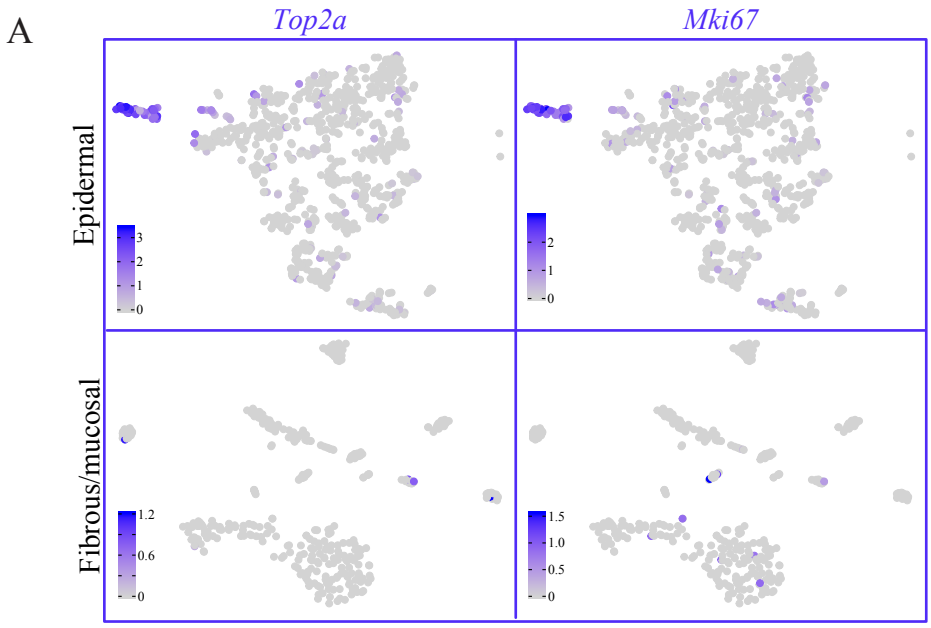


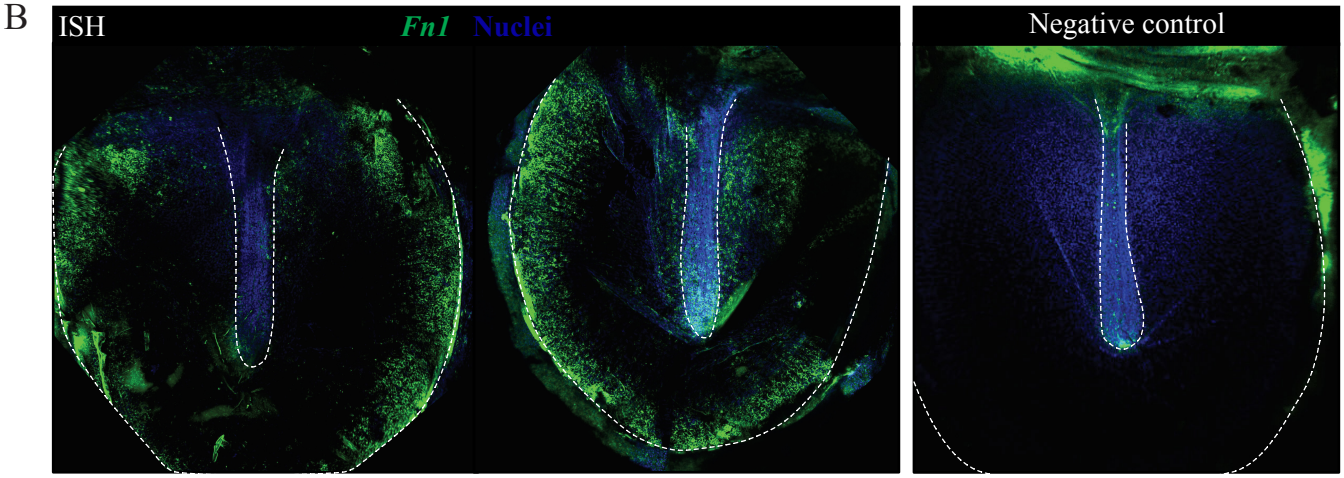


**Figure S1: Proliferating cells in murine scRNA-Seq and *Fn1* expression in whole-mount TMs. Related to Figure 1.** (A) tSNEs from the murine TM epidermis and fibrous/mucosal fraction showing expression of *Top2a* and *Mki67*. (B) Left and middle: ISH for *Fn1* in whole-mount TMs. Right: ISH for negative control probes targeting bacterial transcripts. Images were acquired in tiles and stitched and are maximum projections of z-stacks.

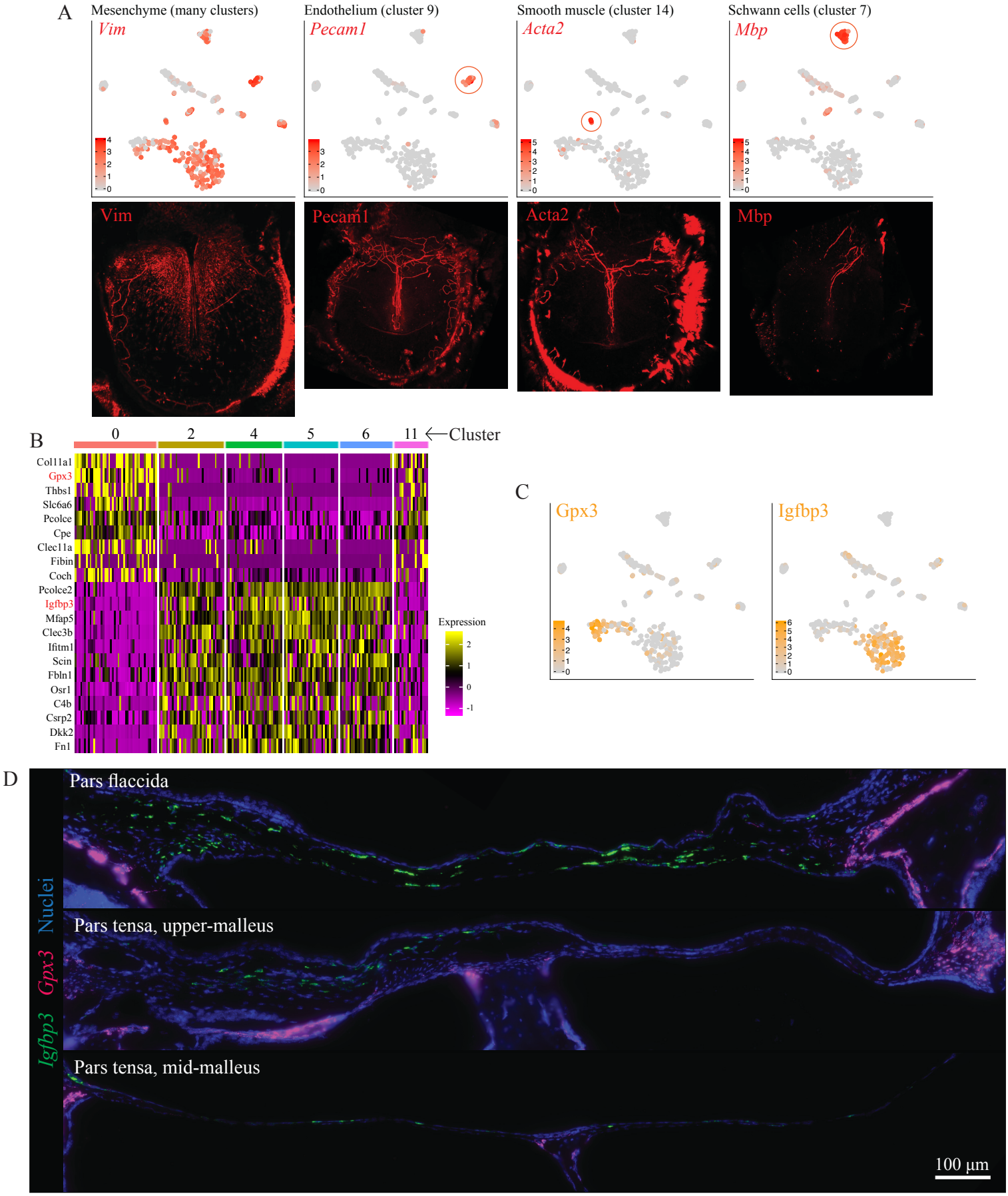


**Figure S2: Mesenchymal cells in the murine fibrous/mucosal scRNA-Seq. Related to Figure 1.** (A) For each population marker, expression tSNE is on top and IF in a whole-mount TM is below. Images are maximum projections of z-stacks. (B) Heat-map showing expression of genes differentially expressed between mesenchyme clusters. (C) tSNEs showing expression of *Gpx3* and *Igfbp3*. (D) ISH for *Gpx3* and *Igfbp3* in TM sections. Epidermis is oriented upward. Images were acquired in tiles and stitched.

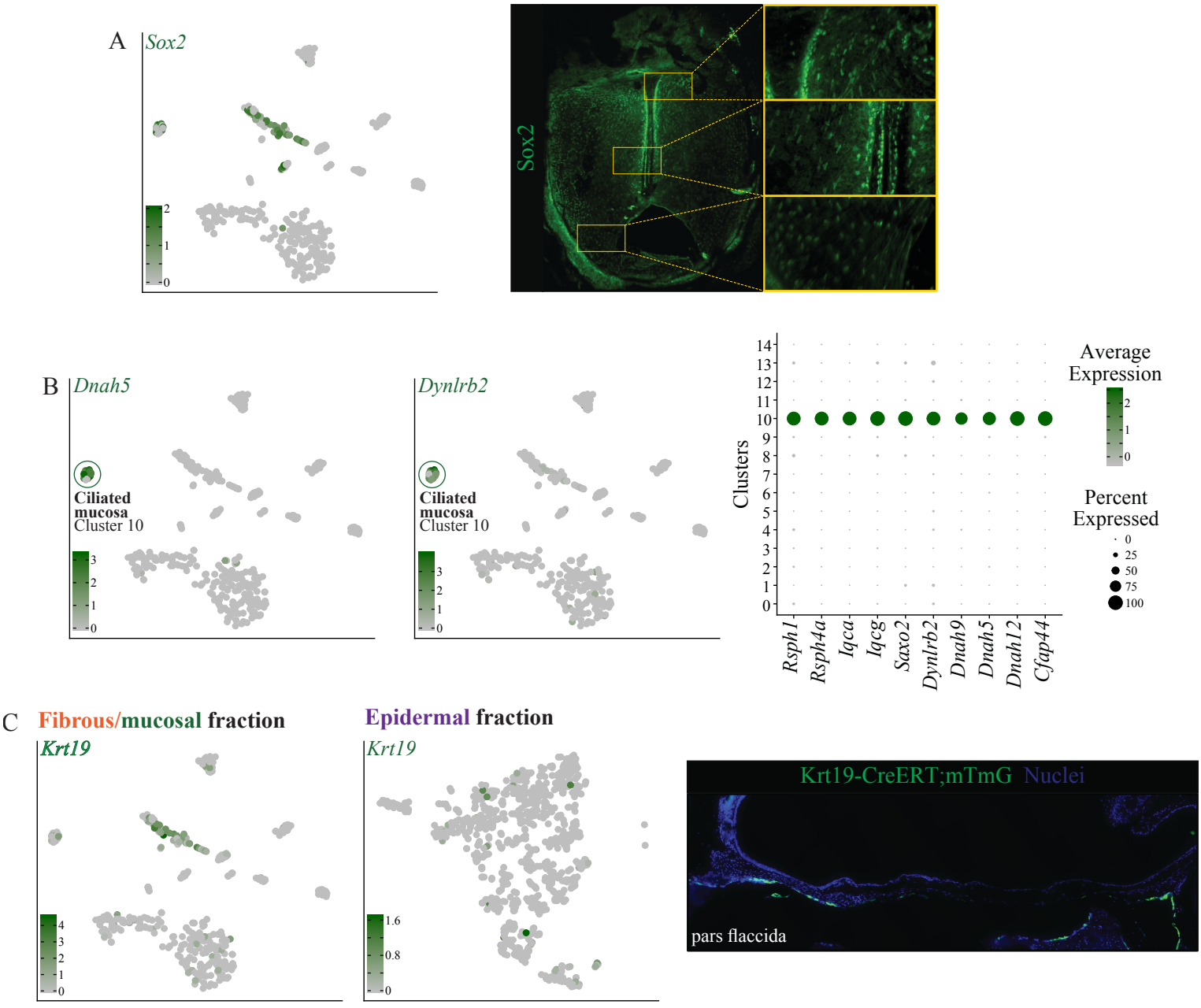


**Figure S3: Mucosal cells in the murine fibrous/mucosal scRNA-Seq. Related to Figure 1.** (A) tSNE showing expression of *Sox2* (left), and IF for Sox2 in a whole-mount TM (right). Image is a maximum projection of a z-stack. (B) tSNEs and dot plot showing expression of genes associated with ciliated epithelium. (C) tSNE of *Krt19* expression in the fibrous/mucosal and epidermal fractions. Right: pars flaccida section from a lineage traced Krt19-CreERT;mT/mG mouse, with epidermis oriented upward. Image was acquired in tiles and stitched.

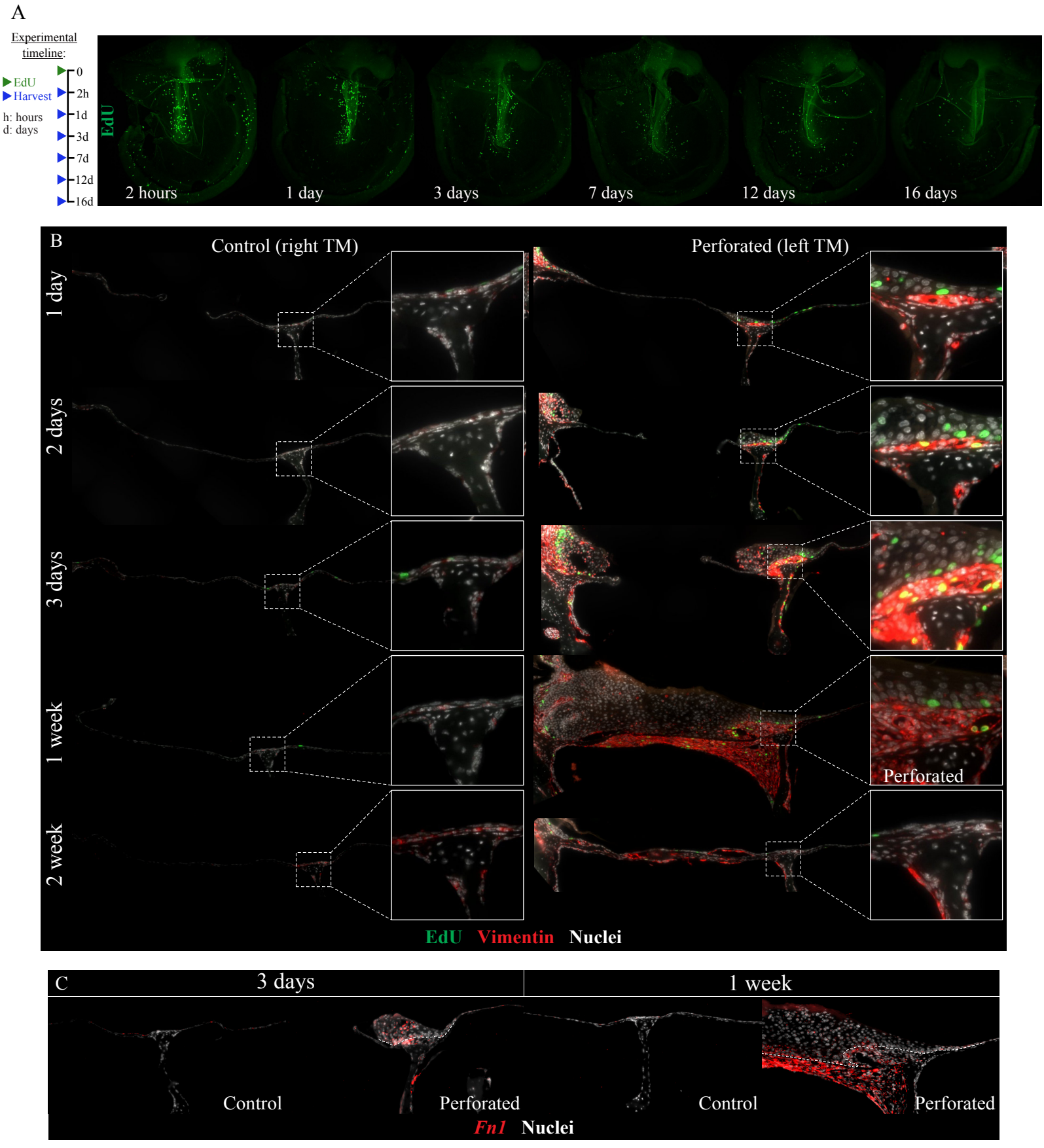


**Figure S4: EdU incorporation demonstrates TM cell proliferation, migration, and response to perforation. Related to Figure 3.** (A) Left: experimental timeline. Mice received a single injection of EdU and TMs harvested 2 hours to 16 days later. Right: whole-mount TMs from the indicated timepoints with EdU labeled. Images are maximum projections of z-stacks. (B) Mid-malleus sections of control and perforated TMs from the same animals at timepoints following wounding, stained for EdU and Vimentin (IF). (C) ISH for *Fn1* in sections at 3 days and 1 week post-perforation. In the perforated TMs, the border between keratinocytes and fibroblasts is indicated with a dashed white line. In panels B and C, the epidermis is oriented upward in all sections, and images were acquired in tiles and stitched.

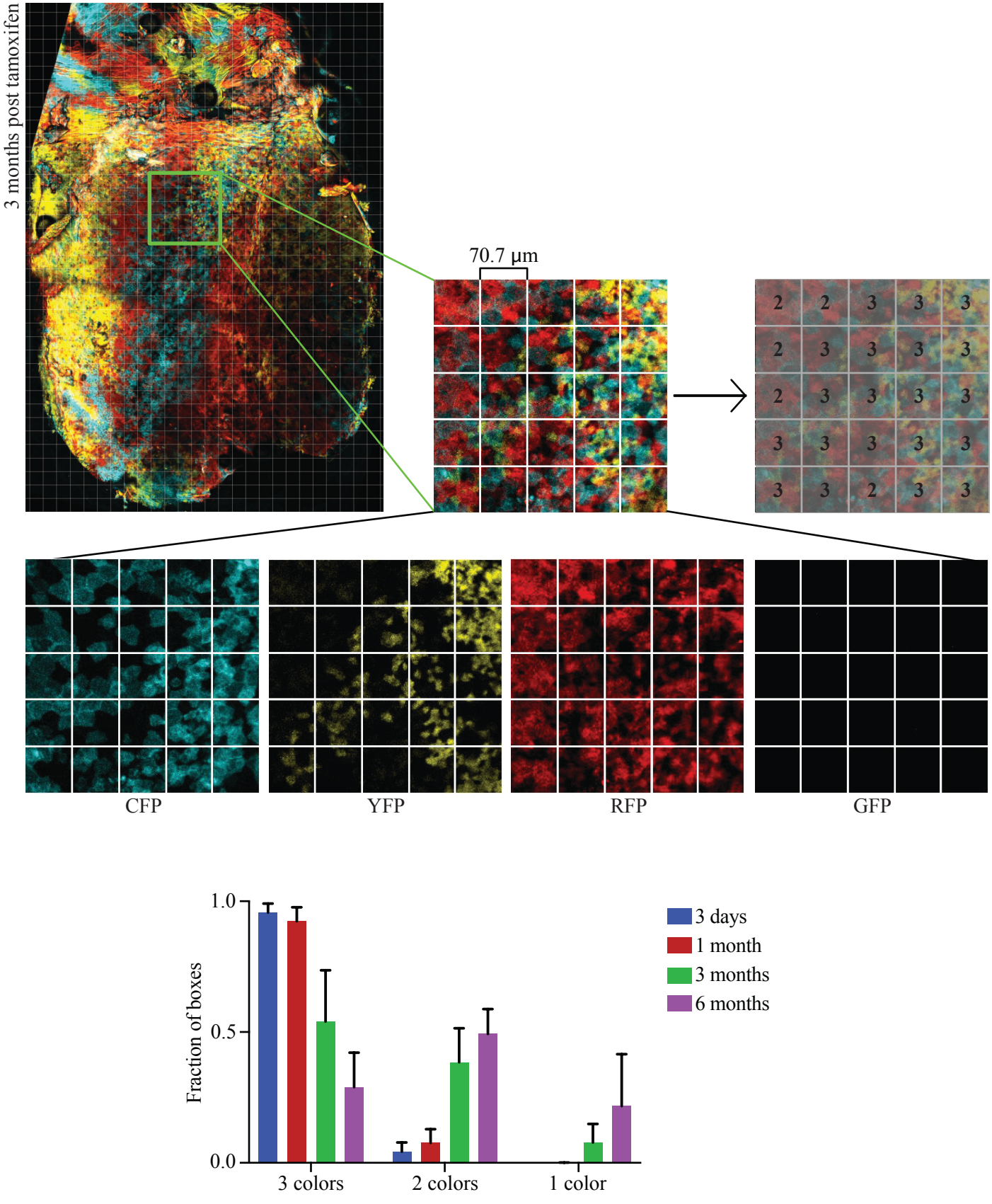


**Figure S5: Quantification of Krt5-CreERT2;R26R-Confetti TMs. Related to Figure 6.** Top: a representative TM harvested 3 months after recombination. The image was acquired in tiles and stitched and is a maximum projection of a z-stack. A grid of 5,000 µm$^2$ boxes was superimposed over the projection and the number of fluorescent proteins (1, 2, or 3) expressed in each box was manually annotated. Bottom: cumulative data. At each timepoint, the numbers of TMs quantified was: 3 days—2 TMs, 1 month—3 TMs, 3 months—3 TMs, 6 months—5 TMs. Histogram shows mean and error bars standard deviation.

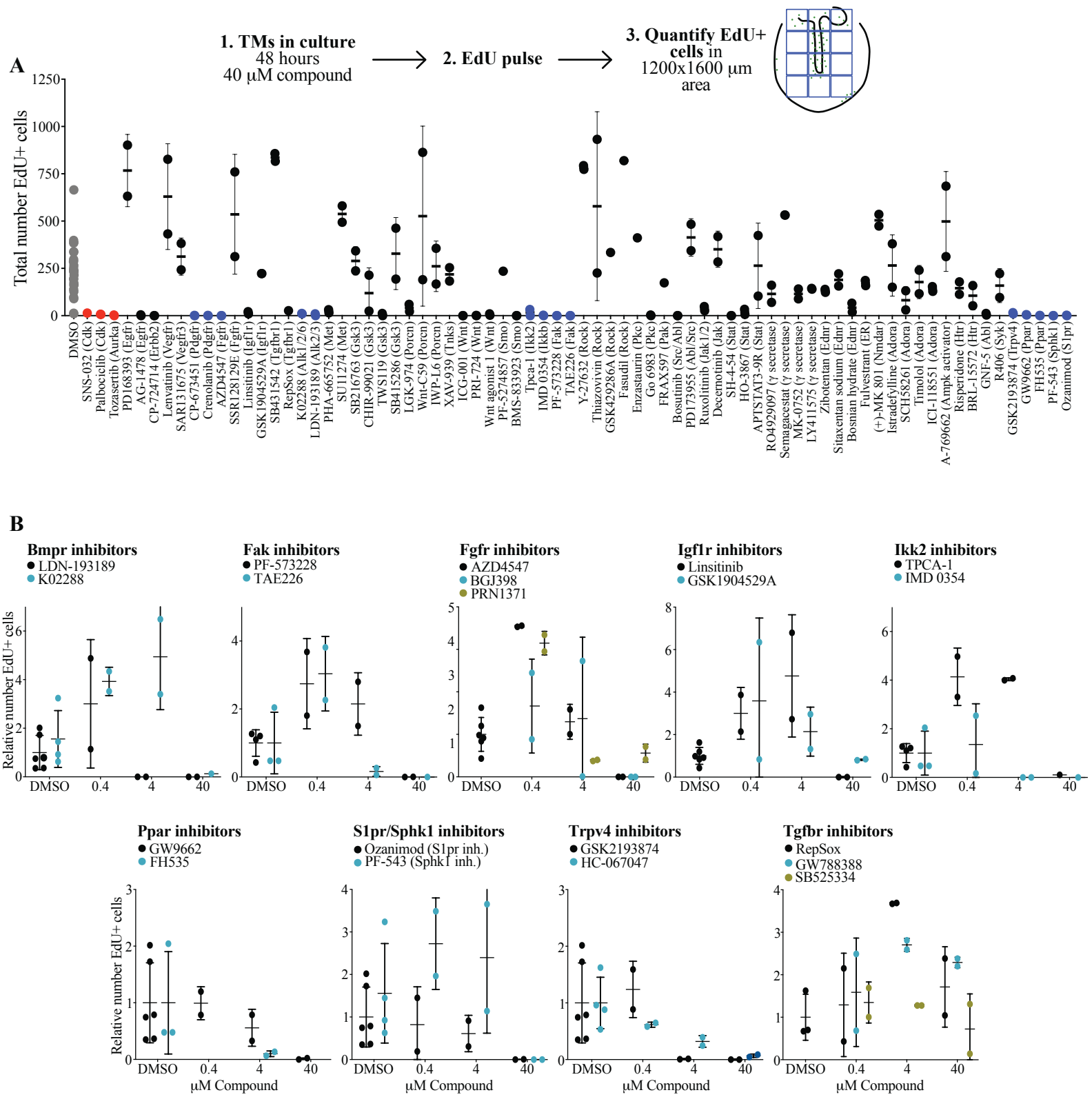


**Figure S6: Compound treatment in TMs explants. Related to Figure 7.** (A) Data from Figure 7B in an expanded format. Positive control compounds are in red. Compounds that ablated proliferation in both experimental TMs are in blue. (B) 48-hour concentration-response experiments for indicated compounds. In both panels, each dot is a TM and error bars show mean and standard deviation.

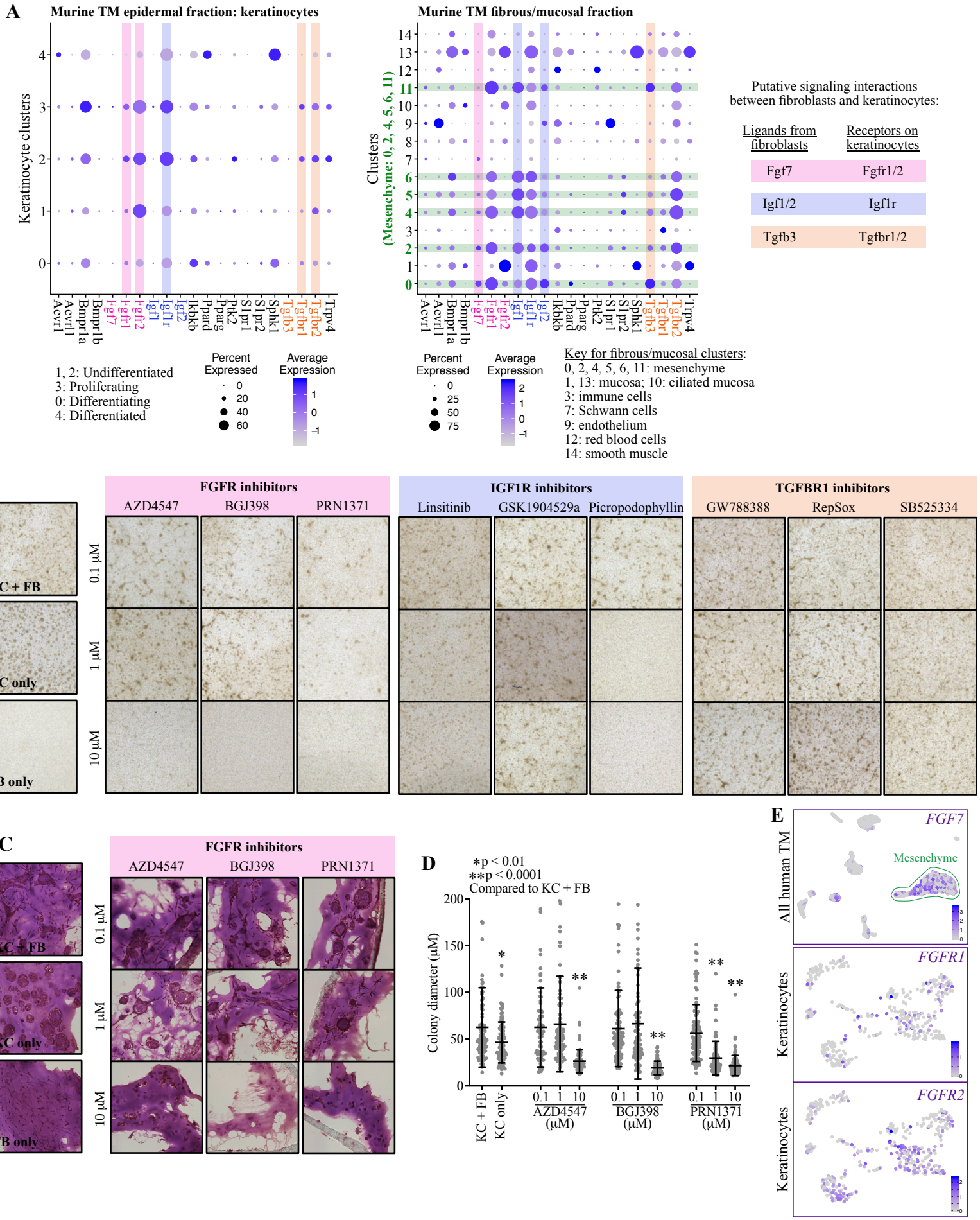


**Figure S7: Signaling mediators in the TM. Related to Figure 7.** (A) Dot plots showing expression of ligands and receptors in the murine TM scRNA-Seq. Fgfr, Igf1r, and Tgfbr mediators are highlighted. (B) Organoid cultures of human TM fibroblasts (FB) and keratinocytes (KC), seeded separately or in co-culture. Co-cultures were treated with the indicated inhibitors. (C) H&E stained sections of the organoids treated with Fgfr inhibitors. Images were acquired in tiles and stitched. (D) Quantification of organoid size. p-values are in comparison to untreated KC+FB organoids. (E) UMAPs showing expression of FGF7 in all human TM and FGFR1/2 in human TM keratinocytes.

**Table S4: Gene sets from MSigDB enriched in human keratinocyte cluster 2 relative to the other keratinocyte clusters. Related to Figure 2.** Genes with at least 1.5-fold increased expression in human keratinocyte cluster 2 relative to the other keratinocyte clusters that met a significance threshold of $p < 0.05$ were used to compute overlaps with gene sets in the Molecular Signatures Database. The top 50 gene sets are shown here. The top 25 are also included in Figure 2C.

| Gene Set Name | p-value |
|---|---|
| PASINI_SUZ12_TARGETS_DN | 1.92E-23 |
| GO_CYTOSKELETON_ORGANIZATION | 1.01E-16 |
| GO_SUPRAMOLECULAR_POLYMER | 1.65E-16 |
| GO_SUPRAMOLECULAR_COMPLEX | 9.22E-16 |
| GO_CYTOSKELETAL_PROTEIN_BINDING | 3.22E-15 |
| CHARAFE_BREAST_CANCER_LUMINAL_VS_BASAL_DN | 1.77E-14 |
| GO_POLYMERIC_CYTOSKELETAL_FIBER | 2.29E-14 |
| GO_ACTIN_FILAMENT_BASED_PROCESS | 3.24E-14 |
| GO_ACTIN_FILAMENT | 4.36E-14 |
| KOINUMA_TARGETS_OF_SMAD2_OR_SMAD3 | 1.29E-13 |
| KIM_WT1_TARGETS_UP | 2.27E-13 |
| GO_ACTIN_BINDING | 2.61E-13 |
| CHICAS_RB1_TARGETS_CONFLUENT | 2.85E-13 |
| LEI_MYB_TARGETS | 3.51E-13 |
| GO_CONTRACTILE_FIBER | 5.04E-13 |
| LIU_PROSTATE_CANCER_DN | 1.30E-12 |
| GO_SUPRAMOLECULAR_FIBER_ORGANIZATION | 2.38E-12 |
| BASAKI_YBX1_TARGETS_UP | 4.20E-12 |
| HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION | 4.90E-12 |
| GO_CELL_SUBSTRATE_JUNCTION | 5.13E-12 |
| APRELIKOVA_BRCA1_TARGETS | 2.10E-11 |
| ONDER_CDH1_TARGETS_2_DN | 2.14E-11 |
| GO_LOCOMOTION | 2.35E-11 |
| GO_ANCHORING_JUNCTION | 3.13E-11 |
| GO_CELL_MOTILITY | 3.68E-11 |
| AMIT_EGF_RESPONSE_480_HELA | 4.29E-11 |
| GO_ACTIN_CYTOSKELETON | 4.67E-11 |
| GO_STRUCTURAL_MOLECULE_ACTIVITY | 4.78E-11 |
| WANG_TUMOR_INVASIVENESS_UP | 6.24E-11 |
| NAKAMURA_TUMOR_ZONE_PERIPHERAL_VS_CENTRAL_UP | 1.12E-10 |
| KARLSSON_TGFB1_TARGETS_UP | 2.45E-10 |
| CHARAFE_BREAST_CANCER_LUMINAL_VS_MESENCHYMAL_DN | 4.10E-10 |
| GO_CELL_JUNCTION | 8.26E-10 |
| DUTERTRE_ESTRADIOL_RESPONSE_24HR_DN | 8.90E-10 |
| GO_ACTOMYOSIN | 9.23E-10 |
| MCBRYAN_PUBERTAL_BREAST_6_7WK_DN | 9.23E-10 |
| WANG_METHYLATED_IN_BREAST_CANCER | 9.60E-10 |
| KEGG_HYPERTROPHIC_CARDIOMYOPATHY_HCM | 1.25E-09 |
| RICKMAN_TUMOR_DIFFERENTIATED_WELL_VS_POORLY_DN | 1.42E-09 |
| REACTOME_SMOOTH_MUSCLE_CONTRACTION | 1.48E-09 |
| LU_AGING_BRAIN_UP | 1.77E-09 |
| KEGG_DILATED_CARDIOMYOPATHY | 2.05E-09 |
| GO_INTERSPECIES_INTERACTION_BETWEEN_ORGANISMS | 2.23E-09 |
| MCBRYAN_PUBERTAL_BREAST_4_5WK_UP | 2.30E-09 |
| REN_ALVEOLAR_RHABDOMYOSARCOMA_DN | 2.64E-09 |
| GO_STRUCTURAL_CONSTITUENT_OF_MUSCLE | 2.82E-09 |
| GO_WOUND_HEALING | 2.99E-09 |
| SMID_BREAST_CANCER_LUMINAL_B_DN | 3.83E-09 |
| GO_MUSCLE_CELL_DEVELOPMENT | 4.18E-09 |
| GO_SARCOMERE_ORGANIZATION | 4.48E-09 |

**Table S5: Compounds evaluated in the explant screen. Related to Figure 7.** A custom library of small molecule inhibitors was purchased from Selleckchem. The inhibitor names and their protein targets. This library was used to treat murine TMs in explant culture and assess for loss of proliferation. The results are in Figure 7B.

| Product Name | Target(s) |
|---|---|
| Risperidone | 5-HT Receptor |
| BRL-15572(dihydrochloride) | 5-HT Receptor |
| Istradefylline | Adenosine Receptor |
| SCH58261 | Adenosine Receptor |
| Timolol Maleate | Adrenergic Receptor |
| ICI-118551 Hydrochloride | Adrenergic Receptor |
| A-769662 | AMPK |
| Tozasertib (VX-680, MK-0457) | Aurora Kinase |
| PD173955 | Bcr-Abl |
| GNF-5 | Bcr-Abl |
| RO4929097 | Beta Amyloid,Gamma-secretase |
| MK-0752 | Beta Amyloid,Gamma-secretase |
| PHA-665752 | c-Met |
| SU11274 | c-Met |
| Palbociclib (PD-0332991) HCl | CDK |
| SNS-032 (BMS-387032) | CDK |
| AG-1478 (Tyrphostin AG-1478) | EGFR |
| PD168393 | EGFR |
| CP-724714 | EGFR,HER2 |
| Bosentan Hydrate | Endothelin Receptor |
| Sitaxentan sodium | Endothelin Receptor |
| Zibotentan (ZD4054) | Endothelin Receptor |
| Fulvestrant | Estrogen/progestogen Receptor |
| PF-573228 | FAK |
| TAE226 (NVP-TAE226) | FAK |
| AZD4547 | FGFR |
| SSR128129E | FGFR |
| LY411575 | Gamma-secretase |
| Semagacestat (LY450139) | Gamma-secretase |
| CHIR-99021 (CT99021) | GSK-3 |
| SB216763 | GSK-3 |
| SB415286 | GSK-3 |
| TWS119 | GSK-3 |
| BMS-833923 | Hedgehog/Smoothened |
| PF-5274857 | Hedgehog/Smoothened |
| GSK1904529A | IGF-1R |
| Linsitinib (OSI-906) | IGF-1R |
| IMD 0354 | IκB/IKK |
| TPCA-1 | IκB/IKK |
| Decernotinib (VX-509) | JAK |
| Ruxolitinib (INCB018424) | JAK |
| (+)-MK 801 maleate | NMDAR |
| FRAX597 | PAK |
| CP-673451 | PDGFR |
| Crenolanib (CP-868596) | PDGFR |
| Enzastaurin (LY317615) | PKC |
| Go 6983 | PKC |
| GW9662 | PPAR |
| FH535 | PPAR,Wnt/beta-catenin |
| Fasudil (HA-1077) HCl | ROCK |
| GSK429286A | ROCK |
| Thiazovivin | ROCK |
| Y-27632 2HCl | ROCK |
| Ozanimod (RPC1063) | S1P Receptor |
| PF-543 | S1P Receptor |
| Bosutinib (SKI-606) | Src |
| APTSTAT3-9R | STAT |
| HO-3867 | STAT |
| SH-4-54 | STAT |
| R406 (free base) | Syk |
| LDN-193189 HCl | TGF-beta/Smad |
| RepSox | TGF-beta/Smad |
| SB431542 | TGF-beta/Smad |
| K02288 | TGF-beta/Smad |
| GSK2193874 | Trpv4 inhibitor |
| SAR131675 | VEGFR |
| Lenvatinib (E7080) | VEGFR |
| ICG-001 | Wnt/beta-catenin |
| IWP-L6 | Wnt/beta-catenin |
| LGK-974 | Wnt/beta-catenin |
| PRI-724 | Wnt/beta-catenin |
| Wnt agonist 1 | Wnt/beta-catenin |
| Wnt-C59 (C59) | Wnt/beta-catenin |
| XAV-939 | Wnt/beta-catenin |