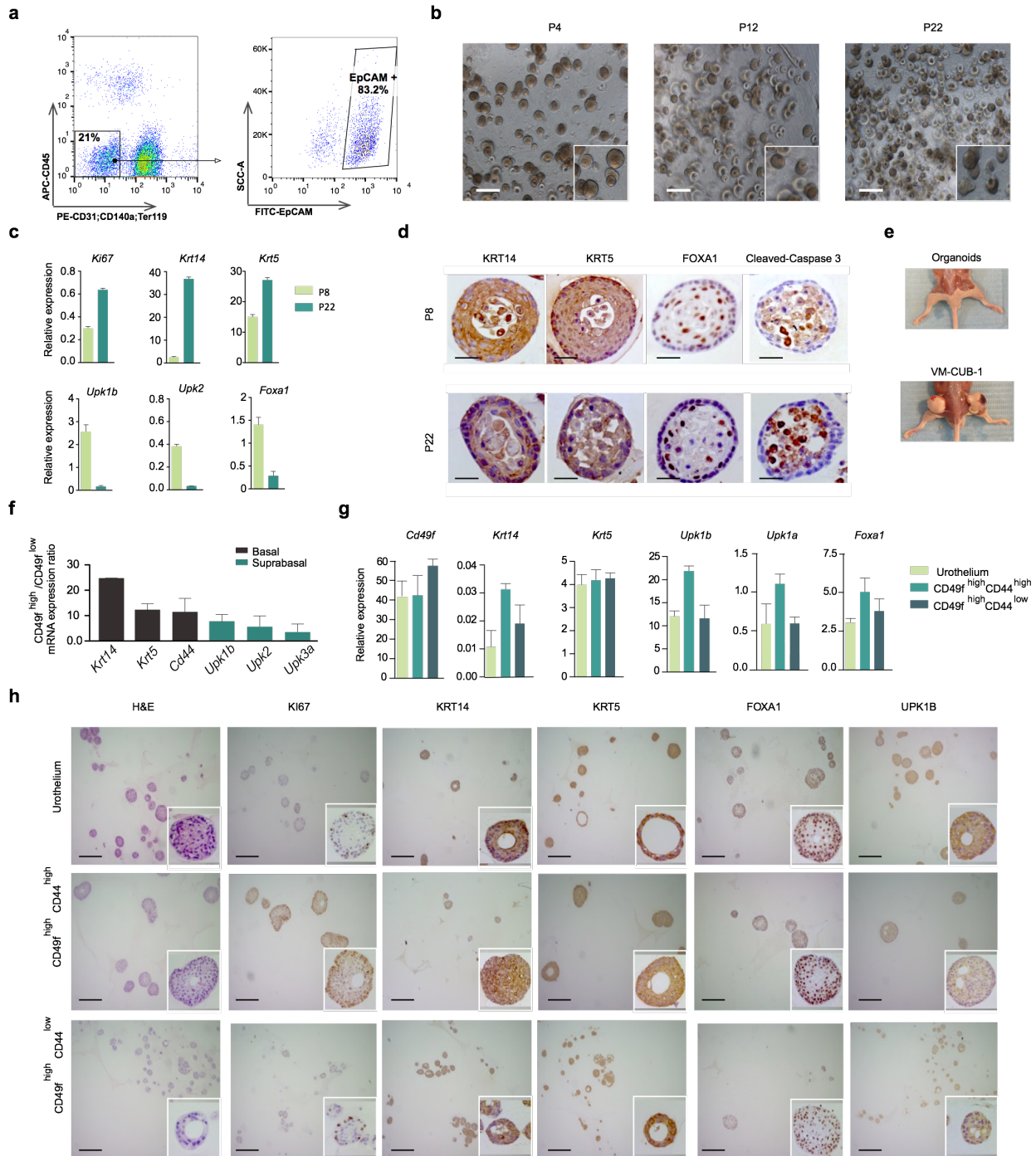
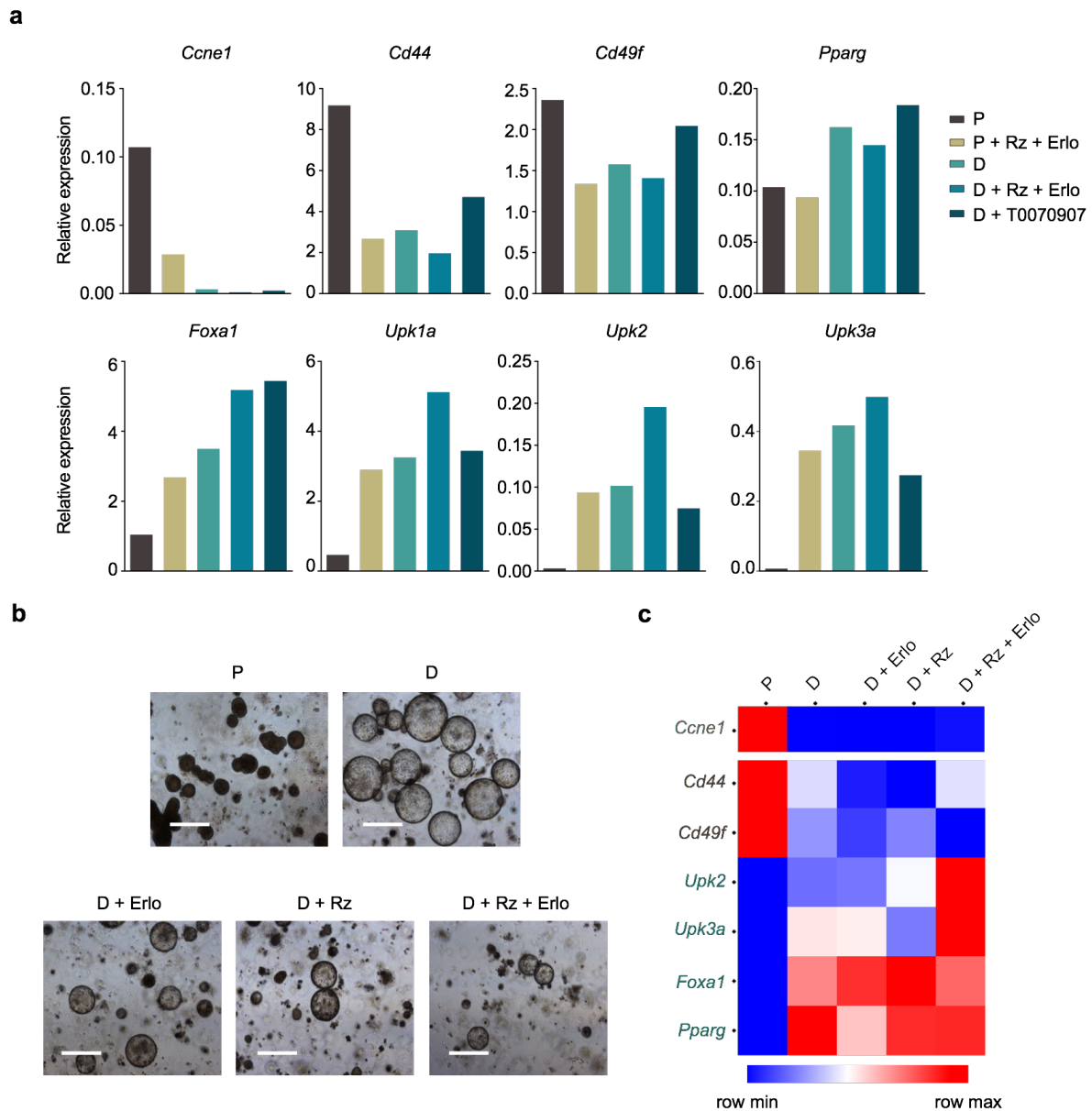


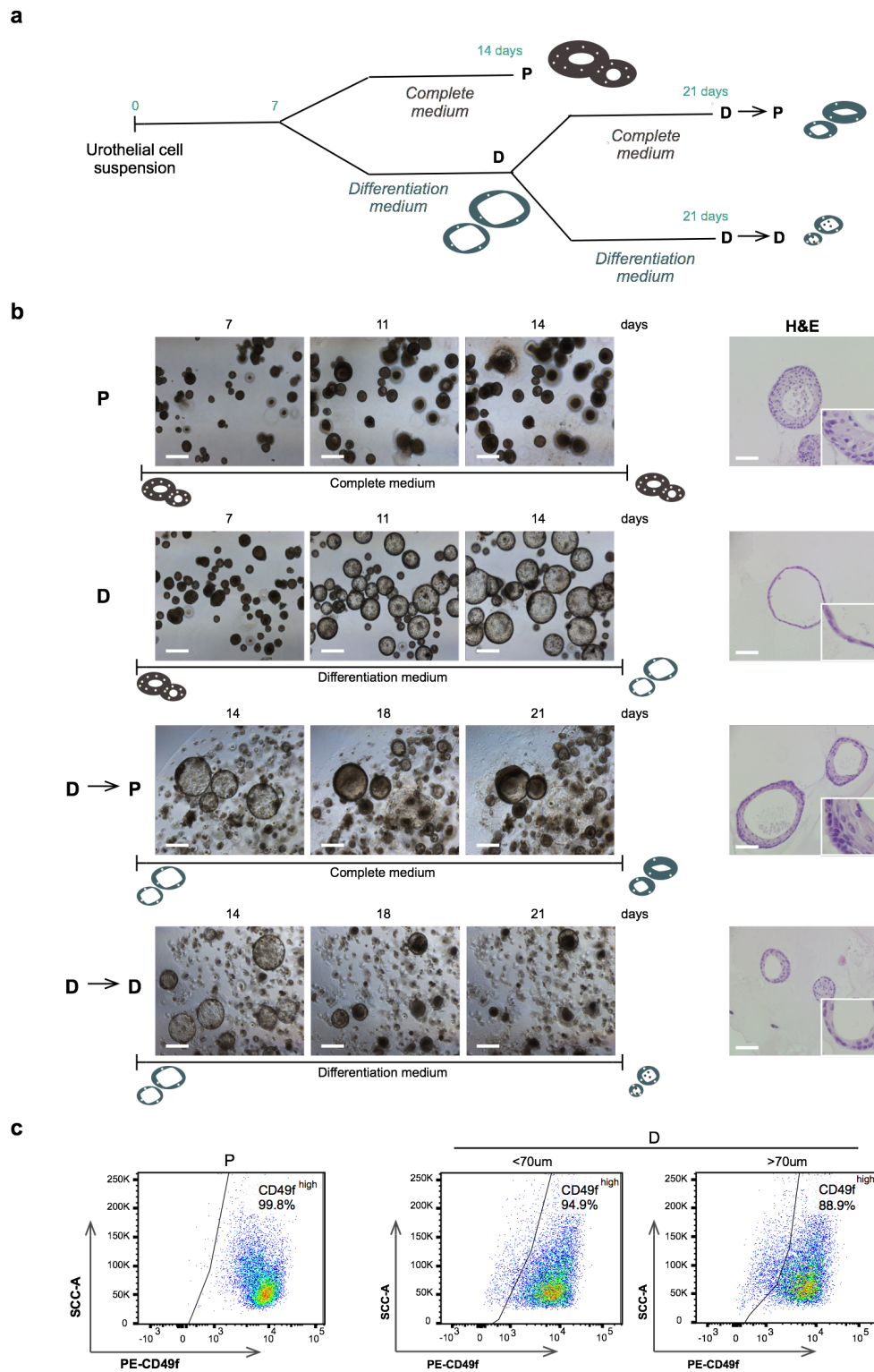
**Supplementary Information - Santos et al.**



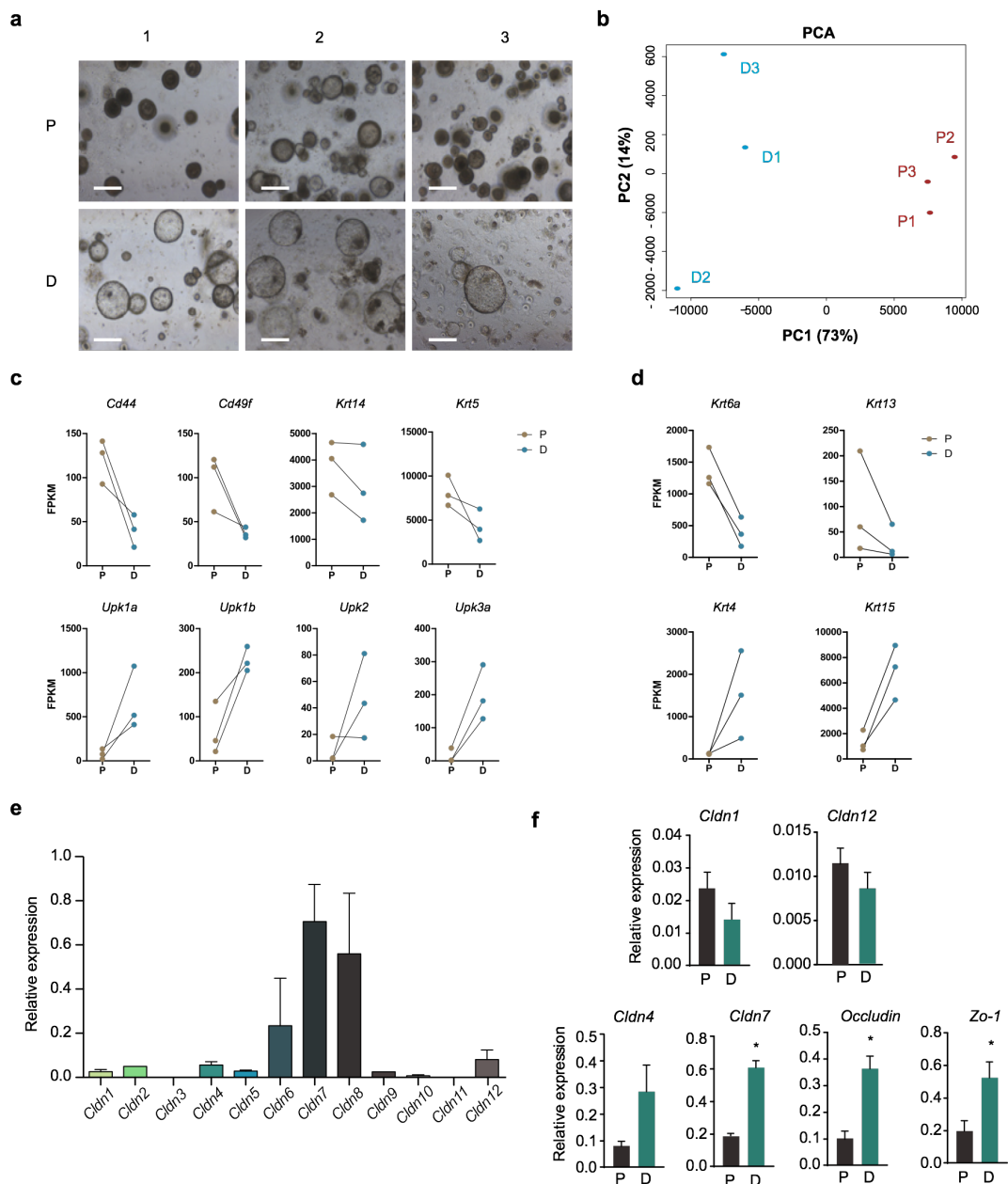
**Supplementary Figure 1. CD49f labels an organoid-forming urothelial cell population with stem cell features.** (a) Flow cytometry analysis reveals that urothelial digests contain ~20% EpCAM<sup>+</sup> epithelial cells with a major contribution of fibroblasts, leukocytes and endothelial cells. (b) Representative phase contrast images of organoids at different passages (P4, P12 and P22) (scale bar, 500 $\mu$ m). (c) RT-qPCR analysis of the expression of urothelial differentiation genes in proliferative (P) organoids at P8 and P22. Continued culture was associated with an enrichment of cells expressing basal markers. (d) Immunohistochemical analysis of urothelial differentiation marker expression in P organoids at P8 and P22 (scale bar, 100 $\mu$ m). (e) Subcutaneous xenotransplantation in the flank of nude mice; VM-CUB-1 urothelial bladder cancer cells were used as a positive control ( $n=6$ ). (f) Relative enrichment of urothelial marker transcripts in CD49f<sup>high</sup> cells compared to CD49f<sup>low</sup> cells. Graph representative of one experiment out of 2; error bars indicate SD. (g) RT-qPCR analysis of the expression of urothelial differentiation markers in organoids derived from FACS-sorted CD49f<sup>high</sup> CD44<sup>high</sup> and CD49f<sup>high</sup> CD44<sup>low</sup> cells compared to those derived from unsorted cells; error bars indicate SEM. (h) H&E and immunohistochemical analysis of Ki67 and urothelial differentiation markers in organoids generated from the indicated FACS populations (scale bar, 750 $\mu$ m).



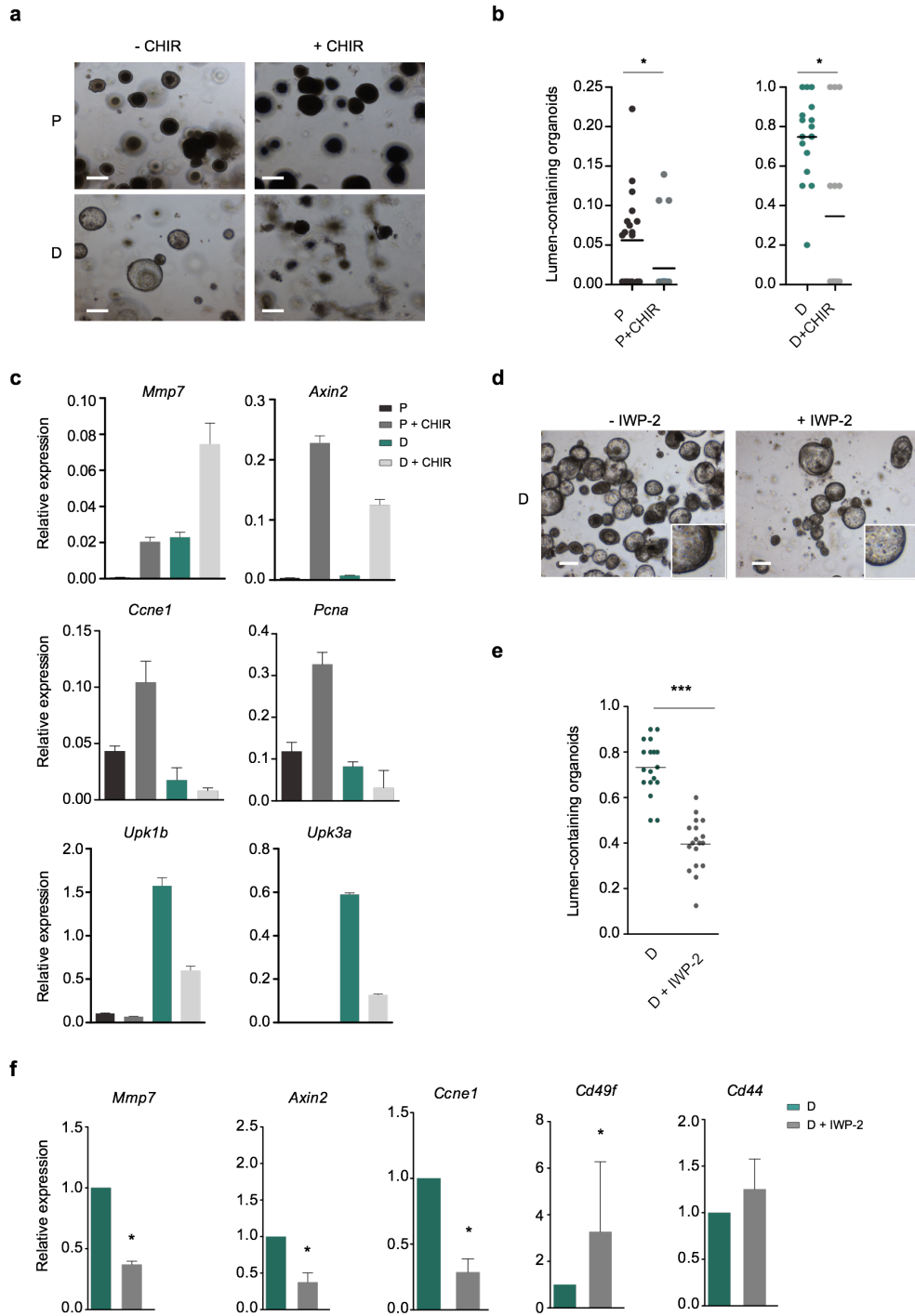
**Supplementary Figure 2. Pharmacological modulation of EGFR and PPAR $\gamma$  activity potentiates organoid differentiation.** (a) RT-qPCR expression analysis of cell cycle and canonical urothelial differentiation markers in proliferative (P) or differentiated (D) organoids treated with Rz+Erlotinib, and with the PPAR $\gamma$  inverse agonist T0070907 (n=2). Data were normalized to *Hprt* expression. (b) Phase contrast images of control (P, D) and drug-treated organoids (scale bar, 500  $\mu$ m). (c) Heatmap representing the expression of proliferation and urothelial differentiation markers in control and drug-treated organoids. P, proliferation; D, differentiation. Er, erlotinib; Rz, rosiglitazone.



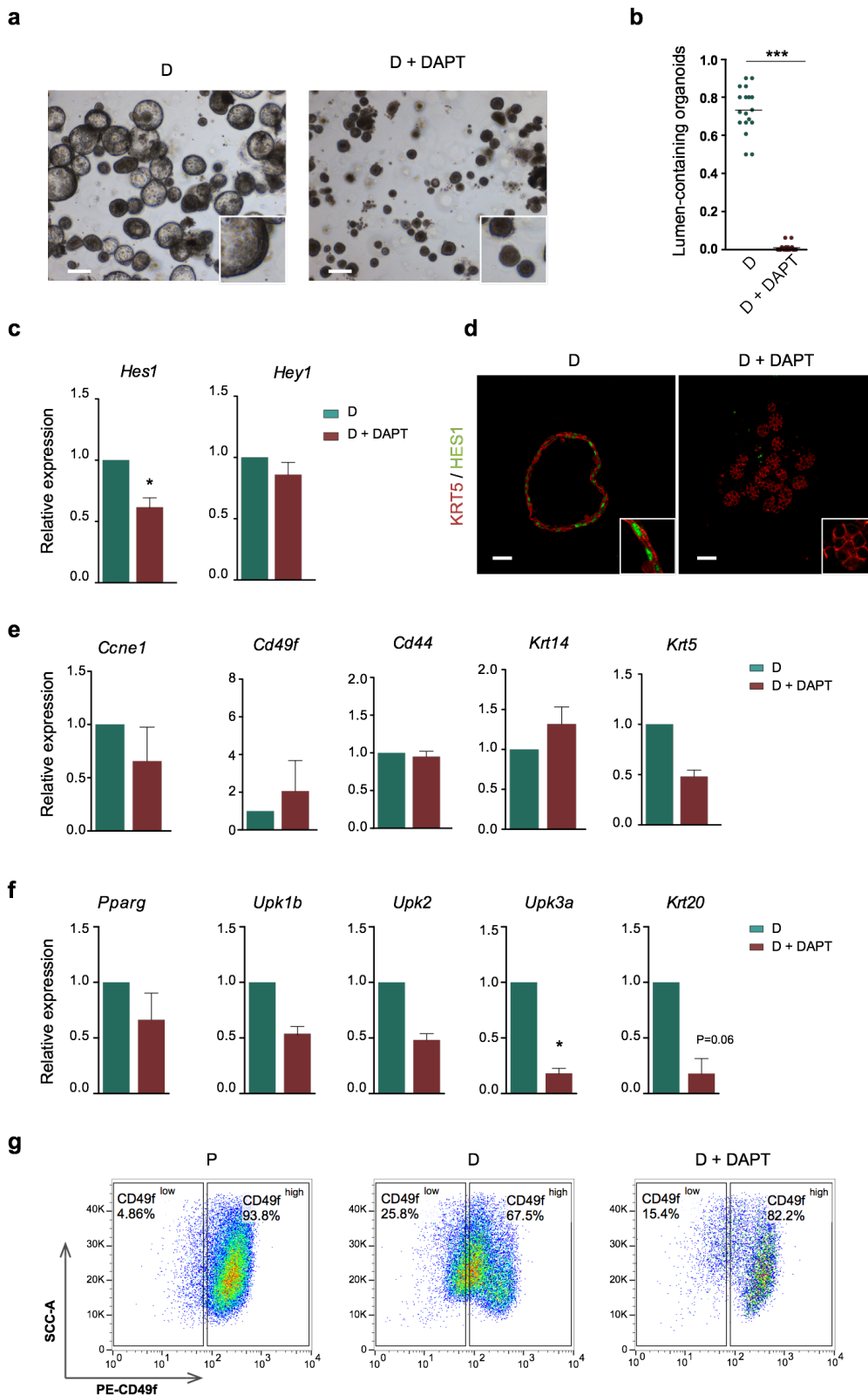
**Supplementary Figure 3. Differentiated (D) organoids are able to re-enter the cell cycle upon exposure to CM. (a)** Experimental design to assess the ability of D organoids to respond to growth factor stimulation. **(b)** Representative phase contrast images of organoids cultured as described in (a) and H&E staining of organoids exposed to complete medium or differentiation medium at day 7 and/or 21 (scale bars 500 μm and 250 μm, respectively). **(c)** After Matrigel removal, proliferative (P) organoids were fractionated according to size using a 70 μm filter, re-embedded in Matrigel, and cultured for an additional 7 days in differentiation medium. Representative flow cytometry plots showing CD49f expression in cells dissociated from P and D organoids (D<70 μm and D>70 μm) (n=2).



**Supplementary Figure 4. Transcriptome analysis reveals the differentiation capacity of organoids and identifies pathways activated during differentiation.** (a) Phase contrast images of proliferative (P) and differentiated (D) organoids used for RNA-seq (n=3) (scale bar, 500 $\mu$ m). (b) Principal component analysis of the transcriptomes of organoids used for RNA-seq showing greater divergence in differentiated conditions. (c) mRNA expression (FPKM values) of basal (top) and luminal/urothelial (bottom) markers in paired samples of P and D organoids showing evidence of urothelial differentiation. (d) mRNA expression (FPKM values) of novel keratin mRNA species with significant differential expression in P and D organoids, providing new markers for to assess the hierarchical urothelial differentiation program. (e) RT-qPCR analysis of claudin transcripts in normal mouse urothelium. Data are normalized to *Hprt* expression. (f) Validation of expression of transcripts coding for tight junction components in P and D organoids. Relative expression to *Hprt* (Mann-Whitney test, error bars SD).

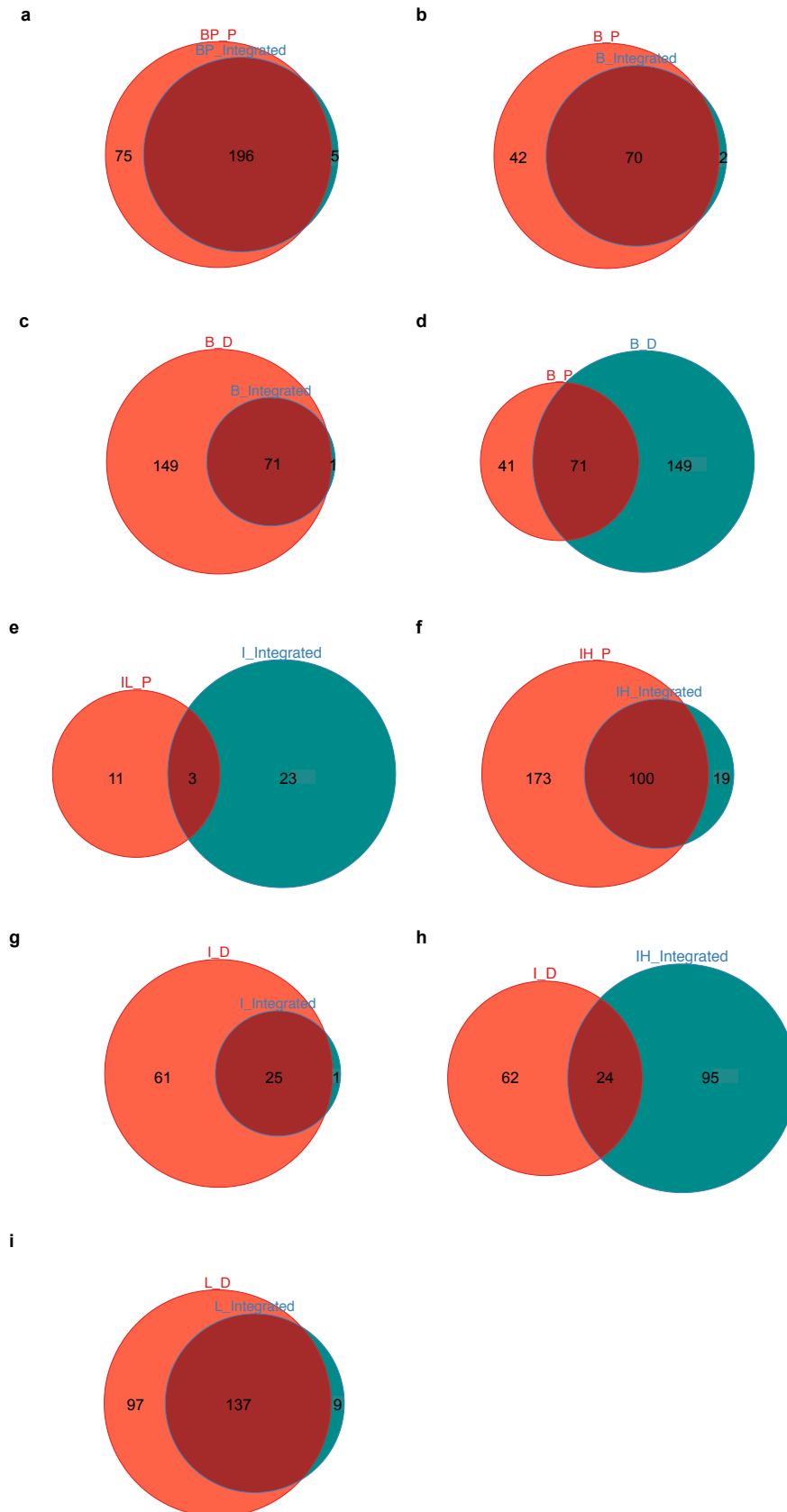


**Supplementary Figure 5. Effect of Wnt agonist CHIR99021 on proliferative (P) and differentiated (D) organoids (n=1 experiment). (a)** Phase contrast images (scale bar, 500  $\mu$ m). **(b)** Quantification of lumen formation. **(c)** RT-qPCR analysis of the expression of Wnt target genes, proliferation and urothelial differentiation markers in P and D organoids treated with CHIR99021. Data are normalized to *Hprt* expression. **(d-f)** Effect of the Wnt processing inhibitor IWP-2 on differentiated organoids (n=3). **(d)** Phase contrast images (scale bar, 500 $\mu$ m). **(e)** Quantification of lumen formation. **(f)** Expression analysis of Wnt target genes and urothelial basal markers using RT-qPCR. Error bars indicate SEM; \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ .

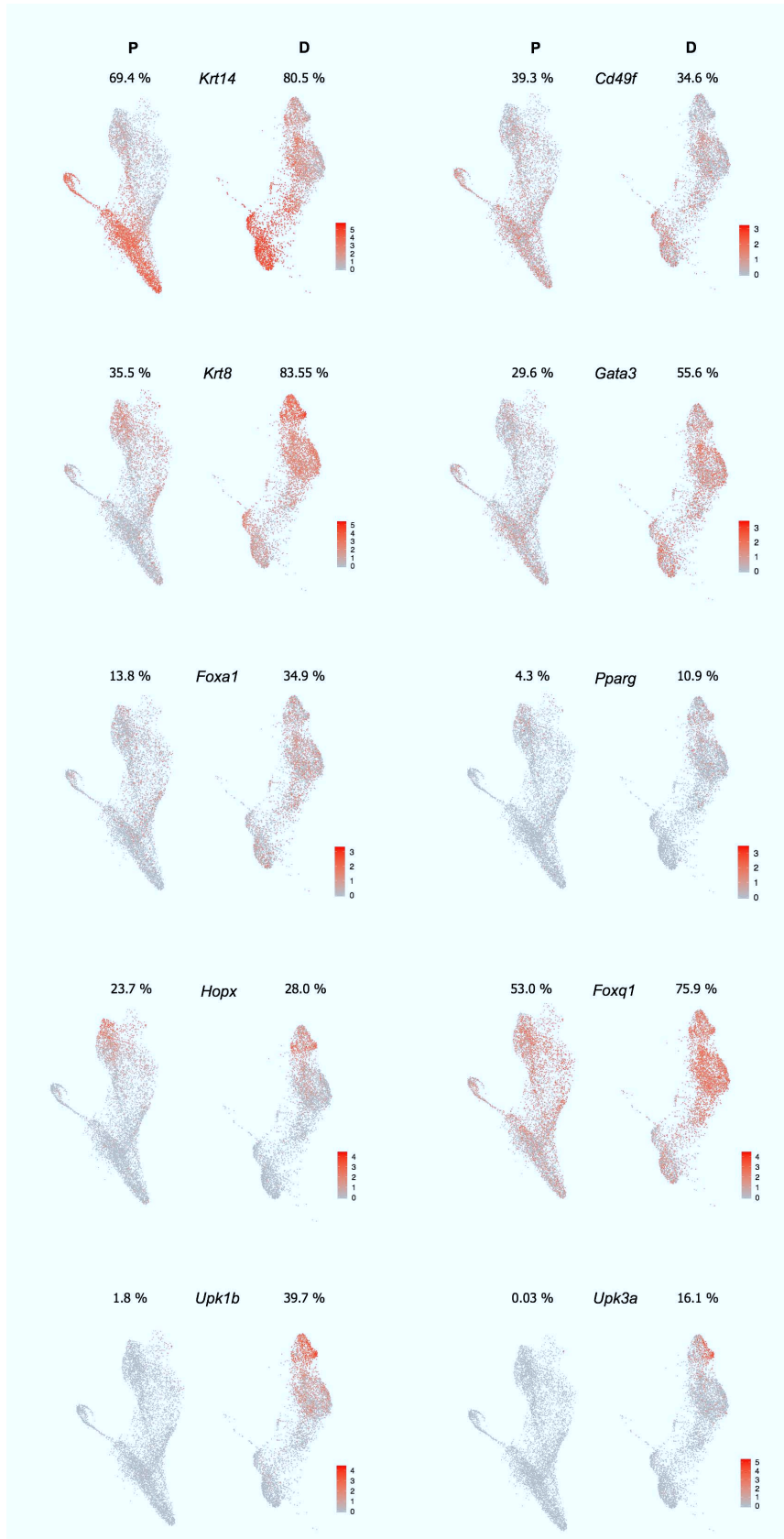


**Supplementary Figure 6. Effect of the pharmacological inhibition of Notch, using the gamma secretase inhibitor DAPT, on urothelial organoid renewal and differentiation (n=5).** (a) Phase contrast images (scale bar, 500 $\mu$ m). (b) Quantification of lumen formation. (c, d) Expression analysis of Notch target genes using (c) RT-qPCR and (d) immunofluorescence (scale bar, 250  $\mu$ m). (e) RT-qPCR analysis of expression of proliferation and basal urothelial markers in differentiated organoids treated with DAPT. (f) RT-qPCR analysis of urothelial markers in differentiated organoids treated with DAPT. (g) FACS plots depicting the analysis of CD49<sup>low</sup> and CD49<sup>high</sup> cell populations in differentiated organoids treated with DAPT. Error bars indicate SEM. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ .

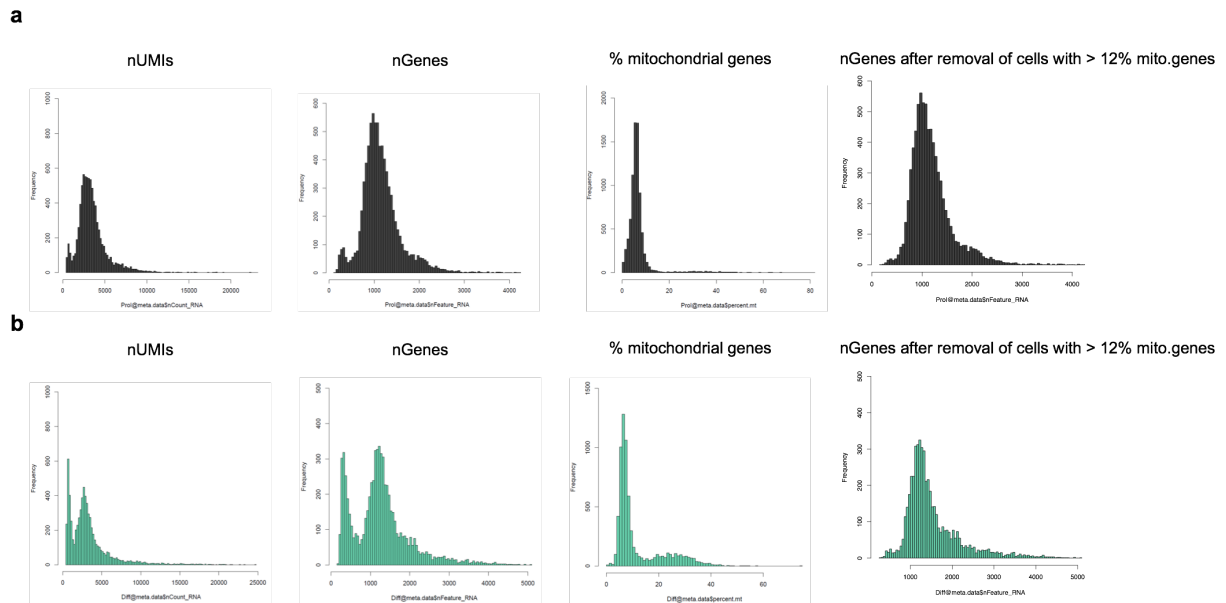




**Supplementary Figure 8. Venn diagrams showing the overlap of cluster markers identified through individual and integrated analyses. (a) Basal-Proliferative P versus Basal-Proliferative Integrated. (b) Basal P versus Basal Integrated. (c) Basal D versus Basal Integrated. (d) Basal P versus Basal D. (e) Intermediate-Low P versus Intermediate Integrated. (f) Intermediate-High P versus Intermediate-High Integrated. (g) Intermediate D versus Intermediate Integrated. (h) Intermediate D versus Intermediate high Integrated. (i) Luminal D versus Luminal Integrated. P, proliferative organoids; D, differentiated organoids.**



**Supplementary Figure 9.** UMAP plots derived from the split Integrated analysis showing the expression of selected basal (*Krt14* and *Cd49f*), intermediate (*Krt8*), and luminal (*Upk1b* and *Upk3a*) markers, three well-established transcription factors involved in urothelial differentiation (*Gata3*, *Foxa1*, *Pparg*), and newly identified candidate transcription factors (*Hopx*, *Foxq1*). The percentage of cells in which each of the markers was detected is shown.



**Supplementary Figure 10. Single-cell RNA-Seq data pre-processing.** Histograms showing the distribution of the number of unique molecular identifiers (nUMIs), genes (nGenes), percentage of mitochondria-encoded genes (% mitochondrial genes), and genes remaining after removal of cells expressing >12% of mitochondria-encoded genes for proliferative (**a**) and differentiated (**b**) datasets.

**Supplementary Table 1. Sequences of the primers used for qRT-PCR.**

<b>Gene name</b>	<b>Forward primer (5'-3')</b>	<b>Reverse primer (5'-3')</b>
<i>Axin2</i>	GAGAGTGAGCGGCAG AGC	CGGCTGACTCGTTCTCCT
<i>Cd44</i>	TGCATTTGGTGAACAAGGAA	GGAATGACGTCTCCAATCGT
<i>Cldn1</i>	AGGTCTGGCGACATTAGTGG	CGTGGTGTGGGTAAGAGGT
<i>Cldn2</i>	GTGGCTGTAGTGGGTGGAGT	CCAAAGAAAACAGGGCTGAG
<i>Cldn3</i>	GCACCCACCAAGATCCTCTA	AGCCTGTCTGTCCTCTTCCA
<i>Cldn4</i>	ATGGCGTCTATGGGACTACA	TTACACATAGTTGCTGGCGG
<i>Cldn5</i>	CCTTCCTGGACCACAACATC	GCCGGTCAAGGTAACAAAGA
<i>Cldn6</i>	GTCCTGTGTGGTTCAGAGCA	GATGATGCCAGAGATGAGCA
<i>Cldn7</i>	TTTCATTGTGGCAGGTCTTG	CCAGAAGGACCAGAGCAGAC
<i>Cldn8</i>	TCCTCGGAATGAAGTGCACC	GCTGACAGGGATGAGCACAA
<i>Cldn9</i>	AACTCCAACACAGTGCTCCC	GGTCATGCCGAGGAGTTCAA
<i>Cldn10</i>	GGCTGTTCCCTGTATGCAAAC	TCTCCGCCTTGATACTTGGTC
<i>Cldn11</i>	CTGGTGGACATCCTCATCCT	ACCCAAGTCAGCAATGTTCC
<i>Cldn12</i>	GCTCCTGTTGCATCTGAGTTC	GGTAATCAGCGTTTCTCCAAGC
<i>Ccne1</i>	TGTTTTTGCAAGACCCAGATGA	GGCTGACTGCTATCCTCGCT
<i>Foxa1</i>	TGGAGTTCATAGAGCCCAGG	CATGAGAGCAACGACTGGAA
<i>Hes1</i>	TCCAAGCTAGAGAAGGCAGACA	CGTTCATGAACTCGCTGAA
<i>Hey1</i>	AAAGACGGAGAGGCATCATCG	TCCCTGCTTCTCAAAGGCACT
<i>Itga6/Cd49f</i>	AGTGCTTCTGCCCGAGGT	GGAGCCTCTTCGGCTTCTC
<i>Ki67</i>	ATCATTGACCGCTCCTTTAGGT	GCTCGCCTTGATGGTTCT
<i>Krt5</i>	ACATTAACAACCTCCGTAGACAG	CGCTTGTTGATCTCATCCTC
<i>Krt14</i>	AGGTGAAGATTCGGGACTGG	GCTCCGTCTCAAACCTTGGTC
<i>Krt20</i>	CCTTGGAGATCAGCTTCCAC	CCTGCGAATTGACAATGCTA
<i>Mmp7</i>	CGGAGATGCTCACTTTGACA	CCAGAGAGTGGCCAAATTCA
<i>Ocln</i>	CCTCCAATGGCAAAGTGAAT	CTCCCCACCTGTCGTGTAGT
<i>Pcna</i>	CGCAGAGGGTTGGTAGTTGT	TGGACATGCTGGTGAGGTTT
<i>Pparg</i>	CGCTGGGGTATTGGGTCG	TTCAAATCTTGTCTGTCACACAGT
<i>Upk1a</i>	GGCCTGACAGCAAATAATGA	GAGAAGCAGGAAGATGGCTT
<i>Upk1b</i>	AATCAACAGGCCCTGGAAG	GAAGAAGGCAGAGGAGACCA
<i>Upk2</i>	GACAGCAGACCAGAGAGGCT	ACACTGCCTGTCCAGACCTT
<i>Upk3a</i>	AGTAGTGCTCAGTGGGACGC	AGCGGCTCTTACGAGGTTTA
<i>Zo1</i>	CCACCTCTGTCCAGCTCTTC	CACCGGAGTGATGGTTTTCT