGGCCATTTCTGT	GACAGGCCAGCTCAGGAA
SNAIL 2	ACCCAATGGCCTCTCTCC	AGCCACTGTGGTCCTTGG
TWIST 1	ACCCAGTCGCTGAACGAG	GCCAGCTTGAGGGTCTGA

Publication: Journal of Cell Science (UK spelling)
Type: Research Article; Open Access: F; Volume: ; Issue:

Table S2. Total number of replicates for ARPE-19A and B qPCR studies. Table displaying the number of replicate experiments (n) performed in triplicate with ARPE-19A and B cells (7 days) to compile real-time quantitative PCR data. Values consist of a combination of technical and biological repeats. NA - not included in this study.

	ARPE-19A							ARPE-19B						
	Con	EXT1 kd	Perl kd	FN kd	Syn-2 kd	Syn-4 kd	PKCα kd	Con	EXT1 kd	Perl kd	FN kd	Syn-2 kd	Syn-4 kd	PKCα kd
Gene														
Fibr-1	10	5	4	5	7	7	4	11	3	3	10	3	3	3
FN	10	5	4	5	7	7	4	11	3	3	10	3	3	3
EXT1	6	6	4	6	5	6	3	4	3	3	3	3	3	3
Perlecan	5	4	5	4	4	4	4	4	3	3	3	3	3	3
PKCα	5	4	5	4	5	4	5	3	3	3	3	3	3	3
Syn-1	6	4	4	6	4	3	3	4	3	3	4	3	3	3
Syn-2	8	5	5	6	7	8	6	5	4	4	4	4	4	4
Syn-3	6	4	4	6	4	4	4	4	3	3	4	3	3	3
Syn-4	9	5	4	7	9	9	3	5	4	4	4	4	4	4
E-cad	12	3	3	9	3	3	3	11	3	3	8	3	3	3
N-cad	10	NA	NA	NA	NA	NA	NA	10	NA	NA	NA	NA	NA	NA
PDGFRβ	9	NA	NA	NA	NA	NA	NA	8	NA	NA	NA	NA	NA	NA
SMA	10	NA	NA	NA	NA	NA	NA	10	NA	NA	NA	NA	NA	NA
SNAI1	8	NA	NA	NA	NA	NA	NA	8	NA	NA	NA	NA	NA	NA
SNAI2	8	NA	NA	NA	NA	NA	NA	8	NA	NA	NA	NA	NA	NA
TWIST 1	7	NA	NA	NA	NA	NA	NA	7	NA	NA	NA	NA	NA	NA

	ARPE-19A							ARPE-19B						
	Con	EXT1 kd	Perl kd	FN kd	Syn-2 kd	Syn-4 kd	PKCα kd	Con	EXT1 kd	Perl kd	FN kd	Syn-2 kd	Syn-4 kd	PKCα kd
Gene														
Fibr-1	10	5	4	5	7	7	4	11	3	3	10	3	3	3
FN	10	5	4	5	7	7	4	11	3	3	10	3	3	3
EXT1	6	6	4	6	5	6	3	4	3	3	3	3	3	3
Perlecan	5	4	5	4	4	4	4	4	3	3	3	3	3	3
PKCα	5	4	5	4	5	4	5	3	3	3	3	3	3	3
Syn-1	6	4	4	6	4	3	3	4	3	3	4	3	3	3
Syn-2	8	5	5	6	7	8	6	5	4	4	4	4	4	4
Syn-3	6	4	4	6	4	4	4	4	3	3	4	3	3	3
Syn-4	9	5	4	7	9	9	3	5	4	4	4	4	4	4
E-cad	12	3	3	9	3	3	3	11	3	3	8	3	3	3
N-cad	10	NA	NA	NA	NA	NA	NA	10	NA	NA	NA	NA	NA	NA
PDGFRβ	9	NA	NA	NA	NA	NA	NA	8	NA	NA	NA	NA	NA	NA
SMA	10	NA	NA	NA	NA	NA	NA	10	NA	NA	NA	NA	NA	NA
SNAI1	8	NA	NA	NA	NA	NA	NA	8	NA	NA	NA	NA	NA	NA
SNAI2	8	NA	NA	NA	NA	NA	NA	8	NA	NA	NA	NA	NA	NA
TWIST 1	7	NA	NA	NA	NA	NA	NA	7	NA	NA	NA	NA	NA	NA