

Epithelial proliferation in inflammatory skin disease is regulated by tetratricopeptide repeat domain 7 (*Ttc7*) in fibroblasts and lymphocytes

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Background: Mutations in tetratricopeptide repeat domain 7A (*TTC7A*) and its mouse orthologue, *Ttc7*, result in a multisystemic disease, mostly affecting the epithelial barriers and immune system. Despite successful hematopoietic stem cell transplantation, ongoing progression of gastrointestinal manifestations can be life-threatening in *TTC7A*-deficient patients.

Objective: We sought to identify whether *TTC7A* mutations dysregulate epithelial cells only or whether a cell-intrinsic defect in lymphocytes or other cells contributes to disease manifestations.

Methods: *Ttc7*-mutated (*Ttc7^{fsn/fsn}*) mice were crossed to generate double-mutant (*Rag2^{-/-}Ttc7^{fsn/fsn}*) and triple-mutant (*Rag2^{-/-}IL2rg^{-/-}Ttc7^{fsn/fsn}*) mice. These models, together with bone marrow chimeras, were used to explore the role of adaptive and innate lymphocytes in the flaky skin phenotype. The effect of the *Ttc7^{fsn/fsn}* mutation on stromal cells was tested in a xenograft model in conjunction with transcriptomic analysis of *Ttc7^{fsn/fsn}* fibroblasts.

Results: We observed that the severity of epithelial hyperproliferation was accentuated by lymphocytes, whereas the phenotype was not induced by transfer of *Ttc7*-mutated hematopoietic cells. Furthermore, mice completely lacking the lymphocytic compartment were not protected from epithelial hyperproliferation. *Ttc7*-mutated mouse fibroblasts expressed increased transcript levels of insulin-like growth factor 1 (*Igf1*) and the antimicrobial protein regenerating islet-derived protein 3γ (*Reg3γ*). In a xenograft model *Ttc7*-mutated fibroblasts markedly increased epithelial proliferation of keratinocytes. Thus *Ttc7*-mutated fibroblasts were identified as potent instigators of epithelial hyperproliferation.

Conclusion: Our results reveal a previously unsuspected fundamental cell-extrinsic role of *Ttc7*. We have identified potential candidates for molecularly targeted treatment strategies that will need to be evaluated in future preclinical studies. (J Allergy Clin Immunol 2018;■■■:■■■-■■■.)

Key words: Primary immunodeficiency, *TTC7A* mutation, fibroblasts, psoriasis, mouse model

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Harmful mutations in the tetratricopeptide repeat domain 7A (*TTC7A*) gene lead to a highly variable multisystem disease that severely affects the immune system and epithelial organs, such as the skin and gut, in particular.¹⁻¹⁰ Even successful hematopoietic stem cell transplantation (HSCT) does not slow progression of the potentially life-threatening gastrointestinal manifestations in this context.^{6,7,10} A cell-intrinsic defect in enterocytes has been suspected based on (1) the disrupted apicobasal enterocyte polarity observed in pathologic assessments of gut biopsy specimens and (2) recapitulation of this inversion in enterocyte polarity in patient-derived organoid cultures.^{8,10} The mechanisms leading to this immunodeficiency remain largely unknown. In particular, it is unclear whether this immunodeficiency is the result of intrinsic T-cell defects, thymic epithelial defects, or both. Thus the value of HSCT in patients carrying *TTC7A* mutations is subject to debate.

Animal models could be of value in providing a deeper understanding of the mechanisms underlying the multisystemic nature of this disease. *TTC7A* deficiency in human subjects shares a number of features with the disease in the flaky skin (*fsn*) mouse (*Ttc7^{fsn/fsn}*), which bear a mutation in the murine orthologue *Ttc7*. Patients' immune defects range from severe combined immunodeficiency to hypogammaglobulinemia and autoimmunity. This is usually combined with epithelial organ involvement, mainly intestinal disease, ranging from intestinal atresia to very early-onset inflammatory bowel disease and skin diseases developing later in life.^{2-4,6-12} *Ttc7^{fsn/fsn}* mice present

Abbreviations used

APC:	Allophycocyanin
DESS:	Dermoepithelial skin substitute
DMEM:	Dulbecco modified Eagle medium
FITC:	Fluorescein isothiocyanate
fsn:	Flaky skin
GO:	Gene Ontology
HSCT:	Hematopoietic stem cell transplantation
Igf1:	Insulin-like growth factor 1
ILC:	Innate lymphoid cell
NGS:	Next-generation sequencing
PE:	Phycoerythrin
qPCR:	Quantitative PCR
Reg3 γ :	Regenerating islet-derived protein 3 γ
STAT3:	Signal transducer and activator of transcription 3
TLR:	Toll-like receptor
TTC7A:	Tetratricopeptide repeat domain 7A
WT:	Wild-type

with severe hyperplastic dermatitis as the main feature of a complex phenotype that includes a hyperproliferative immune disorder (notably splenomegaly and peripheral lymphadenopathy), forestomach papilloma, and anemia.¹³⁻²⁰ The *Ttc7^{fsn/fsn}* mouse has been regarded as a model of human psoriasis because of the patchy, thick, white skin scales; microscopically evident acanthosis, orthokeratosis, and hyperkeratosis; inflammatory dermal infiltrates; and dilated dermal capillaries.^{15,16,19} A comparative study of the skin in patients with *TTC7A* mutations and the *Ttc7^{fsn/fsn}* mouse found that the clinical, functional, and histopathologic features were consistent with abnormal epidermal maturation and epidermal barrier dysfunction.⁵

Here we analyzed the effect of *Ttc7^{fsn/fsn}* mutated hematopoietic cells (particularly lymphocytes) and stromal cells on the hyperproliferative skin phenotype in the *fsn* mouse model. We found that *Ttc7^{fsn/fsn}* hematopoietic cells do not *per se* induce pathologic changes when transferred to wild-type (WT) animals, whereas *Ttc7^{fsn/fsn}* lymphocytes are capable of aggravating epithelial hyperproliferation and inflammation in *Ttc7^{fsn/fsn}* stroma. We identified nonlymphoid *Ttc7^{fsn/fsn}* cells as the major source of IL-22 in these mice. Remarkably, *Ttc7^{fsn/fsn}* fibroblasts induce a disordered proliferation of normal human keratinocytes in dermoepidermal skin cultures. Furthermore, transcriptomic analysis of the fibroblasts unveils alterations in molecular networks required for cell-cell interactions, including regenerating islet-derived protein 3 γ (Reg3 γ). Our results show that *Ttc7* enables fibroblasts to control epithelial proliferation and lymphocytes to decrease inflammation. Thereby *Ttc7* plays a critical role in the crosstalk between cells from different tissues.

METHODS**Mice**

BALB/cBy.A-fsn/+ (CD45.2) mice (termed *Ttc7^{fsn/+}* mice) were obtained from the Jackson Laboratory (Bar Harbor, Me) and maintained under specific pathogen-free conditions at the Laboratory Animal Services Center of the University of Zurich (Zurich, Switzerland). *Ttc7^{fsn/fsn}* and WT mice were generated by mating heterozygous *Ttc7^{fsn/+}* mice. All mice were maintained and handled in accordance with national and institutional policies. CBy.SJL(B6)-Ptpcr<ca>/J (BALB/c; CD45.1) mice were purchased from the Jackson Laboratory and used for adoptive HSCT (license no. 65/2014). C.129S7-Rag2<tm1Mom>(*Rag2^{-/-}*) mice and BALB/c-IL2rg<tm1>B6.129S6-Rag2<tm1Fwa> (*IL2rg^{-/-}* and

Rag2^{-/-}) mice were used for the generation of homozygous double- and triple-mutant mice.

Hematopoietic stem cell transfer

Bone marrow chimeras were generated by transplanting 5×10^6 WT (BALB/c CD45.2) or 5×10^6 *Ttc7^{fsn/fsn}* (BALB/c CD45.2) cells sampled from whole bone marrow. Cells were injected intravenously into total body-irradiated female host BALB/c (CD45.1) mice receiving two 400-rad doses with a 24-hour interdose interval. Mice were allowed to reconstitute for 10 to 13 weeks.

Histopathology

Mice were killed by using CO₂ asphyxiation. Tissues (spleen, axillary, and inguinal lymph nodes and skin from the back) for histopathologic examination were fixed in 4% paraformaldehyde and embedded in paraffin. Deparaffinized sections were stained with either hematoxylin and eosin reagent according to standard protocols or with anti-CD45/LCA (clone 30-F11; BD Biosciences, San Jose, Calif), anti-CD3 (clone SP7; Diagnostic BioSystems, Pleasanton, Calif), or anti-CD19 (clone 6OMP31; Invitrogen BV & Research Genetics, Waltham, Mass), according to the manufacturer's instructions, followed by counterstaining with hematoxylin and the Bond Polymer Refine Detection Kit (Leica Biosystems, Wetzlar, Germany). Images were acquired with a bright-field light microscope (Zeiss Axio Observer; Zeiss, Oberkochen, Germany) at a magnification of $\times 5$ to $\times 10$.

Histopathologic analysis

Acanthosis was quantified in measuring the viable epidermis thickness (including stratum spinosum and stratum granulosum) in 3 different visual fields per mouse (each visual field is divided into 5 quadrants); hyperkeratosis of the stratum corneum was quantified in skin histology by rating the severity per mouse as follows: grade 0, 1 or 2 peeled-off corneal layers at the surface; grade 1, 2 or 3 peeled-off corneal layers; and grade 2, 4 or more peeled-off corneal layers. Inflammation of the dermis was quantified on skin histology by counting the total number of leukocytes and number of mast cells in the dermis underneath the basal layer. Three different images per mouse dermis (magnification $\times 40$) were captured and printed as 13×18 -cm photographs for evaluation.

Cell preparation

Spleen and skin-draining lymph nodes (2 axillary, 2 brachial, and 2 inguinal) were harvested, cut into small pieces, and digested in Hank balanced salt solution (Ca²⁺ and Mg²⁺) supplemented with 10% FBS, 0.2 U/mL Liberase (Roche Applied Science, Mannheim, Germany), and 0.1 mg/mL DNase (Roche Applied Science) for 5 to 10 minutes. Afterward, the tissue fragments were passed through a 40- μ m wire mesh. Erythrocytes in splenocyte and lymph node cell suspensions were lysed with Red Blood Cell Lysis buffer (Sigma-Aldrich, St Louis, Mo), purified, and blocked with anti-mouse CD16/32 (clone 93; BioLegend, San Diego, Calif) before antibody staining for flow cytometric analyses. Cell suspensions from skin and staining for flow cytometry were prepared according to the method of Kulig et al²¹ per 500 mg of skin biopsy specimen.

Flow cytometry

For surface staining, the following dyes and fluorochrome-coupled antibodies were purchased from BioLegend (unless otherwise stated) and used at saturating concentrations: Zombie Aqua, peridinin-chlorophyll-protein complex-anti-CD45.1 (clone A20), allophycocyanin (APC)-anti-CD45.1 (clone A20), Pacific Blue-anti-CD45.2 (clone 104), phycoerythrin (PE)/Cy7-anti-CD19 (clone 6D5), Alexa Fluor 700-anti-CD3 (clone 17A2), fluorescein isothiocyanate (FITC)-anti-CD4 (clone GK1.1; eBioscience, San Diego, Calif), PE-anti-CD8a (clone 53-6.7; eBioscience), FITC-anti-CD11b (clone M1/70), PE-anti-Ly6G (clone 1A8), APC-Cy7-anti-Ly6C (clone HK1.4), Biotin-anti-Siglec-F (Miltenyi Biotec, Bergisch Gladbach,

Germany), Alexa Fluor 700–streptavidin (Milenyi Biotec), and APC–anti-F4/80 (clone BM8). Measurements were performed with a Gallios flow cytometer (Beckman Coulter, Fullerton, Calif), and data were analyzed with FlowJo software (Version 10.2; GraphPad Software, La Jolla, Calif).

Cytokine measurements

Color-coded beads precoated with analyte-specific capture antibodies for IL-4, IL-5, IL-6, IL-10, IL-17, IL-22, TNF- α , and IFN- γ were used to measure serum cytokine levels with a Guava easyCyte Flow Cytometer (Merck Millipore, Billerica, Mass).

Primary mouse fibroblast cell cultures for use in dermoepithelial skin substitutes

Mouse skin samples were cut into small (1-cm²) pieces and digested overnight at 4°C in 12 U/mL Dispase (Corning, Corning, NY) to isolate fibroblasts. Next, the dermis and epidermis were separated with forceps. The dermal compartment was digested further with 2 mg/mL collagenase for 1 hour at 37°C. Isolated fibroblasts were then seeded on tissue-culture plates and grown in Dulbecco modified Eagle medium (DMEM; Gibco, Invitrogen, Carlsbad, Calif) supplemented with 10% FBS, 1% HEPES, and 5 mg/mL gentamicin.

Primary human keratinocyte cell culture for use in dermoepithelial skin substitutes

Foreskin samples were cut into small (1-cm²) pieces and digested overnight at 4°C in 12 U/mL Dispase (Corning) to isolate human keratinocytes. Afterward, the dermis was separated from the epidermis with forceps. The epidermal compartment of the skin was further digested in 0.5% trypsin (Thermo Fisher, Waltham, Mass) for 2 minutes at 37°C. Isolated keratinocytes were cultured in serum-free medium (CellnTec, Bern, Switzerland) supplemented with 25 μ g/mL bovine pituitary extract, 0.2 ng/mL epidermal growth factor, and 5 μ g/mL gentamicin.

Preparation of dermoepithelial skin substitutes containing skin fibroblasts isolated from *Ttc7^{fsn/fsn}* and WT mice

Dermoepithelial skin substitutes (DESSs) were prepared by using 3 mL of bovine collagen type I (5 mg/mL; Symatase Biomateriaux, Chaponost, France) mixed with 2×10^5 mouse fibroblasts isolated from *Ttc7^{fsn/fsn}* (*Ttc7^{fsn/fsn}* DESSs) or WT skin (WT DESSs) and cultured in 6-well cell-culture inserts (pore size, 3.0 μ m; BD Biosciences). The prepared dermal equivalents were cultured in DMEM (Thermo Fisher) supplemented with 10% FBS, 1% HEPES, and 5 μ g/mL gentamicin for 7 days after seeding of 7×10^5 keratinocytes isolated from human foreskin. DESSs were cultured for 5 additional days and subsequently transplanted into athymic nude rats (*Foxn1^{nu/nu}*; Charles River Laboratories, Wilmington, Mass).

DESS transplantation

Athymic nude female rats aged 8 to 10 weeks (Harlan Laboratories, Indianapolis, Ind) were prepared and anesthetized, as previously described.²² DESSs were transplanted into full-thickness skin wounds created surgically on the animal's back. Protection from wound closure was provided by suturing steel surgical rings (diameter, 26 mm) to the skin defects with nonabsorbable polyester sutures (Ethibond; Ethicon, Somerville, NJ). DESSs were covered with silicon foil (Silon-SES; Bio Med Sciences, Allentown, Pa), a polyurethane sponge (Ligasano; Ligamed, Haiming Ötztal-Bahnhof Austria), a bandage (Sincohaft; Theo Frey AG, Bern, Switzerland), and tape as a wound dressing. Photographs were taken weekly when the wound dressing was changed. Three weeks after transplantation, the DESSs were excised and processed for cryosection analysis.

Immunofluorescence staining of transplanted DESSs

Twelve-micrometer cryosections were fixed with 1:1 acetone/methanol for 5 minutes at -20°C , air-dried, washed 3 times in PBS, blocked in 2% BSA in PBS solution for 30 minutes, and incubated overnight at 4°C with diluted primary antibodies. Thereafter, slides were washed 3 times in PBS, blocked for an additional 15 minutes, and incubated with an appropriate secondary antibody for 45 minutes at room temperature. Lastly, the slides were incubated for 5 minutes in PBS containing 1 μ g/mL Hoechst reagent (Sigma-Aldrich, St Louis, Mo), washed once with PBS, mounted with mounting medium (Dako, Baar, Switzerland), and covered with coverslips. Immunofluorescence pictures were taken with a DXM1200F digital camera connected to a Nikon Eclipse TE2000-U inverted microscope (Nikon, Tokyo, Japan) equipped with Hoechst 33342, FITC, and tetramethylrhodamine isothiocyanate filter sets (Nikon ACT-1 software, version 2.70; Nikon AG). Images were processed with Photoshop software (version 10.0; Adobe Systems, San Jose, Calif). The following primary antibodies were used: anti-loricrin (polyclonal, 1:200; Abcam, Cambridge, United Kingdom), Alexa Fluor 488–anti-E-cadherin (clone 67A4; BD PharMingen), and anti-Ki67 (clone SP6, Abcam).

Quantification of cells proliferating in DESSs *in vivo*

Transplanted skin grafts were stained with an anti-Ki-67 antibody (clone SP6; Abcam). Numbers of Ki-67⁺ cells in the basal cell layer were quantified in 3 separate areas of 2 representative sections from each DESS.

Quantification of proliferating A375 cells

WT and *Ttc7^{fsn/fsn}* fibroblast were seeded at 1×10^5 cells/mL and cultured for 4 days in DMEM 10%–FBS. Supernatant was collected, filtered sterile through 0.2- μ m PES filters, and added to cultures of A375 cells (human melanoma skin epithelial cell line) at a 1:1 ratio with DMEM 10%–FBS. After 48 hours, proliferation was measured with alamarBlue (Thermo Fisher), according to the manufacturer's protocol.

Isolation of RNA from primary fibroblast cultures from *Ttc7^{fsn/fsn}* and WT mice

RNA was isolated from primary mouse fibroblast cultures from WT and *Ttc7^{fsn/fsn}* mice. Fibroblasts were lysed and stored in TRIzol Reagent (Thermo Fisher), and total RNA was isolated with a PureLink RNA Mini kit (Thermo Fisher), according to the manufacturer's instructions.

Next-generation sequencing of RNA from mouse fibroblasts from *Ttc7^{fsn/fsn}* and WT mice

Next-generation sequencing (NGS) of purified total RNA isolated from fibroblasts from WT and *Ttc7^{fsn/fsn}* mice was performed by the Functional Genomic Center Zurich (ETH Zurich and University of Zurich, Zurich, Switzerland). The TruSeq stranded mRNA Sample Prep Kit (Illumina, San Diego, Calif) was used with a starting amount of 500 ng of total RNA per sample. Libraries were sequenced on HiSeq 4000 in single-read mode at 1×125 bp, and NGS reads were quality checked with FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Reads were trimmed with Trimmomatic (version 0.33; 4 bases hard trimming from the start and adapter trimming at the end). We aligned the trimmed reads to the reference genome and transcriptome (FASTA and GTF files, respectively; Ensembl GRCm38) with STAR version 2.5.1b software. Gene expression was quantified with RSEM (version 1.2.22). To detect differentially expressed genes, we applied the count-based negative binomial model implemented in the R/Bioconductor package edgeR (R version 3.3.2, edgeR version 3.16.5), in which the normalization factor was calculated by using the trimmed mean of M values method.²¹ The unsupervised hierarchical cluster analysis of RNA sequencing data is based on normalized counts after log transformation with an offset of 10 counts added. The 2500 genes with the biggest variance across all samples were used.

Statistics

Differences between 2 groups or 2 data sets were evaluated by using an unpaired 2-tailed Student *t* test or a Mann-Whitney test. Data are provided as means $\pm$ SEMs or means $\pm$ SDs. Correlation between 2 parameters was estimated by calculating the Pearson *r* value. All statistical analyses were performed with Prism 6 software (Version 6; GraphPad Software, La Jolla, Calif).

Data availability

Sequence data that support the findings of this study have been deposited in the European Nucleotide Archive with the primary accession ID PRJEB21496. The authors declare that all other data supporting the results of this study are available on request.

Study approval

Human keratinocytes were isolated from foreskin samples from children 1 to 16 years old. This part of the study was performed in accordance with the tenets of the Declaration of Helsinki and with permission by the Ethic Commission of the Canton Zurich. Informed consent for skin sampling was given by parents, patients, or both. *In vivo* animal experiments were approved by the veterinary office of the Canton of Zurich (approval nos. ZH65/2014 for mouse experiments and ZH090/15 for rat experiments).

RESULTS

Ttc7^{fsn/fsn} mice present with hyperplastic dermatitis, lymphadenopathy of skin-draining lymph nodes, and T_H2 cytokinemia

Given that the skin of *Ttc7^{fsn/fsn}* mice appears normal at birth and becomes hyperplastic only over the course of the first weeks of life, we were interested in investigating which cell types support *Ttc7^{fsn/fsn}* keratinocytes in causing development of hyperplastic dermatitis. Therefore we analyzed skin-infiltrating leukocytes just before and during emergence of hyperplastic dermatitis. Ruffled fur and thick white scales are visible in *Ttc7^{fsn/fsn}* mice from a postnatal age of 6 to 8 weeks onward. Histologic analysis of sections of these lesions revealed acanthosis (thickening of the viable epidermis), hyperkeratosis, and infiltration of the dermis by leukocytes (Fig 1, A). Skin thickness, hyperkeratosis severity, and inflammatory cell counts were significantly more severe in *Ttc7^{fsn/fsn}* mice than in control WT mice (Fig 1, B-E). In particular, and consistent with previous observations in *Ttc7^{fsn/fsn}* mouse skin, we noted increased numbers of mast cells, although we found that this difference only became evident beyond 10 weeks of age.

When comparing 4-week-old mice with 10-week-old mice, the difference in dermal infiltration between *Ttc7^{fsn/fsn}* and WT mice was more pronounced than the difference in the severity of acanthosis and hyperkeratosis. Increased numbers of leukocytes (CD45⁺ cells) within the skin of 4-week-old *Ttc7^{fsn/fsn}* mice were also detectable by using flow cytometry (Fig 1, F). CD4⁺ T lymphocytes were identified as the major population contributing to this increase by using flow cytometric analysis of skin from 4-week-old and histopathologic analysis of skin from 10-week-old *Ttc7^{fsn/fsn}* mice (Fig 1, F, and see Fig E1 in this article's Online Repository at www.jacionline.org). No differences in myeloid cell counts were noted at this early stage.

Ttc7^{fsn/fsn} mice present with massively enlarged skin-draining lymph nodes (brachial, axillary, and inguinal lymph nodes; Fig 1, G).¹⁷ In histopathologic analysis we observed that (1) follicular areas were smaller in *Ttc7^{fsn/fsn}* mice than in WT mice and (2) the

cortex and paracortical area were markedly enlarged (Fig 1, H). When comparing 4-week-old mice with 10-week-old mice, an increase in lymph node weight was observed as the mice aged (Fig 1, I). Cell counts of the various leukocyte subpopulations were found to be increased in *Ttc7^{fsn/fsn}* lymph nodes relative to WT control values (Fig 1, J-L).

To assess whether skin infiltrates would be accompanied by systemic inflammation, we measured serum cytokine levels. Consistent with previous reports,¹⁷ serum levels of IL-4 and IL-5 were significantly greater in *Ttc7^{fsn/fsn}* mice than in WT littermates (Fig 1, M). IL-6 and IL-10 were also present at higher levels than in WT mice, whereas IFN- γ and TNF- α levels were less than the corresponding assay's limit of detection (Fig 1, M, and data not shown). Thus the hyperplastic dermatitis seen in *Ttc7^{fsn/fsn}* mice develops with age, consists of a mixed leukocytic infiltrate with predominance of CD4⁺ T lymphocytes and mast cells, and is accompanied by local lymphadenopathy and a T_H2 cytokine pattern.

Nonlymphoid cells are critical for the development of flaky skin disease, whereas lymphocytes aggravate the phenotype

To further analyze the influence of lymphocytes on the progression of hyperplastic skin disease in *fsn* mice, we analyzed the skin phenotypes of single mutants (*Ttc7^{fsn/fsn}*), double mutants (*Rag2^{-/-}Ttc7^{fsn/fsn}*), and triple mutants (*Rag2^{-/-}IL2rg^{-/-}Ttc7^{fsn/fsn}*). The double mutant lacks lymphocytes from the adaptive immune system, whereas the triple mutant lacks lymphocytes from both the adaptive and innate immune systems. All 3 mutant mouse strains had ruffled fur and white scaly skin (see Fig E2 in this article's Online Repository at www.jacionline.org). This indicates that *Ttc7* in nonlymphoid cells plays an important role in regulating skin homeostasis. Consistently, histologic analysis revealed acanthosis, hyperkeratosis, and infiltration of the dermis by leukocytes in all 3 mutant mouse strains (Fig 2, A). Histologic examination revealed significant increases in skin thickness, severity of hyperkeratosis, and inflammatory cell counts (including the mast cell counts) in the 3 mutant strains (Fig 2, B-E) when compared with that seen in control WT mice (Fig 1, B-E). Also, the number of mast cells in the dermis of 10-week-old WT mice ($n = 1.8 \pm 0.45$; Fig 1, E) was significantly lower than in the dermis of single-mutant ($n = 9 \pm 2.24$, $P < .01$; Fig 1, E), double-mutant ($n = 5.6 \pm 2.41$, $P < .01$; Fig 2, E), and triple-mutant ($n = 4 \pm 0.71$, $P < .01$; Fig 2, E) mice. However, notably, all these changes were less severe in double- and triple-mutant mice than in single-mutant mice other than hyperkeratosis (compared with Fig 1, B-E), suggesting that *Ttc7^{fsn/fsn}* lymphocytes are involved in aggravating the skin phenotype. As expected, the skin-draining lymph nodes were smaller in double- and triple-mutant strains than in single-mutant (*Ttc7^{fsn/fsn}*) mice but were still markedly larger than in the original *Rag2^{-/-}* and *Rag2^{-/-}IL2rg^{-/-}* strains (in which the lymph nodes could not be seen macroscopically). In double- and triple-mutant mice the skin-draining lymph nodes were filled with nucleated cells that stained positive for CD45 (Fig 2, F). Eosinophilic and neutrophilic granulocyte, monocyte, and macrophage cell counts were lower in double- and triple-mutant mice when compared with single-mutant mice (Fig 1, K and L, and Fig 2, G). In double-mutant mice eosinophil and monocyte counts were increased relative to WT control values, and in contrast to the high proportion of CD11b^{low} macrophages seen in single-mutant mice, lymph nodes

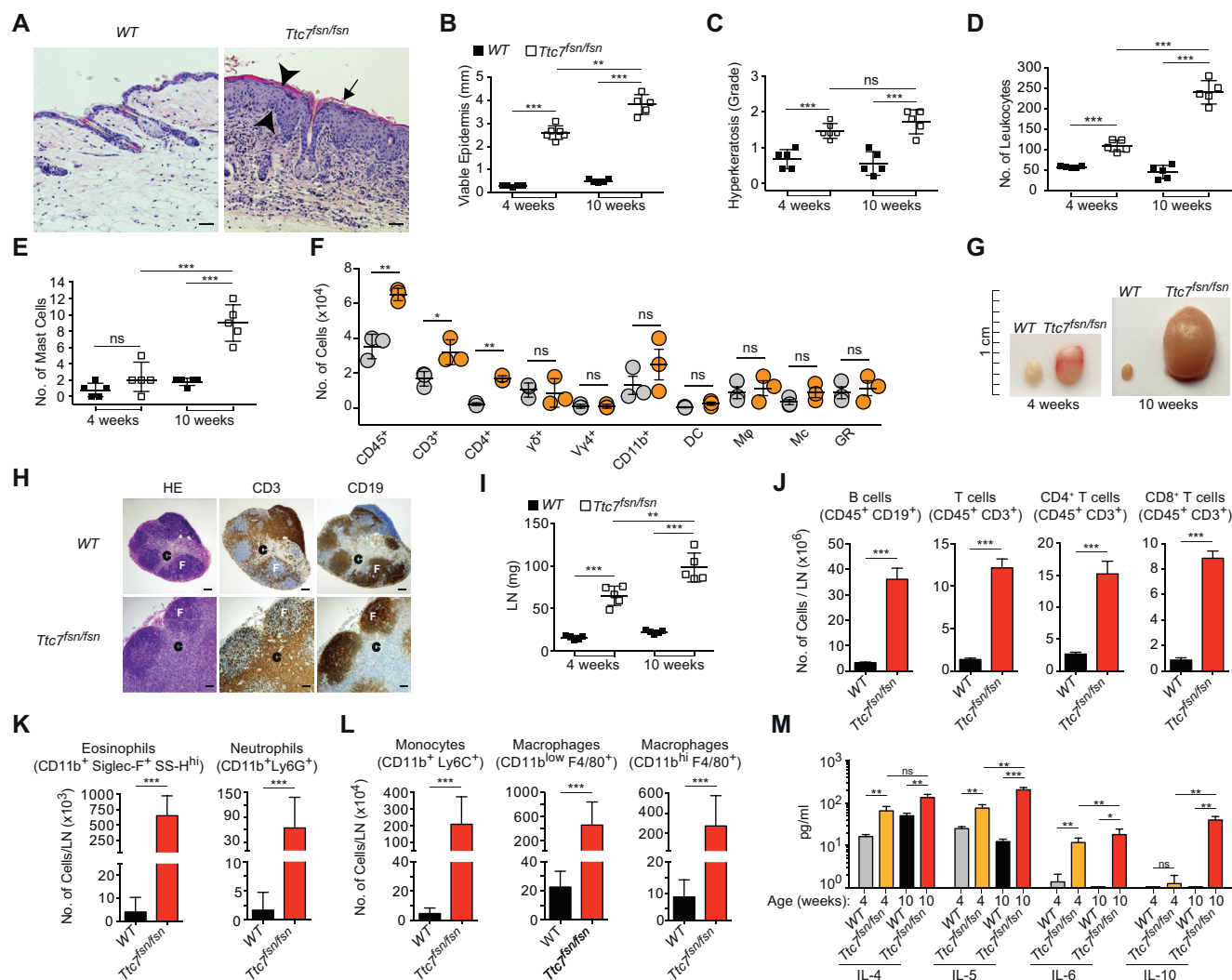


FIG 1. Fsn mice have hyperplastic dermatitis with lymphadenopathy of skin-draining lymph nodes and T_H2 cytokinemia. **A**, Acanthosis in 10-week-old *Ttc7^{fsn/fsn}* mice, which was visible as thickening of the viable epidermis (arrowheads) and ablation of keratin scales (arrows). Scale bar = 500 μ m. Hematoxylin and eosin staining; magnification $\times 10$. **B**, Acanthosis quantification: 15 measurements per mouse. **C-E**, Severity of hyperkeratosis per mouse (Fig 1, C) and dermal inflammation quantified by leukocyte (Fig 1, D) and mast cell (Fig 1, E) counts. **F**, Flow cytometric analysis of skin from 4-week-old mice. WT mice, Gray; *Ttc7^{fsn/fsn}* mice, orange. Each data point represents an individual mouse ($n = 3$). DC, Dendritic cells; GR, granulocytes; M Φ , monocytes; MC, mast cells. **G**, Representative photographs of axillary lymph nodes from 4- and 10-week-old female WT and *Ttc7^{fsn/fsn}* mice. **H**, Axillary lymph node stained with hematoxylin and eosin (HE). Magnification $\times 5$. Scale bars = 100 μ m. **I**, Average weight of axillary lymph node from WT and *Ttc7^{fsn/fsn}* mice ($n = 5$ mice). LN, Lymphoid node. **J-L**, Analysis of WT and *Ttc7^{fsn/fsn}* lymph nodes by using flow cytometry. Average numbers of cells per lymph node are shown. **M**, Serum levels of IL-4, IL-5, IL-6, and IL-10. Fig 1, J, Bar plots indicate means $\pm$ SEMs ($n = 8$ mice). Fig 1, B-F, I, and K-M, Bar plots indicate means $\pm$ SDs. Fig 1, K-M, $n = 8$ mice. * $P < .05$, ** $P < .01$, and *** $P < .001$. ns, not significant.

of double-mutant mice showed markedly increased levels of CD11b^{hi} macrophages when compared with WT control mice.

Ttc7^{fsn/fsn} mice present with IL-22 cytokinemia and IL-22⁺ mast cells

We next investigated whether nonlymphoid *Ttc7^{fsn/fsn}* cells were sufficient to promote a systemic increase in levels of proinflammatory cytokines during skin inflammation. As depicted in Fig 2, H, we observed increased serum levels of IL-17 and IL-22 in *Ttc7^{fsn/fsn}* mice when compared with levels in WT

littermates. IL-17 serum cytokine levels were significantly lower in double-mutant mice when compared with those in single-mutant mice and were not detected in triple-mutant mice, revealing that flaky skin can develop in the absence of systemic IL-17. IL-1 β , IL-23, and TGF- α levels were less than the limit of detection in serum samples from single-, double-, and triple-mutant mice and corresponding WT mice. IL-4, IL-5, IL-6, IL-10, IFN- γ , and TNF- α levels were less than the corresponding assay's limit of detection in double and triple mutants (data not shown). Remarkably, increased serum IL-22 levels were observed in all 3 *Ttc7^{fsn/fsn}* mutants, even in the double

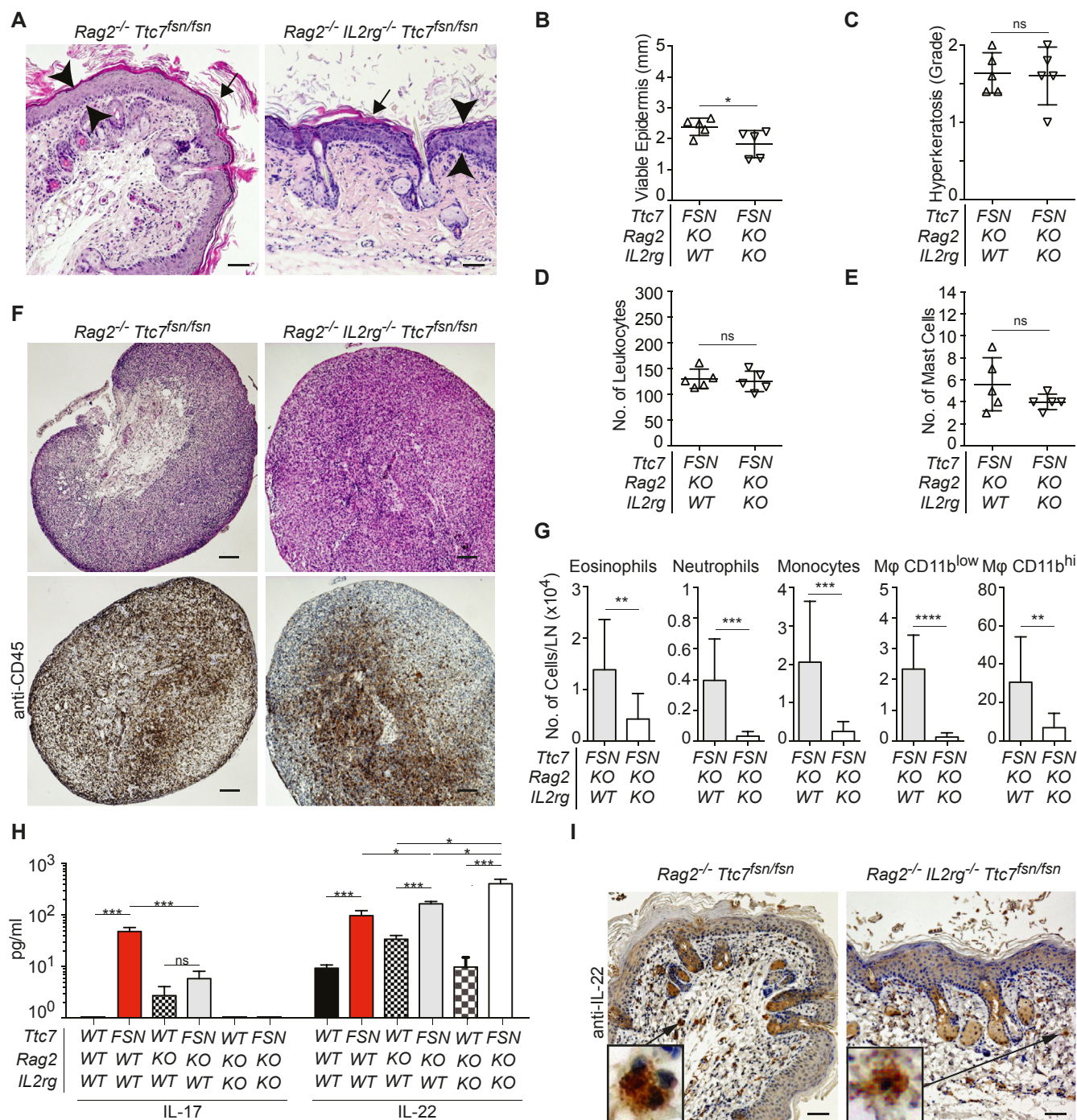


FIG 2. Flaky skin phenotype and IL-22 hypercytokinemia develop independently of lymphocytes in *Ttc7*^{fsn/fsn} mice, whereas the presence of *Ttc7*^{fsn/fsn} lymphocytes aggravates hyperkeratosis and dermal inflammation. **A**, Skin sections of female mice aged 10 weeks showing acanthosis (arrowheads) and ablation of keratin scales (black arrows). Scale bar = 500 μm. Hematoxylin and eosin staining; magnification ×10. **B-E**, Acanthosis (Fig 2, B), hyperkeratosis (Fig 2, C), total number of leukocytes in the dermis (Fig 2, D), and number of mast cells in the dermis (Fig 2, E) of *Rag2*^{-/-} *Ttc7*^{fsn/fsn} and *Rag2*^{-/-} *IL2rg*^{-/-} *Ttc7*^{fsn/fsn} mice. **F**, Axillary lymph node sections stained with hematoxylin and eosin reagent above and with anti-CD45 below (magnification ×5). Scale bars = 100 μm. **G**, Leukocytes isolated from peripheral lymph nodes were pooled separately from each mouse and analyzed by using flow cytometry. **H**, Serum levels of IL-17 and IL-22 measured in sex-matched WT, *Ttc7*^{fsn/fsn}, *Rag2*^{-/-}, *Rag2*^{-/-} *Ttc7*^{fsn/fsn}, *Rag2*^{-/-} *IL2rg*^{-/-}, and *Rag2*^{-/-} *IL2rg*^{-/-} *Ttc7*^{fsn/fsn} mice. **I**, Skin sections of female mice aged 10 weeks stained for IL-22-expressing cells. Scale bar = 500 μm. Magnification ×10, inset magnification ×50. Fig 2, G and H, Bar plots indicate means ± SDs (n = 8 mice). Fig 2, B-E, G, and H, The genotype of each mouse is shown below the graph. FSN, *Ttc7*^{fsn/fsn}; KO, knockout. Statistical significance was evaluated by using the unpaired 2-tailed Student t test: *P < .05, **P < .01, and ***P < .001. ns, Not significant.

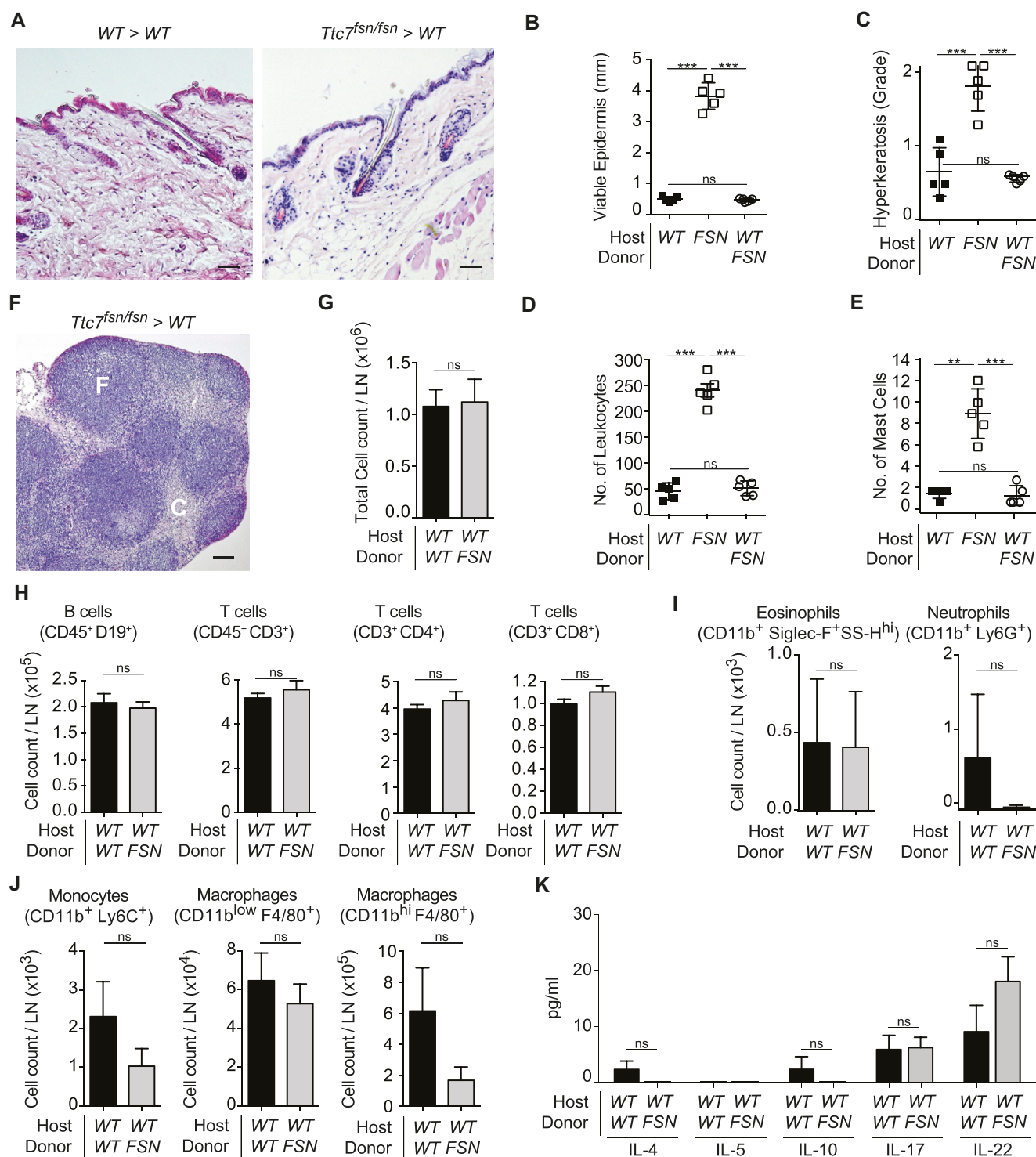


FIG 3. The flaky skin phenotype is not transferred by HSCT. **A**, Skin sections of female chimeras analyzed 10 to 13 weeks after HSCT. Scale bar = 500 μ m. Hematoxylin and eosin staining: magnification $\times 10$. **B-E**, Acanthosis (Fig 3, **B**), hyperkeratosis (Fig 3, **C**), total number of leukocytes in the dermis (Fig 3, **D**), and number of mast cells in the dermis (Fig 3, **E**) in skin histology of chimera mice were quantified. The same data presented in Fig 1 for WT and *Ttc7^{fsn/fsn}* mice are shown here for clarity. **F**, Histologic analysis of an axillary lymph nodes. Scale bars = 100 μ m. **F**, Follicle; **C**, cortex. Hematoxylin and eosin staining: magnification $\times 5$. **G**, Average cell number of cells per peripheral lymph node. **H-J**, Flow cytometry of skin-draining lymph nodes. **K**, Serum levels of IL-4, IL-5, IL-10, IL-17, and IL-22 measured in sex-matched mice 10 to 13 weeks after HSCT. Bar plots indicate means $\pm$ SDs ($n = 7$ mice). Fig 3, **B-E** and **G-K**, The genotype of acceptor (host) and donor mice is shown below the graph. *FSN*, *Ttc7^{fsn/fsn}*. Fig 3, **H**, Bar plots indicate means $\pm$ SEMs. Fig 3, **I** and **J**, Bar plots indicate means $\pm$ SDs. Statistical significance was evaluated by using an unpaired, 2-tailed Student *t* test: * $P < .05$, ** $P < .01$, and *** $P < .001$. *ns*, Not significant.

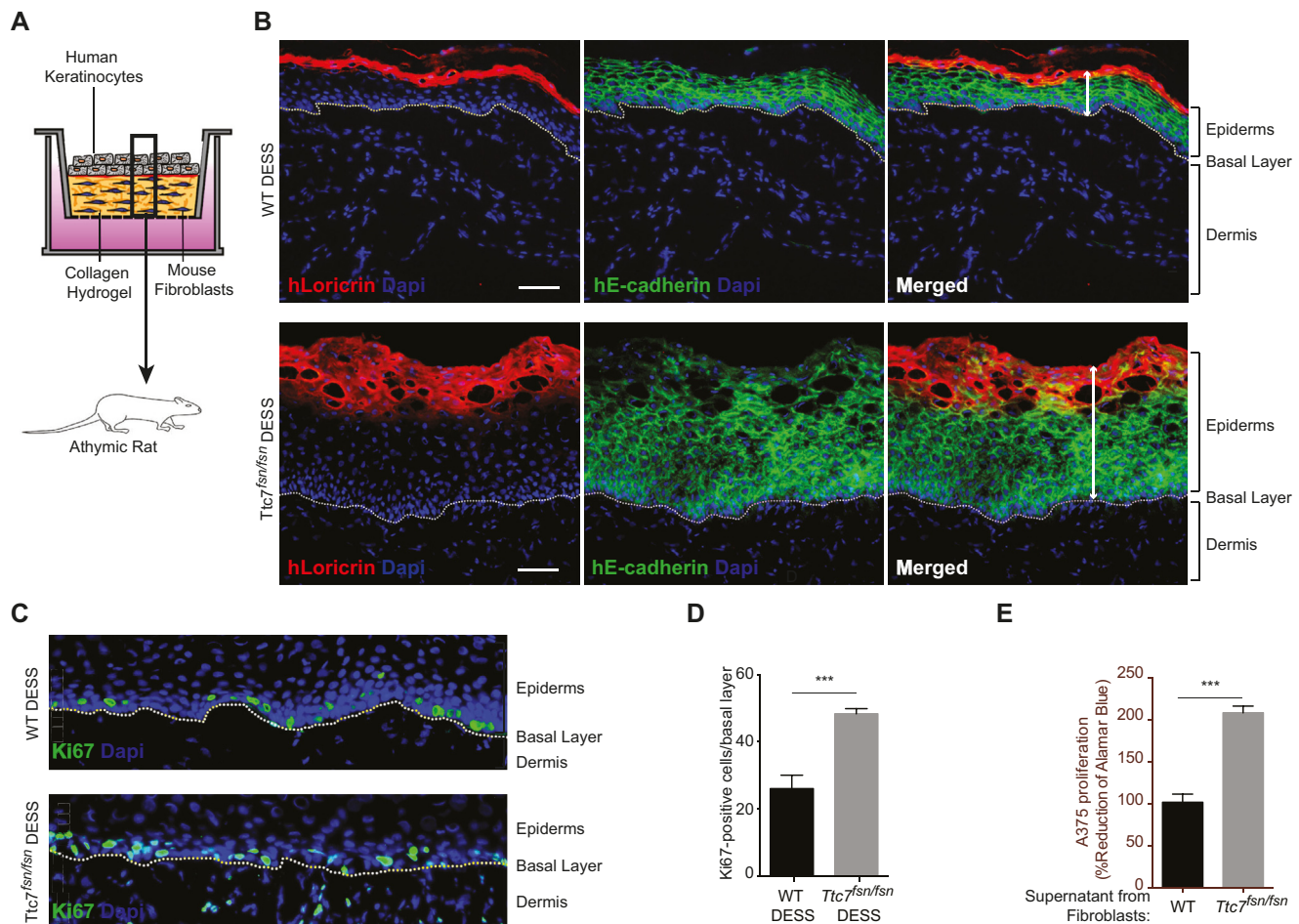


FIG 4. *Ttc7^{fsn/fsn}* fibroblasts induce hyperproliferation of human keratinocytes in tissue-engineered skin. **A**, Schematic structure of the *in vitro* culture system of DESSs. **B**, DESSs were cultured *in vitro* with human keratinocytes and fibroblasts from WT (WT DESS) or *Ttc7^{fsn/fsn}* (*Ttc7^{fsn/fsn}* DESS) mice and subsequently transplanted onto full-thickness skin wounds of athymic nude rats. On day 21 after transplantation onto athymic nude rats, sections from *Ttc7^{fsn/fsn}* and WT DESSs were stained with antibodies against loricrin (red) and E-cadherin (green) for immunohistochemistry; white arrows indicate thickness of the epidermis. **C**, On day 21 after transplantation onto athymic nude rats, sections from *Ttc7^{fsn/fsn}* and WT DESSs were also stained with antibodies against Ki-67 (green) for immunohistochemistry; white dotted lines indicate the dermoepidermal junction. **D**, Ki-67⁺ cells in the basal cell layer of WT and *Ttc7^{fsn/fsn}* DESSs were quantified. Scale bar = 100 μ m. **E**, Proliferation of A375 cells (human melanoma skin epithelial cell line) when incubated with supernatants derived from either cultures of *Ttc7^{fsn/fsn}* or WT fibroblasts. Proliferation is shown as percentage reduction of alamarBlue reagent after 48 hours of culture. Statistical significance was evaluated by using an unpaired, 2-tailed Student *t* test: ****P* < .001.

(*Rag2^{-/-}Ttc7^{fsn/fsn}*) and triple (*Rag2^{-/-}IL2rg^{-/-}Ttc7^{fsn/fsn}*) mutants, relative to corresponding control values (Fig 2, H). The highest IL-22 serum levels were measured in triple-mutant mice.

Immunohistochemistry of skin samples from double- and triple-mutant mice was performed to determine the source of IL-22 (Fig 2, I). Mast cells clearly stained positive for IL-22. Although other potential IL-22 producers (eg, fibroblasts) stained negative, we could not definitively rule out the presence of IL-22-producing neutrophils. Thus T cells, B cells, natural killer cells, and other innate lymphoid cells are not essential in driving the hyperplastic dermatitis seen in *Ttc7^{fsn/fsn}* mice. In this murine model IL-22 is not secreted by stromal cells but by leukocytes, such as mast cells.

HSCT does not transfer the flaky skin phenotype

We have identified *Ttc7^{fsn/fsn}* leukocytes as the source of high levels of IL-22. Therefore the skin phenotype of WT mice having received bone marrow transplants from *Ttc7^{fsn/fsn}* mice (*Ttc7^{fsn/fsn}* > WT chimeras) was analyzed to test whether hematopoietic cells alone are capable of causing hyperplastic dermatitis. The skin of these mice did not display any macroscopic lesions (data not shown). Acanthosis, hyperkeratosis, and inflammatory infiltrates were absent (Fig 3, A-E), even though the mean level of chimerism 84 days after bone marrow transfer was 67.5% (range, 40% to 95%). We observed that HSCT from *Ttc7^{fsn/fsn}* to WT mice was not associated with modification of the axillary lymph node architecture or an enlargement of the skin-draining lymph nodes (Fig 3, F and G). *Ttc7^{fsn/fsn}* > WT chimeras and

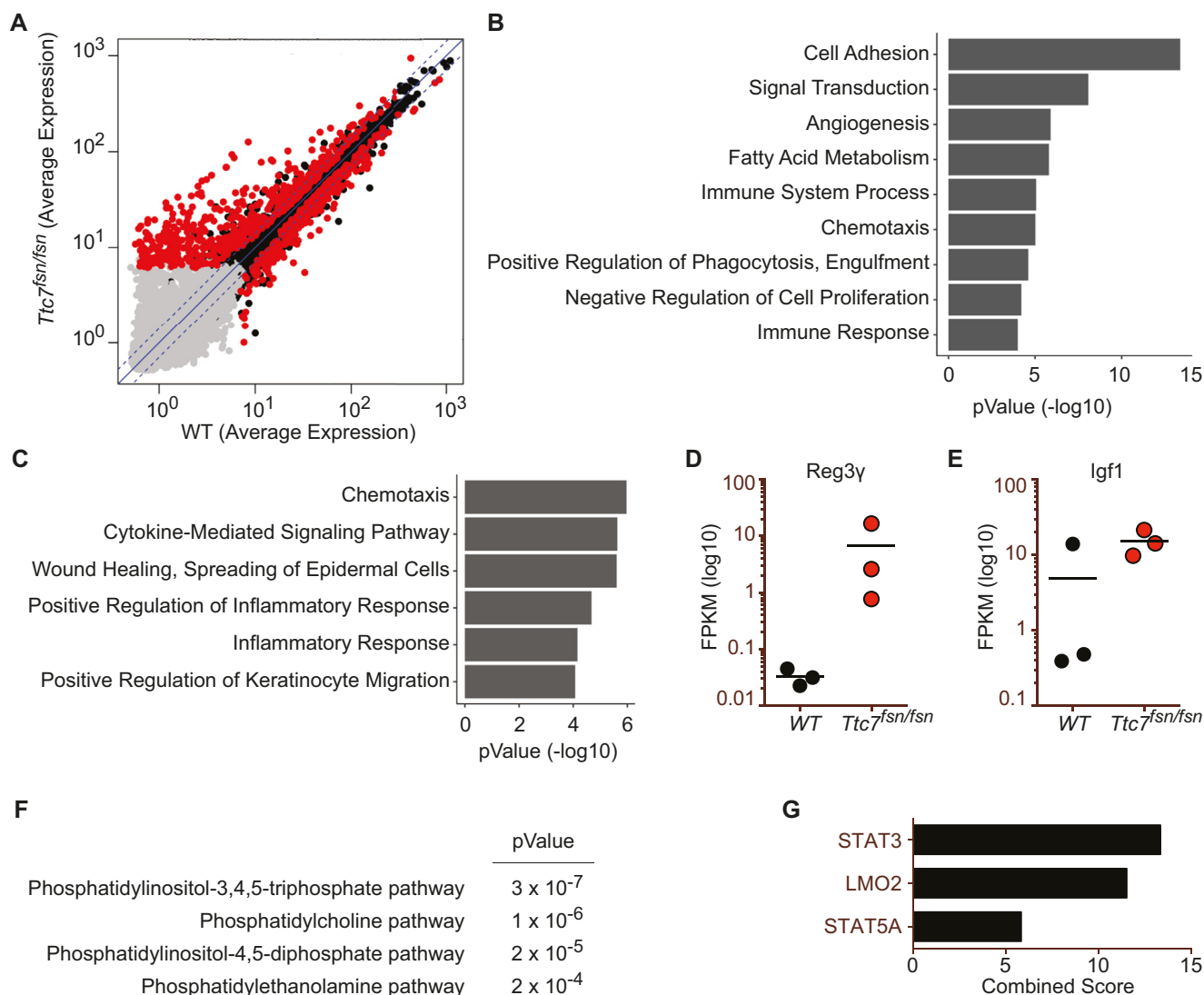


FIG 5. *Ttc7* modulates the fibroblast transcriptome. **A**, Scatterplot showing differentially expressed genes (red) in *Ttc7*^{fsn/fsn} versus WT fibroblasts on an exponential scale measured by using RNA sequencing and a false discovery rate *P* value of .01 or less (*n* = 3). **B**, Bar chart showing enriched GO categories based on the biological process of the top 1000 differentially expressed genes presented on a *P* value ($-\log_{10}$) scale. For simplicity, GO terms were clustered by using Revigo.²⁴ **C**, Detailed breakdown of the chemotaxis GO term cluster generated with Revigo presented on a *P* value ($-\log_{10}$) scale. Within this cluster, genes associated with the GO term “positive regulation of keratinocyte migration” according to the GO terms available at the mouse genome informatics²⁵ were analyzed further (see Fig E3). **D** and **E**, Dot plots showing the expression level (fragments per kilobase of transcript per million mapped reads [FPKM]) of *Reg3γ* (Fig 5, D) and *Igf1* (Fig 5, E) genes in fibroblasts from WT (black) and *Ttc7*^{fsn/fsn} mice (red) measured by using RNA sequencing. **F**, Top 4 metabolic pathways derived from analysis of RNA sequencing data using MetaCore (Clarivate Analytics, Philadelphia, Pa). **G**, Top 3 transcription factors predicted by using the transcription factor protein-protein interactions (PPIs) in Enrichr²⁷ to control expression of the 1000 differentially expressed genes.

control chimeras had the same numbers of B cells, total T cells, CD4⁺ T cells, and CD8⁺ T cells in the lymph nodes (Fig 3, H). Also, the number of eosinophilic and neutrophilic granulocytes, monocytes, and macrophages did not differ significantly between *Ttc7*^{fsn/fsn} > WT and control chimeras (Fig 3, I and J). Furthermore, serum levels of IL-4, IL-5, IL-10, IL-17, IL-22, IFN- γ , and TNF- α did not differ from control chimeras (Fig 3, K, and not shown). These results indicate that the hematopoietic system is not critical for inducing flaky skin disease.

Ttc7^{fsn/fsn} fibroblasts drive keratinocyte hyperproliferation

The fact that skin dysfunction in the *Ttc7*^{fsn/fsn} mouse is not initiated by the hematopoietic system is suggestive of a skin-endemic defect. To test whether the skin compartment alone drives the phenotype, we made use of an *in vitro* coculture system of stromal and epithelial skin cells. Tissue-engineered DESSs (based on collagen type I hydrogels; Fig 4, A) were populated with either WT or *Ttc7*^{fsn/fsn} fibroblasts and seeded with human

keratinocytes. DESSs were then transplanted onto full-thickness skin wounds of athymic nude rats. A hydrophobic, opaque stratum corneum was visible 3 weeks after transplantation of the DESSs. Analysis of the architecture of the skin on DESSs revealed that the epidermis on DESSs produced with *Ttc7^{fsn/fsn}* fibroblasts was markedly thicker than those produced with the WT counterpart (Fig 4, B). Immunofluorescence staining was used to detect loricrin (a major component of the cornified cell envelope) and E-cadherin (a transmembrane protein with important functions in morphogenesis and cell migration). Cell layers coexpressing loricrin and E-cadherin were considerably thicker in *Ttc7^{fsn/fsn}* DESSs than in WT DESSs. Despite this difference, the distributions of loricrin and E-cadherin were similar in *Ttc7^{fsn/fsn}* and WT DESSs. In both cases loricrin was most prominently expressed in the granular layer of the epidermis in terminally differentiated keratinocytes, and E-cadherin was most prominently expressed in the viable epidermis (as is observed in healthy skin *in vivo*). Remarkably, the proliferation marker Ki-67 was expressed in both dermal and epithelial layers in *Ttc7^{fsn/fsn}* DESSs (Fig 4, C); this contrasted with its restriction to the basal layers in WT DESSs. Moreover, human epidermal cells in the basal layer of *Ttc7^{fsn/fsn}* DESSs proliferated more than the equivalent cells in WT DESSs (Fig 4, D). Accordingly, the Ki-67⁺ cell count within the skin's basal layer was greater in *Ttc7^{fsn/fsn}* mice than in WT mice (see Fig E3 in this article's Online Repository at www.jacionline.org). Supernatants isolated from *Ttc7^{fsn/fsn}* fibroblast cultures were also able to induce twice as much proliferation of A375 (a human melanoma skin epithelial cell line; Fig 4, E) and HEK293 (a human embryonic kidney cell line, data not shown) cells when compared with supernatants from WT fibroblast cultures. Thus *Ttc7^{fsn/fsn}* fibroblasts were identified as key players in hyperproliferation of different cell types originating from the ectodermal layer.

***Ttc7^{fsn/fsn}* fibroblasts show altered expression of signal transducer and activator of transcription 3-regulated genes accompanied by defective expression of phospholipid synthetizing genes and enhanced transcription of *Reg3γ* and insulin-like growth factor 1 (*Igf1*)**

Ttc7^{fsn/fsn} fibroblasts were able to induce human melanoma A375 and human kidney HEK293 cell hyperproliferation, suggesting that mutations in *Ttc7* alter the expression of regulatory molecules in fibroblasts. Therefore NGS was used to analyze and compare the transcriptomes of *Ttc7^{fsn/fsn}* and WT fibroblasts. This method revealed that expression of more than 1000 genes was significantly altered in *Ttc7^{fsn/fsn}* fibroblasts. Within these, there were more upregulated genes than downregulated genes (false discovery rate, 0.5; log fold change, >1; Fig 5, A). Cluster and MetaCore analyses of the top 10% of differentially regulated genes revealed a high degree of variance between *Ttc7^{fsn/fsn}* and WT fibroblasts (see Fig E4 in this article's Online Repository at www.jacionline.org). Manual targeted analysis of transcript levels of genes known to be important in patients with psoriasis (such as Toll-like receptor [TLR] 1, TLR2, TLR4, TLR5, TLR7, TLR8, and TLR9)²³ showed no significant differences (data not shown). Gene Ontology (GO) classification of the genes with significantly altered expression by using Revigo²⁴ identified genes associated with cell adhesion as being the largest

differentially regulated cluster in *Ttc7^{fsn/fsn}* fibroblasts (Fig 5, B).²⁴ Significantly increased expression of genes involved in cell adhesion and signal transduction processes was also identified together with a significant increase in the expression of genes involved in angiogenesis, fatty acid metabolism, and immune system processes. Within the clusters created by using Revigo, the chemotaxis cluster was found to include expression of genes involved in wound healing, spreading of epidermal cells, and positive regulation of keratinocyte migration (Fig 5, C).²⁵ Particular attention was dedicated to this cluster because these GO terms encompass hyperproliferation of keratinocytes. Annotation of genes associated with the GO term "positive regulation of keratinocyte migration" (see Fig E5 in this article's Online Repository at www.jacionline.org) revealed that *Reg3γ* expression was upregulated (Fig 5, D). This gene has been previously associated with hyperproliferation of keratinocytes in patients with psoriasis.²⁶ When screening for other factors that might drive keratinocyte proliferation, we found a partial increase in expression of *Igf1* (Fig 5, E). *Igf1* is also known to induce keratinocyte proliferation. Expression of other growth factors (including fibroblast growth factors 3, 7, and 10; epidermal growth factors; and others) was downregulated (data not shown).

To analyze the secretion of *Igf1* and *Reg3γ*, their protein levels were measured in supernatants of *Ttc7^{fsn/fsn}* and WT fibroblast cultures (see Fig E6, A, in this article's Online Repository at www.jacionline.org). *Igf1* levels were significantly greater in supernatants of fibroblasts isolated from *Ttc7^{fsn/fsn}* mice relative to their WT counterparts. Although *Reg3γ* levels were less than the corresponding assay's limit of detection in the supernatants of fibroblasts from both mouse strains (data not shown), we observed greater, although not significantly increased, plasma levels in *Ttc7^{fsn/fsn}* mice than in their WT counterparts (see Fig E6, B).

By performing a MetaCore analysis for metabolic networks altered in *Ttc7^{fsn/fsn}* fibroblasts compared with their WT counterparts, we found that expression of genes involved in several phospholipid metabolism pathways was significantly altered. The most affected metabolic pathway was the phosphatidylinositol-3,4,5-triphosphate synthesis pathway (Fig 5, F).

We next analyzed which transcription factor could be responsible for the transcriptional signature seen in *Ttc7^{fsn/fsn}* fibroblasts. Feeding of transcriptomic data into Enrichr²⁷ revealed that a vast majority of genes with altered expression in *Ttc7^{fsn/fsn}* fibroblasts are controlled by the transcription factor signal transducer and activator of transcription 3 (STAT3; Fig 5, G).²⁷ This increased transcriptional activity found by using transcriptomic analysis was confirmed by using quantitative PCR (qPCR) analysis of a representative set of STAT3-regulated genes in *Ttc7^{fsn/fsn}* fibroblasts (see Fig E7, A and B, in this article's Online Repository at www.jacionline.org). Because STAT3 is important in T_H17 polarization, immunohistochemistry was conducted for IL-17 in the skin of *Ttc7^{fsn/fsn}* mice, in which an increased infiltration of IL-17-producing cells was found (see Fig E7, C).

These data show that *Ttc7* plays a central role in fibroblast biology. The *Ttc7^{fsn/fsn}* mutation affects these cells at different levels. Phospholipid metabolism is severely affected. This is accompanied by hyperactivity of STAT3 and transcription of *Reg3γ* and *Igf1*, factors with the potential to regulate keratinocyte proliferation.

DISCUSSION

When managing patients with an immunodeficiency, it is essential to know whether the underlying genetic defect also leads to major defects in nonhematopoietic cells. In the case of *TTC7A* defects, there is an ongoing debate whether *TTC7A* mutations dysregulate epithelial cells, as well as lymphocytes, or uniquely affect epithelial cells. Studies of murine models of *TTC7A* deficiency (eg, the *Ttc7^{fsn/fsn}* mouse) have revealed the *Ttc7* gene's important functional roles within a number of organs. Therefore we set out to characterize the cellular determinants of the flaky skin phenotype in the *Ttc7^{fsn/fsn}* mouse.

Our results show that in a murine model of *TTC7A* deficiency, nonlymphoid cells are critical for epithelial hyperproliferation, whereas lymphocytes aggravate the phenotype. Our work calls the prevailing pathogenic model of *TTC7A* deficiency into question by showing that mouse fibroblasts critically alter the proliferative behavior of epidermal cells. Thus dysfunctional crosstalk between stromal cells adds a new dimension to the possible pathogenesis of the multisystem disease associated with *TTC7A* deficiency in human subjects.

It is important to note that other researchers have reported involvement of fibroblasts in keratinocyte hyperproliferation. In cocultures with dermal fibroblasts from psoriatic patients, keratinocytes from healthy donors showed hyperproliferation on *in vitro* skin equivalents.²⁸ This observation points to a diffusible factor from fibroblasts that might also be involved in our system, irrespective of the question whether the *fsn* mouse model should be regarded as a model for psoriasis or a disease in the group of syndromic ichthyoses.^{5,15} There is evidence of a growth factor–mediated crosstalk between keratinocytes and fibroblasts, which becomes particularly important during cutaneous tissue repair aiming at restoring the barrier function of the skin.²⁹ With a view to identifying such factors in *Ttc7^{fsn/fsn}* fibroblasts, we compared the latter's transcriptome with that of WT counterparts. Overall, our transcriptomic analysis revealed that several cellular functions in the fibroblasts themselves are altered. With regard to factors that might influence the growth of keratinocytes, we found that transcription of the *Reg3γ* and *Igf1* genes was markedly upregulated in *Ttc7^{fsn/fsn}* fibroblasts.

In view of additional factors that might influence the growth of keratinocytes, we have found that IL-22 is present in skin mast cells and at increased levels in the sera of *Ttc7^{fsn/fsn}* mice. This inflammatory interleukin is mainly produced by lymphoid cells.^{30,31} However, we observed increased serum IL-22 levels in all mouse strains with *Ttc7^{fsn/fsn}* germline mutations, even in *Ttc7^{fsn/fsn}* mice crossed with *Rag2^{-/-}* and *Rag2^{-/-}IL2rg^{-/-}* mice. It is striking that IL-22 serum levels were particularly increased in the mice lacking T and B cells. In accordance with previous reports,³² our data point to mast cells as the nonlymphoid source of IL-22 in the *Ttc7^{fsn/fsn}* strains. It is noteworthy that within the heterodimeric IL-22 receptor, specifically IL-22 receptor 1 has a tightly regulated expression and is predominantly found on cells of epithelial origin, including intestinal and lung epithelial cells and keratinocytes.³³ Based on the known function of IL-22 in upregulating antibacterial molecules, such as *Reg3γ*, defensins, and lipocalin,^{34–36} skin inflammation in *Ttc7^{fsn/fsn}* mice might be a consequence of abnormal epidermal barrier dysfunction caused by hyperproliferation. Subsequently, IL-22–modulated inflammation could perpetuate keratinocyte proliferation in the presence of *Reg3γ*. Hence IL-22 (but not IL-17) might have a key role in modifying

hyperproliferative skin disease in the flaky skin model. Nevertheless, IL-17 might worsen the phenotype.

In a recent immunofluorescence analysis, increased numbers of Langerhans cells and Thy-1⁺ dendritic epidermal cells were observed in skin sections of *Ttc7^{fsn/fsn}* mice, suggesting an immune system–related abnormality.³⁷ We cannot rule out an effect of Langerhans cells on the skin phenotype in our mouse models. However, the results of our *Rag2^{-/-}* crossing experiments confirmed that γδ T-cell receptor–expressing Thy-1⁺ dendritic epithelial cells are not the main drivers. Although the skin of *Rag2^{-/-} Ttc7^{fsn/fsn}* mice displayed an increased Thy-1⁺CD45⁺ cell count (corresponding to innate lymphoid cells), this population is not relevant to the onset of the skin defect, as evidenced by development of hyperproliferative skin disease in *Rag2^{-/-} IL2rg^{-/-}* mice that lack ILCs.

Genetic diseases with multisystemic manifestations, such as those caused by *TTC7A* deficiencies, require target–designed therapies. Such therapies have to be able to act on multiple organs. The target of these therapeutic agents should be a regulator of cellular functions that are activated or downregulated to compensate for altered functionality of the affected gene. RhoA kinase inhibitors represent interesting candidates because reversion of abnormal polarity of epithelial cells in organoid cultures has been observed by addition of a RhoA kinase inhibitor. Another possibility could be compounds restoring proper phosphatidylinositol phosphorylation at the inner leaflet of the plasma membrane. Further potential therapeutic targets could eventually be found at the crosstalk between affected cell types. Our effort at finding such potential targets has led us to the observation that *Ttc7^{fsn/fsn}* fibroblasts express increased levels of *Igf1* and *Reg3γ* transcripts. Studies of the cytokine signatures in the different mouse strains has also highlighted that IL-4, IL-17, and IL-22 are the main factors characterizing the hyperproliferative immune response in *Ttc7^{fsn/fsn}* mice. However, further research will be needed to investigate the translatability of these findings to human disease.

It would be expected that defects in a protein central to so many cellular activities would have much more profound effects in multiple organs. As such, we report multiple-organ defects in *Ttc7^{fsn/fsn}* mice, although the severity of these defects varies depending on the organ. This brings forward the possibility of yet unknown compensatory mechanisms that could be more active in some cell types than others. Human *TTC7A* mutations cause developmental defects in the form of atresia, whereas in the *Ttc7^{fsn/fsn}* mouse model, skin disease develops later. In addition to supporting the possibility of the cross-species differential function of *TTC7A*/*Ttc7*, it also strengthens the possibility of compensatory mechanisms that could be more active during development in mice.

The suggested role of *TTC7A* in controlling localization of the synthesis of phosphatidylinositol-4-phosphates to the plasma membrane brings forward several possibilities about the molecular function of *TTC7A*. One possibility is that its function regulates the activity of signaling pathways by controlling the spatiotemporal synthesis of phosphatidylinositols. Another possibility is that dynamic cellular activities and polarization supported by the localization of phosphatidylinositols could be affected by *TTC7A*. This could include cytoskeletal rearrangement for podia formation or control of cellular polarity by regulation of tubulin and actin anchoring to the plasma

membrane. In line with this possibility, our transcriptomic analysis revealed that several cellular functions in the fibroblasts themselves are disrupted.

It has been suggested that the function of human TTC7A and the murine orthologue *Ttc7* diverged during evolution.⁷ Therefore we have performed purification studies (according to Glatter et al³⁸), which showed that mouse *Ttc7* expressed in HEK293 cells copurified with phosphatidylinositol 4-kinase III α and FAM126A (data not shown). This finding suggests that the functions of human TTC7A and murine *Ttc7* have been conserved during evolution.

Inverted apicobasal polarity of patients' enterocytes in organoid cultures, disturbed proliferation, and increased apoptosis have emerged as disease hallmarks in the context of inaccurate localization of phosphatidylinositol 4-kinase III α and increased phosphorylation of ezrin/radixin/moesin proteins.^{6,8,10,11} In addition to disrupted cell-intrinsic sensing, altered homotypic and heterotypic cell-cell interactions could induce this aberrant multicellular behavior. Based on our data, we opt for a multifaceted interaction network consisting of fibroblasts, epithelial cells, and hematopoietic cells as the driving force in the pathogenesis of TTC7A deficiency.

In conclusion, our present results show that mutated *Ttc7* is associated with fibroblast-driven keratinocyte hyperproliferation and lymphocyte-propagated skin inflammation in the presence of the proliferation-inducing factors IL-22, Reg3 γ , and Igf-1. Our findings highlight a previously unsuspected function of *Ttc7* in fibroblasts in controlling cellular behavior and epithelial cell proliferation. *Ttc7* appears to have a central role in the crosstalk between cells from different tissues. This suggested mechanism might not only explain why HSCT only partially relieves the multisystem genetic disease seen in patients with TTC7A deficiency but also provides potential new molecular targets for achieving systemic therapeutic effects.

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Clinical implications: Our murine studies shift the focus of research on TTC7A deficiency from epithelial and immune cells to fibroblasts. The novel transacting role of *Ttc7* implies targeted therapeutic approaches to be evaluated in future studies.

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METHODS

ELISA

ELISA was conducted on undiluted fibroblast supernatants, as described by the manufacturer's guidelines. Igf1 was from Thermo Fisher Scientific, and REG3 γ was from LifeSpan BioScience (Seattle, Wash).

qPCR

Fibroblasts were harvested and moved to TRIzol. RNA was isolated as described in the Pure link RNA Mini Kit (Invitrogen) with on-column DNase application. For qPCR, total RNA was reverse transcribed with oligo-dT AMV Reverse Transcriptase (Roche). Genes expression was determined by using real-time PCR with the TaqMan Gene expression Assay, as described by the

manufacturer (Thermo Fisher). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene. Fluorescence during PCR and subsequent dissociation was measured in quadruplicates on a 7900ht Fast Real Time PCR System and analyzed with Applied Biosystems SDS2.4 software (Applied Biosystems, Foster City, Calif). TaqMan probes with FAM for Kit (Mm00445212_m1), Syk (Mm01333032_m1), Ptpn6 (Mm00469153_m1), Apex1 (Mm00507805_g1), and Coro1a (Mm00486998_m1) were used.

Immunohistochemistry

Additional antibodies used were CD4 (clone 4SM95; eBioscience), Ki67 (clone SP6; Lifescreen; Cell Marque, Sigma-Aldrich), and IL-17 (Abcam).

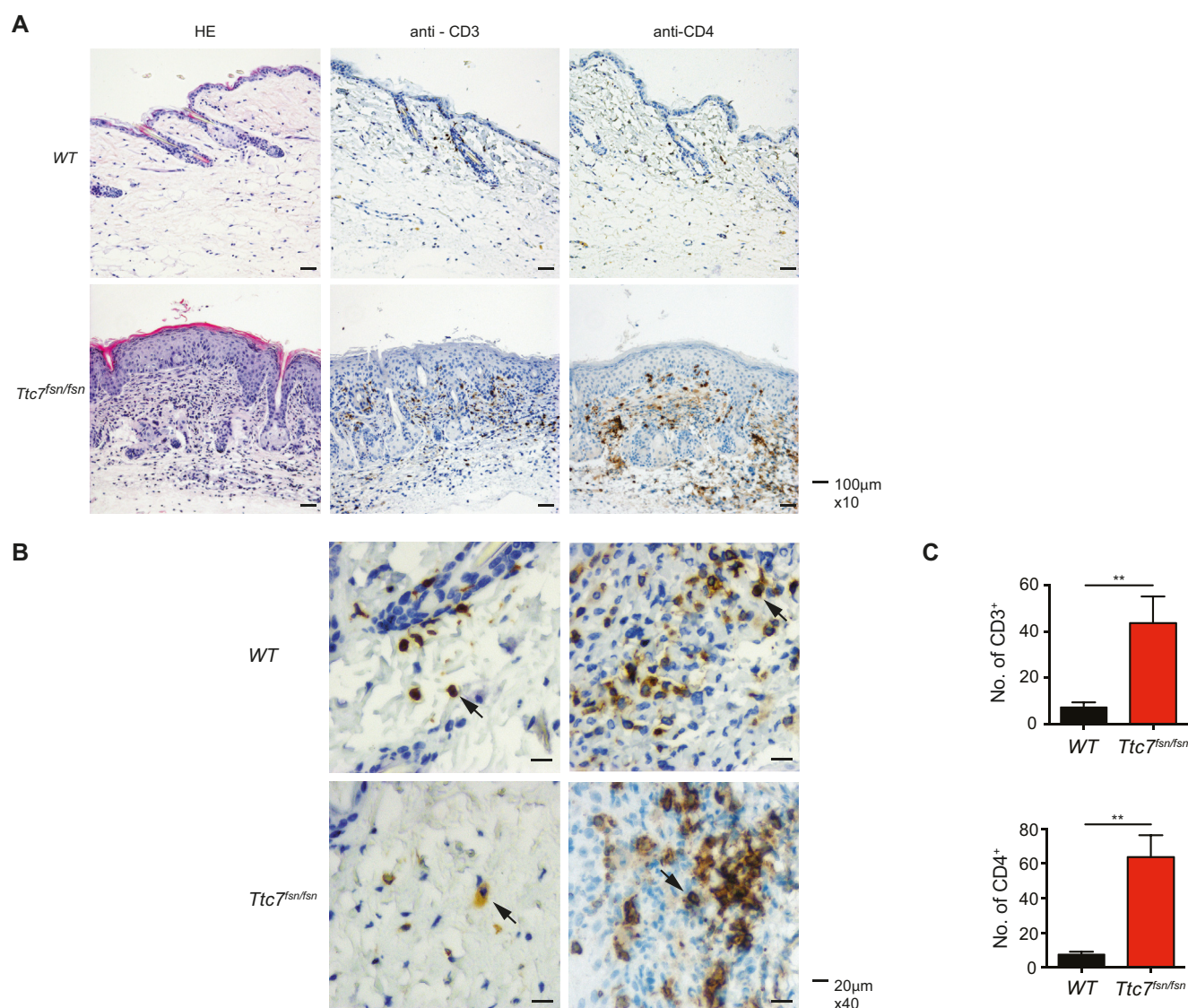
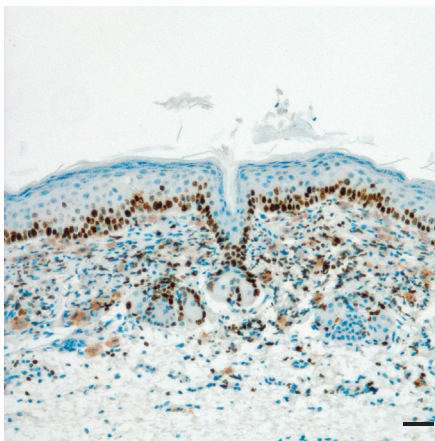
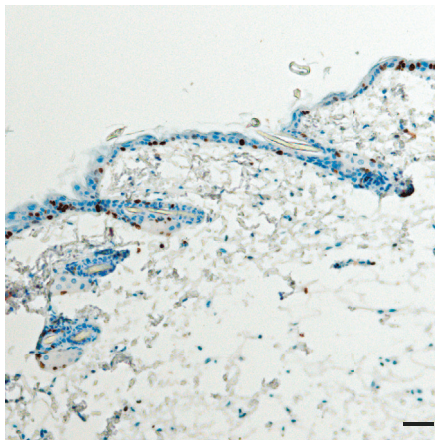


FIG E1. Dermal CD3⁺ and CD4⁺ cell infiltrates. Hematoxylin and eosin (HE) staining (**A**) and immunohistochemistry for CD3 and CD4 on *Ttc7^{fsn/fsn}* and WT skin (**A** and **B**). Skin is from the back of 10-week-old female mice. Scale bar = 100 µm. Magnification ×10 (Fig E1, **A**) and ×40 (Fig E1, **B**). **C**, CD3 and CD4 cells in the dermis were quantified in *Ttc7^{fsn/fsn}* and WT skin (n = 1 mouse per group). ***P* < .01.



FIG E2. Flaky skin phenotype. Representative photographs of flaky skin on the back of female single-mutant (*Ttc7^{fsn/fsn}*), double-mutant (*Rag2^{-/-} Ttc7^{fsn/fsn}*), and triple-mutant (*Rag2^{-/-} IL2rg^{-/-} Ttc7^{fsn/fsn}*) mice (age 10 weeks).

Ki67



— 100μm
x10

FIG E3. Ki67 expression in the skin. Immunohistochemical analysis of Ki67-expressing cells in skin sections of *Ttc7^{fsn/fsn}* and WT mice is shown. Skin from the back of 10-week-old female mice was analyzed. Scale bar = 100 μm. Magnification ×10.

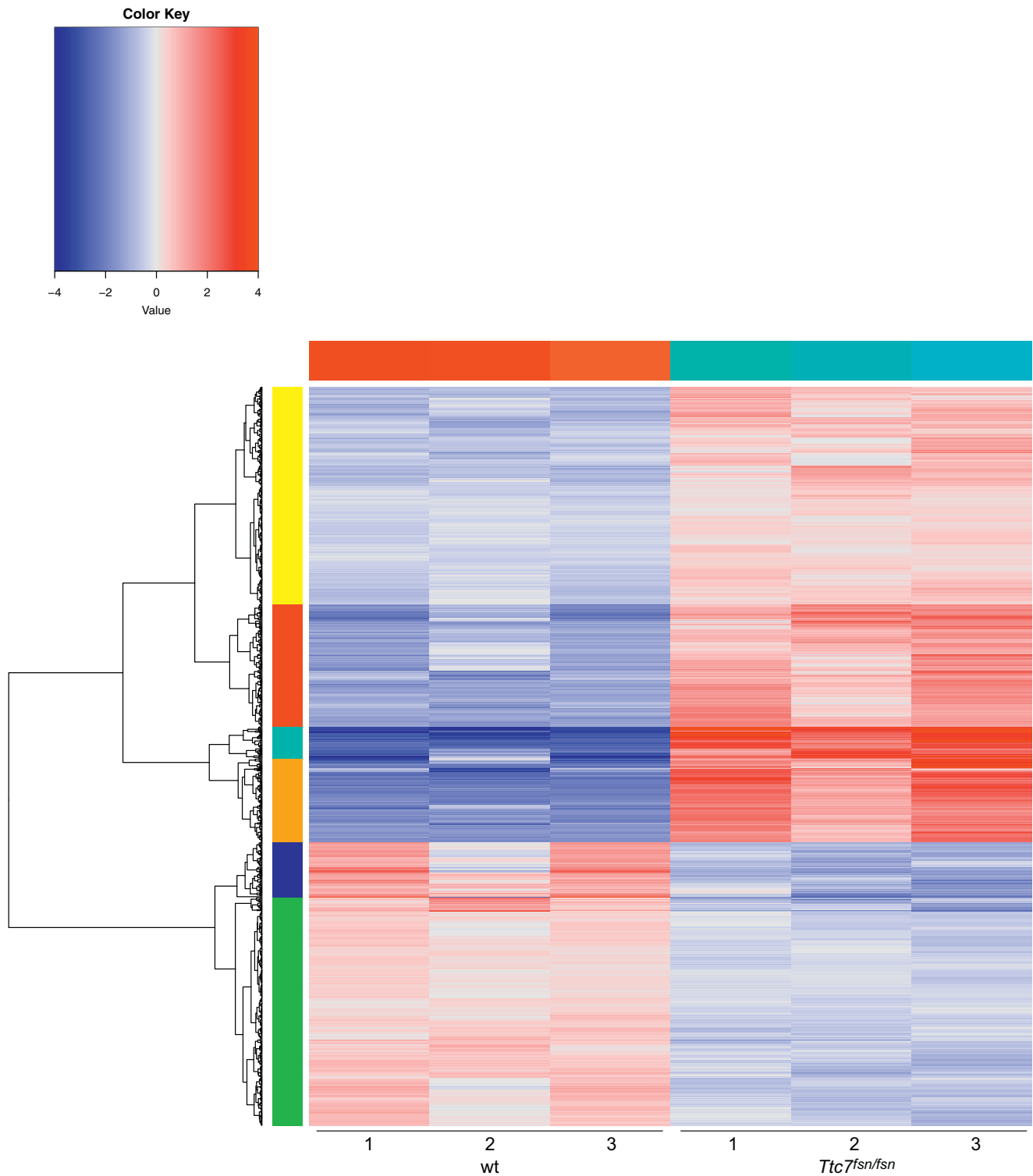


FIG E4. Clustering of differentially expressed genes. Heat map showing expression patterns for the 1000 top-ranked genes in 6 different clusters. Log₂-transformed signals are shown according to the *scale bar*. Each *column* represents an experimental sample, and each *row* represents a gene. Red corresponds to high expression, and blue corresponds to low expression.

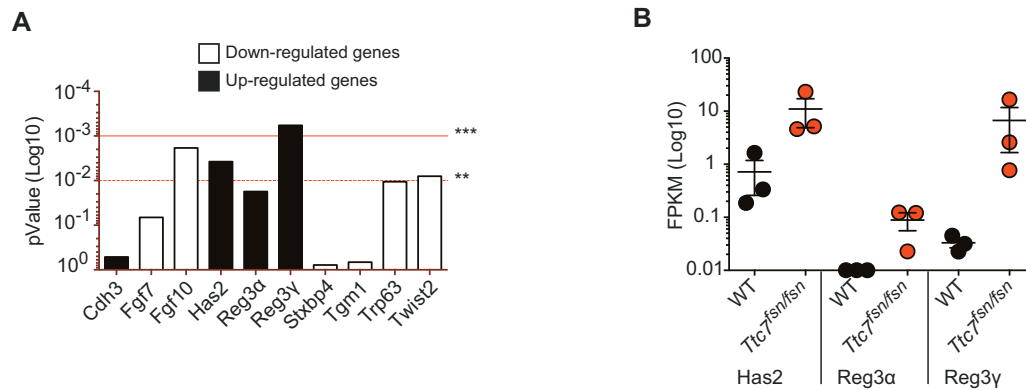


FIG E5. Expression of genes belonging to the GO term “positive regulation of keratinocytes migration measured by RNAseq.” Genes were selected according to the functional annotation by using the GO terms available at the mouse genome informatics. **A**, Differential expression of genes belonging to the GO term “positive regulation of keratinocytes migration in WT and *Ttc7^{fsn/fsn}* fibroblasts” is shown as a *P* value. White bars show *P* values of genes that are downregulated in *Ttc7^{fsn/fsn}* fibroblasts. Black bars show *P* values of genes that are upregulated in *Ttc7^{fsn/fsn}* fibroblasts. **B**, Expression level (shown as fragments per kilobase of transcript per million mapped reads [FPKM]) of genes found to be upregulated in *Ttc7^{fsn/fsn}* fibroblasts with a *P* value close to or greater than statistical significance. Fig E5, A: red dotted line, ***P* = .01; red line, ****P* = .001. In Fig E5, B, each dot represents 1 experiment, and the black line indicates means ± SEMs.

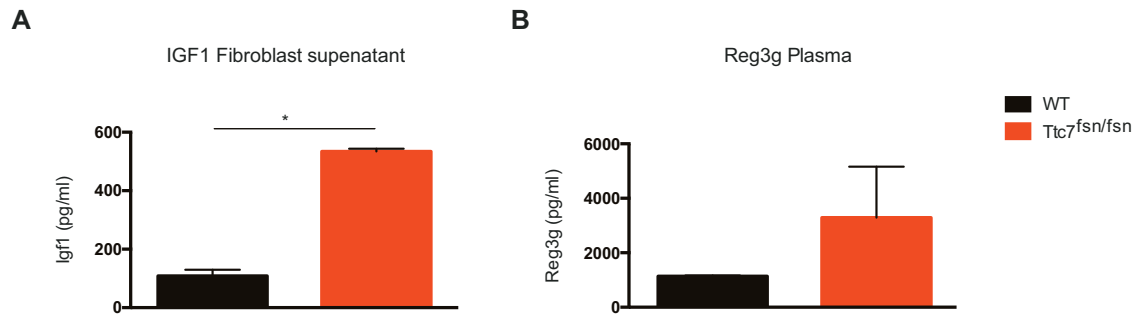


FIG E6. Igf1 and Reg3 γ protein levels. **A**, *Ttc7^{fsn/fsn}* and WT fibroblasts were cultured to 70% confluence, and supernatants were collected. Igf1 levels were increased significantly in supernatants, whereas Reg3 γ levels were less than the assay's detection limit (not shown). **B**, Reg3 γ levels in plasma of *Ttc7^{fsn/fsn}* mice increased, although not significantly ($P = .25$). Bar plots indicate means $\pm$ SDs: * $P < .05$ ($n = 3$ mice per group).

A

Fibroblasts

STAT3 Target genes	p-Value
CSF2RB	6.72E-07
KIT	8.39E-07
PRKCB	3.08E-06
PTPN6	1.49E-05
BTK	3.48E-05
GATA1	7.80E-05
CCR1	0.000117
CORO1A	0.000352
CISH	0.000391
GATA2	0.000973
GHR	0.001155
SYK	0.001283
TSHR	0.00154
IL6ST	0.001671
FES	0.003171
STAT1	0.005746
PPP2R1B	0.006193
APEX1	0.006253
SLC40A1	0.007732
NCOA1	0.007857
PDGFRA	0.009721

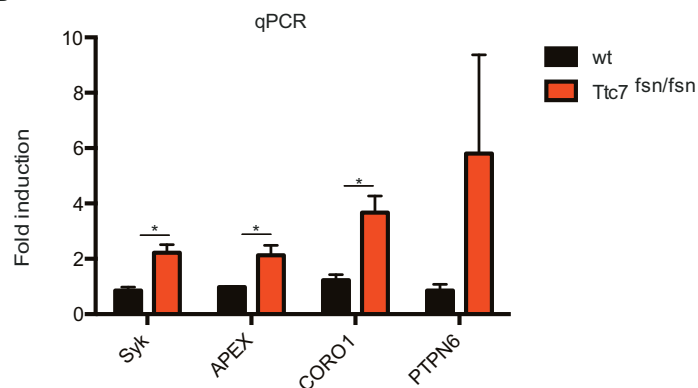
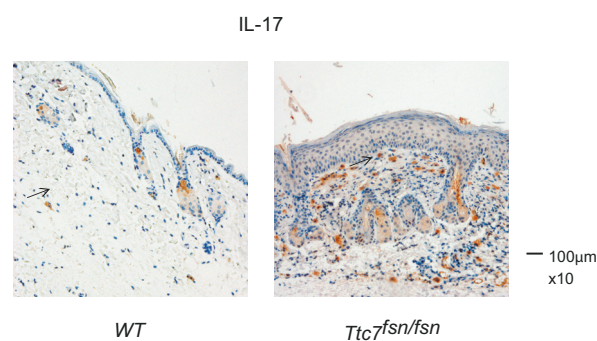
B**C**

FIG E7. STAT3 target genes. Analysis of significantly upregulated and downregulated genes by using Enrichr identified STAT3 as the most likely transcription factor underlying a transcriptomic profile. **A**, STAT3 target genes that are significantly upregulated or downregulated and used to define STAT3 as the most significant transcription factor. **B**, Real-time PCR of representative STAT3 target genes. Bar plots indicate means $\pm$ SDs ($n = 3$ mice per group). An unpaired t test corrected for multiple comparisons by using the Holm-Sidak method: $*P < .05$. **C**, Immunohistochemical analysis of IL-17-expressing cells in skin sections of *Ttc7^{fsn/fsn}* and WT mice. Skin from the backs of 10-week-old female mice was analyzed. Black arrow, Example of positive cell. Scale bar = 100 μ m. Magnification $\times 10$.