Psoriasis is a common chronic skin disorder characterized by keratinocyte hyperproliferation with altered differentiation accompanied by inflammation and increased angiogenesis. It remains unclear whether the first events that initiate psoriasis development occur in keratinocytes or inflammatory cells. Here, using different psoriasis mouse models, we showed that conditional deletion of Flt1 or Nrp1 in epidermal cells inhibited psoriasis mediated by Vegfa overexpression or c-Jun/JunB deletion. Administration of anti-Nrp1 antibody reverted the psoriasis phenotype. Using transcriptional and chromatin profiling of epidermal cells following Vegfa overexpression together with Flt1 or Nrp1 deletion, we identified the gene regulatory network regulated by Vegfa/Nrp1/Flt1 during psoriasis development and uncovered a key role of Fosl1 in regulating the chromatin remodeling mediated by Vegfa overexpression in keratinocytes. In conclusion, our study identifies an epidermal autonomous function of Vegfa/Nrp1/Flt1 that mediates psoriatic-like disease and demonstrates the clinical relevance of blocking Vegfa/Nrp1/Flt1 axis in psoriasis.

INTRODUCTION
Psoriasis is a frequent skin inflammatory disorder affecting approximately 3% of the world population (1). Psoriasis vulgaris is the most common type and is characterized by erythematous and scaly plaques of the skin. Psoriasis vulgaris typically occurs in the elbows, knees, or scalp regions. In addition to the skin lesions, several other organs can be affected in psoriasis, including arthritis, metabolic syndrome, or bowel inflammatory disease, which all contribute to the disease burden (1). Histologically, psoriasis is characterized by a hyper-thickening of the skin epidermis with increased vascularization and immune infiltration of the dermis (2). Although psoriasis is an autoimmune disease, the skin epidermis is also an important player in the initial pathogenesis of psoriasis and contributes to the recruitment of inflammatory cells, and the cross-talk between epidermal cells and immune cells is likely to be important for the pathogenesis of psoriasis (2).

Vascular endothelial growth factor A (VEGFA), the main pro-angiogenic factor, is overexpressed in human psoriasis skin and is correlated to disease severity (3). A role for VEGFA in psoriasis was suggested by VEGFA genomic location in close proximity to PSORS1, one of the psoriasis susceptibility locus 1, and the correlation of single nucleotide polymorphism in VEGFA with psoriasis severity (4). Administration of bevacizumab, a monoclonal antibody targeting VEGFA, for the treatment of solid cancers has been associated with improvement of psoriatic lesions (5). Experimental studies also demonstrated the effectiveness of VEGFA blocking therapy in improving the skin phenotype in mouse models of psoriasis-like disease (6–8).

Transgenic mouse models overexpressing Vegfa in keratinocytes lead to the development of an inflammatory skin condition recapitulating the main hallmarks of human psoriasis, supporting a key role of Vegfa expressed by keratinocytes in promoting psoriasis-like disease (8–10). However, the precise roles played by VEGFA in mediating psoriasis are poorly understood. VEGFA mediates its effects by binding to tyrosine kinase receptors Flt1 (VEGFR1) and Flk1 (VEGFR2) (11). Neuropilin 1 (Nrp1) acts as a VEGFA coreceptor amplifying VEGFA signaling by promoting VEGFR receptor signaling in the same cells (cis effect) or presenting VEGFA to neighboring cells (trans effect) (12).

Despite the well-known role of VEGFA in psoriasis, the molecular mechanism by which VEGFA promotes psoriasis is not well understood. It remains unclear on which cells VEGFA acts to promote psoriasis and the molecular mechanisms downstream of VEGFA in this process. Does VEGFA act only on blood vessels or macrophages, as it has been previously suggested (13), which in turn mediates recruitment of inflammatory cells and causes the defect of keratinocyte differentiation, or does VEGFA also act directly on epidermal cells in an autocrine or a paracrine manner to orchestrate the changes associated with psoriasis, as it has been suggested during tumorigenesis (14, 15)?

Using genetically engineered psoriasis mouse models, we assessed the role of Nrp1 or Flt1 expression by epidermal cells to induce psoriasis development in a cell autonomous manner. Unexpectedly, we found that deletion of Nrp1 or Flt1 in the skin epidermis completely prevents the development of psoriasis following Vegfa overexpression. In addition, epidermal deletion of Flt1 in mice with c-Jun/JunB deletion, one of the best-studied mouse models of psoriasis (16), also leads to a notable improvement of the psoriasis lesions. We showed that therapeutic administration of Nrp1-blocking antibodies reverts the development of psoriatic lesions induced by Vegfa.

Combination of RNA sequencing (RNA-seq) and assay for transposase-accessible chromatin sequencing (ATAC-seq) on fluorescence-activated cell sorting (FACS)–isolated epidermal cells following Vegfa overexpression in the presence or in the absence of Flt1 or Nrp1 allowed the identification of the gene regulatory network downstream of Flt1/Nrp1 in keratinocytes that control the development of Vegfa-induced psoriasis. Together, our results unravel a novel cell autonomous function of Flt1 and Nrp1 in epidermal cells that promotes Vegfa-induced psoriasis and opens...
the way for new therapeutic opportunities for the treatment of psoriatic disease.

**RESULTS**

**Epidermal autonomous expression of Flt1 is essential for psoriasis development induced by Vegfa**

As previously reported, Vegfa overexpression in mouse epidermis using K14-Cre/Rosa-Vegfa (K14-Vegfa) induces a psoriatic-like disease (8), which appears 1 month postnatally and characterized macroscopically by red skin, scaly lesions in the ear and the tail, as well as severe erythema and edema of the oral mucosa (fig. S1, A and B), and microscopically by hyperplasia of the epidermis, abnormal keratinocyte differentiation, increase in epidermal cell proliferation, immune infiltration, and blood vessel density (fig. S1, C to J). These macroscopic and microscopic features represent the hallmark of psoriatic skin lesions in humans (17). In this mouse model, different populations of cells express Vegfa receptors and coreceptors. Endothelial cells (ECs) express VEGFR2/Flk1 (18), VEGFR1/Flt1 (19), and its coreceptor Nrp1 (20). Flt1 is also expressed by different immune cells (21), but the Vegfa receptors expressed by keratinocytes are still a matter of discussion (22, 23). Nrp1 is a Vegfa coreceptor, which promotes Vegfa signaling in trans by presenting Vegfa to other immune and ECs, or in cis by promoting Vegfa signaling in a cell autonomous manner (12). Using RNA-seq or quantitative reverse transcription polymerase chain reaction (qRT-PCR), we found that basal keratinocytes expressed VEGFR1/Flt1 and Nrp1, but not VEGFR2/Flk1 (fig. S1K).

To assess the cell autonomous role of Flt1 expression by keratinocytes in Vegfa-mediated psoriasis, we deleted Flt1 exclusively in the epidermis using K14-Cre/Rosa-Vegfa/Flt1 flox/flox mice (K14-Vegfa/Flt1 cKO) (Fig. 1A). Vegfa mRNA expression was comparable in K14-Vegfa and K14-Vegfa/Flt1 cKO mice (Fig. 1B), whereas Flt1 expression was virtually abolished at the mRNA and protein levels in K14-Vegfa/Flt1 cKO epidermis (Fig. 1, B to D). Epidermal thickness, which was increased by threefold in K14-Vegfa epidermis, was normalized to the control level in K14-Vegfa/Flt1 cKO epidermis (Fig. 1, F and G).

The hyperplasia of the epidermis in psoriatic skin is associated with increased proliferation of basal keratinocytes (2). Whereas Vegfa overexpression increased basal keratinocyte proliferation [51% of EdU (5′-ethynyl-2′-deoxyuridine)–positive cells in K14-Vegfa versus 17% for control mice], the deletion of Flt1 prevented the increase in cell proliferation induced by Vegfa (19% of EdU–positive cells) (Fig. 1, H and I).

Psoriatic skin induced by Vegfa overexpression is also characterized by an infiltration of immune cells (2). To define whether Flt1 expression in keratinocytes controls the immune infiltration induced by Vegfa overexpression, we performed immunostaining of CD45, a pan-leucocyte marker in the skin epidermis of control, K14–Vegfa, and K14–Vegfa/Nrp1 cKO mice. Flt1 deletion in the epidermis completely prevented the increase in dermal immune infiltrate following Vegfa overexpression (Fig. 1, J and K). CD19–positive B lymphocytes and F4/80 macrophages were the main immune cell populations increased in the dermis of K14–Vegfa mice and were decreased upon epidermal Flt1 deletion (fig. S2, B and C).

Neangiogenesis is another important hallmark characterizing psoriatic skin (24). To determine the role of epidermal Nrp1 in the regulation of neangiogenesis induced by Vegfa expression by keratinocytes, we performed CD31 immunostaining in the skin epidermis and quantified the microvascular density in the mice expressing or not expressing Flt1 in the epidermis. Whereas Vegfa expression by epidermal cells increased the microvascular density, the deletion of Flt1 in keratinocytes normalized the microvascular density to the level found in control mice (Fig. 1, L and M).

**Epidermal expression of Nrp1 is required for psoriasis development mediated by Vegfa**

To investigate whether the expression of Nrp1 by epidermal cells regulates Vegfa-mediated psoriatic-like disease, we overexpressed Vegfa and deleted Nrp1 specifically and exclusively in the skin epidermis using K14-Cre/Rosa-Vegfa/Nrp1flox/flox mice (K14-Vegfa/Nrp1 cKO) (Fig. 2A). The level of Vegfa mRNA expression in basal epidermal cells was comparable between K14-Vegfa and K14-Vegfa/Nrp1 cKO mice (Fig. 2B), whereas Nrp1 expression was virtually undetectable in the epidermis of K14–Vegfa/Nrp1 cKO mice both at the mRNA and protein levels (Fig. 2, B to D). The deletion of Nrp1 in keratinocytes in K14–Vegfa/Nrp1 cKO mice was sufficient to completely block the development of the macroscopic psoriasis phenotype (erythema and scaly skin) in the tail and the ear epidermis induced by Vegfa overexpression (Fig. 2E). The deletion of Nrp1 in the epidermis also prevented the development of microscopic alterations that characterized psoriatic skin, including the epidermal hyperthickening, the increase in basal keratinocyte proliferation, the immune infiltration, and microvascular density associated with Vegfa overexpression (Fig. 2, F to I).

The psoriasis-like disease induced by Vegfa was unaffected by the deletion of a single allele of either Nrp1 or Flt1, whereas compound heterozygous mice (K14–Vegfa/Nrp1+/cKO/Flt1+/cKO) did not present a psoriatic phenotype and were indistinguishable from the complete deletion of both Nrp1 and Flt1 (fig. S3, A to H), showing that Flt1 and Nrp1 interact genetically in an epistatic manner to promote Vegfa-induced psoriasis. Together, these data clearly demonstrate the essential cell autonomous function of Flt1 and Nrp1 in promoting psoriasis development induced by Vegfa overexpression.

**Administration of function-blocking anti-Nrp1 antibody improves psoriasis phenotype induced by Vegfa overexpression**

To assess whether inhibiting Nrp1/Vegfa interaction can be of therapeutic relevance for the treatment of psoriasis, we administrated to K14–Vegfa mice an anti-Nrp1 antibody that blocks the binding of Vegfa (Nrp1b antibody) or the binding of semaphorins (Nrp1a antibody) (fig. S3, A to C) (25, 26).

The epidermal thickness was rapidly normalized following the administration of anti-Nrp1 antibody that blocks Nrp1/Vegfa interaction (Fig. 3, D and E). Likewise, keratinocyte hyperproliferation, immune infiltration, and increase in microvascular density induced by Vegfa overexpression were also all rapidly normalized following the administration of anti-Nrp1 antibody that blocks Vegfa interaction, whereas no improvement of psoriasis was observed after the administration of Nrp1 antibody that blocks semaphorins interaction (Fig. 3, F to K). These data demonstrate the therapeutic benefit of blocking Vegfa/Nrp1 interaction for the treatment of psoriasis.

**Epidermal autonomous expression of Flt1 is essential for psoriasis development induced by c-Jun/JunB deletion**

We then assessed whether the essential role of Flt1 in epidermal cells is conserved across different mouse models of psoriasis. To this
Fig. 1. Flt1 expression by keratinocytes is essential for Vegfa-induced psoriasis. (A) Strategy to constitutively activate Vegfa and inhibit Flt1. (B) Vegfa and Flt1 mRNA expression by qRT-PCR on FACS-isolated keratinocytes (n = 3) (means ± SEM, Mann-Whitney). (C) Flt1 expression assessed by Western blot on FACS-isolated basal keratinocytes. (D) Flt1 protein expression (n = 3) (means ± SEM, Mann-Whitney). (E) Naso-oral region, ear, and tail. (F) Hematoxylin and eosin (H&E) on tail skin. Scale bars, 50 μm. (G) Epidermal tail thickness measured microscopically (n = 10) (means ± SEM, Student’s t test). (H) K14/EdU staining. Scale bars, 50 μm. (I) Percentage of EdU-positive basal cells (BCs) in interfollicular epidermis (IFE) [n = 398 (Ctrl), n = 436 (K14-Vegfa), n = 422 (K14-Vegfa/Flt1 cKO)] total BCs, n = 10 mice] (mean ± SEM, Student’s t test). (J) K14/CD45 staining. Scale bars, 50 μm. (K) Density of CD45-positive cells in dermal IFE area (represents the dermal area just beneath the IFE) of 300,565 μm² (Ctrl), 289,678 μm² (K14-Vegfa), and 278,767 μm² (K14-Vegfa/Flt1 cKO); n = 10 mice. Number of CD45-positive cells per 10,000 μm² (means ± SEM, Student’s t test). (L) K14/CD31 staining. Scale bars, 50 μm. (M) Number of CD31-positive cells (microvascular density) calculated in dermal IFE area of 324,567 μm² (Ctrl), 345,234 μm² (K14-Vegfa), and 342,356 μm² (K14-Vegfa/Flt1 cKO); n = 10 mice. Number of CD31-positive cells per 10,000 μm² (means ± SEM, Student’s t test). Photo credit: Benhadou Farida, Laboratory of Stem Cells and Cancer.
Fig. 2. Cell autonomous function of Nrp1 in the epidermis is critical for Vegfa-induced psoriasis. (A) Strategy to constitutively activate Vegfa and delete Nrp1 expression in epidermis. (B) Vegfa and Nrp1 mRNA expression by qRT-PCR on FACS-isolated keratinocytes (n = 3) (means ± SEM, Mann-Whitney test). (C) Nrp1 expression assessed by Western blot performed on FACS-isolated basal keratinocytes. (D) Nrp1 protein expression (n = 3) (means ± SEM, Mann-Whitney test). (E) Naso-oral region, ear, and tail. (F) H&E on tail skin. Scale bars, 50 μm. (G) Epidermal tail thickness measured microscopically (n = 10) (mean ± SEM, Student’s t test). (H) K14/EdU staining. Scale bars, 50 μm. (I) Percentage of EdU-positive BCs [n = 507 (Ctrl), n = 487 (K14-Vegfa), n = 490 (K14-Vegfa/Nrp1 cKO)] total BCs; n = 10 mice (means ± SEM, Student’s t test). (J) K14/CD45 staining. Scale bars, 50 μm. (K) Density of CD45-positive cells in the dermal IFE area of 344,965 μm² (Ctrl), 449,687 μm² (K14-Vegfa), and 423,876 μm² (K14-Vegfa/Nrp1 cKO); n = 10 mice. CD45-positive cells per 10,000 μm² (means ± SEM, Student’s t test). (L) K14/CD31 staining. Scale bars, 50 μm. (M) Number of CD31-positive cells calculated in a dermal IFE area of 409,560 μm² (Ctrl), 432,890 μm² (K14-Vegfa), and 428,532 μm² (K14-Vegfa/Nrp1 cKO); n = 10 mice. Number of CD31-positive cells per 10,000 μm² (means ± SEM, Student’s t test). Photo credit: Benhadou Farida, Laboratory of Stem Cells and Cancer.
Fig. 3. Function-blocking anti-Nrp1 antibodies improve Vegfa-induced psoriasis. (A to C) Naso-oral region, ear, and tail. (D) H&E on tail skin. Scale bars, 50 μm. (E) Measures of epidermal thickness (n = 3) (means ± SEM, Mann-Whitney). (F) K14/EdU staining. Scale bars, 50 μm. (G) Percentage of EdU-positive BCs [n = 387 (isotype, day 0), n = 354 (isotype, day 15), n = 442 (Nrp1 b AB, day 0), n = 409 (Nrp1 b AB, day 15), n = 391 (Nrp1 a AB, day 0), n = 421 (Nrp1 a AB, day 15) total BCs, n = 3] (means ± SEM, Mann-Whitney). (H) K14/CD45 staining. Scale bars, 50 μm. (I) Density of CD45-positive cells in the dermal IFE area of 254,342 μm² (isotype, day 0), 298,567 μm² (isotype, day 15), 267,890 μm² (Nrp1 b AB, day 0), 287,908 μm² (Nrp1 b AB, day 15), 257,560 μm² (Nrp1 a AB, day 0), 294,901 μm² (Nrp1 a AB, day 15); n = 10. Number of CD45-positive cells per 10,000 μm² (means ± SEM, Mann-Whitney). (J) K14/CD31 staining. Scale bar, 50 μm. (K) Microvascular density in dermal IFE area of 267,980 μm² (isotype, day 0), 234,589 μm² (isotype, day 15), 222,370 μm² (Nrp1 b AB, day 0), 223,456 μm² (Nrp1 b AB, day 15), 200,154 μm² (Nrp1 a AB, day 0), and 212,980 μm² (Nrp1 a AB at day 15). Number of CD31-positive cells per 10,000 μm² (means ± SEM, Mann-Whitney). Photo credit: Benhadou Farida, Laboratory of Stem Cells and Cancer.
end, we induced the deletion of Flt1 in the skin epidermis of c-Jun/JunB cKO mice, one of the most commonly used mouse models of psoriasis (16, 27). In this model, mice develop a strong psoriasis-like disease 2 weeks after c-Jun/JunB conditional deletion (fig. S4, A and B) (16).

Epidermal deletion of Flt1 in this model (c-Jun/JunB/Flt1 triple cKO) decreased the severity of psoriasis lesions both macroscopically and microscopically with a decrease in epidermal thickness (fig. S4, C and D). The immune infiltrate within the epidermis, which consists mainly of neutrophils in this model, was normalized upon epidermal Flt1 deletion (fig. S4, E to G). The microvascular density was also normalized upon Flt1 deletion (fig. S4, F to I). These data demonstrate the conserved function of Flt1 in epidermal cells to initiate psoriasis development across different psoriasis mouse models.

Transcriptional landscape associated with Vegfa/Flt1/Nrp1 signaling in psoriasis

To define the molecular mechanisms that regulate the cell autonomous function of Flt1 and Nrp1 in epidermal cells to initiate psoriasis formation, we first defined the transcriptional signature of epidermal cells induced by Vegfa in the presence or absence of Flt1 and Nrp1. To this end, we performed RNA sequencing on FACS-isolated basal keratinocytes from control, K14-Vegfa, K14-Vegfa/Nrp1 cKO, and K14-Vegfa/Flt1 cKO mice.

The majority of the genes up-regulated by Vegfa [382 of 968 genes (40%), \( P = 10^{-17} \)] were no longer up-regulated following either Flt1 or Nrp1 deletion, demonstrating that the core of the transcriptional changes that occur following Vegfa overexpression in epidermal cells is coregulated by Flt1 and Nrp1 (Fig. 4A). In contrast, 243 of the 968 genes up-regulated by Vegfa [25%, \( P = 10^{-12} \)] were up-regulated by Vegfa in all conditions irrespective of Nrp1 and Flt1 expression by keratinocytes, and thus, represent the genes that are regulated by Vegfa in keratinocytes independently of Flt1/Nrp1 expression in epidermal cells (Fig. 4A). Last, 226 of 968 genes [23%, \( P = 10^{-12} \)] up-regulated by Vegfa were no longer up-regulated by Vegfa following Flt1 deletion in K14-Vegfa/Flt1 cKO but were normally up-regulated following Nrp1 deletion, and 117 of 968 genes [12%, \( P = 10^{-11} \)] were no longer up-regulated in the absence of Nrp1 but normally regulated in the absence of Flt1 (Fig. 4A). These data indicate that the vast majority of the genes up-regulated by Vegfa in the epidermis were dependent on Flt1 and Nrp1 expression by keratinocytes.

Gene ontology (GO) of the genes up-regulated by more than twofold by Vegfa overexpression and depending on Flt1 and Nrp1 expression by keratinocytes (382 of 968 genes) showed enrichment for transcripts regulating immunity (\( P = 10^{-8} \)), proliferation (\( P = 10^{-8} \)), and epidermal differentiation (\( P = 10^{-8} \)), as previously reported in human psoriasis and other murine models of psoriasis-like disease (Fig. 4B). We summarized the top up-regulated genes in table S1. Some of these genes were known to be involved in psoriasis, whereas many others have not been previously implicated in psoriasis but regulate cell functions potentially relevant for psoriasis development, such as the regulation of proliferation and epidermal differentiation.

In addition to the up-regulated genes, our analysis also showed that Vegfa overexpression also induced the down-regulation of 1225 genes by more than twofold. The majority of the down-regulated genes [602 of 1225 genes (49%), \( P = 10^{-17} \)] were dependent on the expression of both Nrp1 and Flt1 by keratinocytes, and an additional 227 of 1225 genes (19%, \( P = 10^{-10} \)) of the genes down-regulated by Vegfa were Flt1 dependent but Nrp1 independent (Fig. 4D). Only 8% (\( P = 10^{-7} \)) of the down-regulated genes were blocked by Nrp1 deletion, but not Flt1 deletion (Fig. 4D). In addition, 24% of these genes (296 of 1225, \( P = 10^{-8} \)) were down-regulated by Vegfa independently of the expression of either Nrp1 or Flt1 (Fig. 4D). GO of the genes down-regulated by more than twofold by Vegfa in an Flt1- and Nrp1-dependent manner (602 of 1225 genes) showed enrichment for transcripts inhibiting proliferation (\( P = 10^{-21} \)), regulating the extracellular matrix (ECM) (\( P = 10^{-8} \)) and epidermal differentiation (\( P = 10^{-8} \)) (Fig. 4E). We summarized the top down-regulated genes in table S2.

Together, our data demonstrated that most genes up-regulated and down-regulated by Vegfa overexpression in epidermal cells were dependent on the expression of both Flt1 and Nrp1 in keratinocytes, suggesting that the core of the transcriptional changes associated with psoriasis development induced by Vegfa is dependent on a cell autonomous function of Flt1 and Nrp1 in epidermal cells.

Chromatin remodeling associated with Vegfa/Flt1/Nrp1 signaling in psoriasis

To understand more globally the changes in the chromatin landscape that occur during Vegfa-mediated psoriasis-like disease and identify the transcription factors (TFs) and gene regulatory networks that control the changes in gene expression associated with psoriasis development, we FACS-isolated basal keratinocytes from control, K14-Vegfa, K14-Vegfa/Nrp1 cKO, and K14-Vegfa/Flt1 cKO mice and performed ATAC-seq, a technique that allows the mapping of the open chromatin regions with high definition and predicts the TFs that regulate chromatin remodeling (28). We first defined the chromatin remodeling associated with Vegfa-mediated psoriasis by assessing the chromatin regions (ATAC-seq peaks) that are changed by more than twofold between K14-Vegfa and control mice. We found that 16,863 chromatin regions were more open (\( P = 10^{-11} \)), and 5829 chromatin regions were more closed upon Vegfa overexpression (\( P = 10^{-11} \)). Among the peaks that were up-regulated upon Vegfa overexpression, we found that 9964 peaks (59%, \( P = 10^{-12} \)) were no more up-regulated upon Flt1 or Nrp1 deletion, 2703 peaks (16%, \( P = 10^{-10} \)) were no more up-regulated upon Flt1 deletion only, 2366 peaks (14%, \( P = 10^{-11} \)) were no more up-regulated upon Nrp1 deletion only, and 1830 peaks (11%, \( P = 10^{-7} \)) were unchanged following Nrp1 or Flt1 deletion (Fig. 5A).

Among the peaks that were down-regulated upon Vegfa overexpression, we found that 1723 peaks (30%, \( P = 10^{-12} \)) were no more down-regulated upon Flt1 and Nrp1 deletion, 1444 peaks (25%, \( P = 10^{-12} \)) were no more up-regulated upon Flt1 deletion only, 595 peaks (10%, \( P = 10^{-8} \)) were no more up-regulated upon Nrp1 deletion only, and 2067 peaks (35%, \( P = 10^{-13} \)) were unchanged upon Flt1 or Nrp1 deletion (Fig. 5B). We performed TF binding site motif analysis on the peaks that were up-regulated upon Vegfa overexpression in an Flt1/Nrp1-dependent manner. We found an enrichment in TF motifs corresponding to Gata TF (26%, \( P = 10^{-185} \)), TEAD TF (28%, \( P = 10^{-41} \)), Egr1 (14%, \( P = 10^{-23} \)), AP-1 (32%, \( P = 10^{-26} \)), basic helix-loop-helix (bHLH) TF (38%, \( P = 1 \times 10^{-21} \)), and Slug (14%, \( P = 10^{-11} \)) (Fig. 5C). Among the peaks that were down-regulated upon Vegfa overexpression in an Flt1/Nrp1-dependent manner, we found an enrichment in TF motifs corresponding to Klf TF (33%, \( P = 10^{-19} \)) and homeobox TF (30%, \( P = 10^{-19} \)) (Fig. 5D).

After assigning the genes associated with each of these peaks, we assessed which of these peaks up-regulated by Vegfa were associated with an increase in gene expression by more than twofold (peaks up genes up) (Fig. 5E). We found that 937 genes fell into this category.
Fig. 4. Transcriptional landscape associated with Nrp1/Flt1/Vegfa signaling in psoriasis. (A) Pie chart showing the percentage of total up-regulated genes in K14-Vegfa and depending either on common Nrp1/Flt1 (=Nrp1/Flt1 dependent) or specifically on Nrp1 (=Nrp1 dependent) or Flt1 (Flt1 dependent) expression. Genes with unchanged expression after Nrp1 or Flt1 epidermal ablation were also represented (=Nrp1/Flt1-independent up genes). (B) GO analysis of up-regulated genes in Vegfa overexpression in an Nrp1- and Flt1-dependent manner. (C) mRNA relative expression of up-regulated genes by RNA-seq in Vegfa overexpression in FACS-isolated basal keratinocytes (n = 2) (means ± SEM). (D) Pie chart showing the percentage of total down-regulated genes in K14-Vegfa and depending either on common Nrp1/Flt1 expression (=Nrp1/Flt1 dependent) or specifically on Nrp1 (=Nrp1 dependent) or Flt1 (Flt1 dependent) expression. Genes with unchanged expression after Nrp1 or Flt1 epidermal ablation were also represented (=Nrp1/Flt1-independent down genes). (E) GO analysis of down-regulated genes in Vegfa overexpression in an Nrp1- and Flt1-dependent manner. (F) mRNA relative expression of down-regulated genes by RNA-seq in Vegfa-overexpressing mice and depending on Nrp1 or Flt1 expression in FACS-isolated keratinocytes (n = 2) (means ± SEM).
Fig. 5. Chromatin landscape associated with Nrp1/Flt1/Vegfa signaling in psoriasis. (A) Percentage of total up-regulated peaks in K14-Vegfa and depending either on Nrp1/Flt1 (−Nrp1/Flt1 dependent) or specifically on Nrp1 (−Nrp1 dependent) or Flt1 (Flt1 dependent) or independent of Nrp1 or Flt1 expression (−Nrp1/Flt1 independent). (B) Percentage of down-regulated peaks and their distribution in the different categories. (C) Enriched TF motifs found in the peaks that were up- or down-regulated in an Nrp1/Flt1-dependent manner. P value of enrichment of the motif in peaks compared with background and percentage of peaks containing the motif. (D) Percentage of total up-regulated peaks in K14-Vegfa overlapping with up-regulated genes and depending either on Nrp1/Flt1 expression (−Nrp1/Flt1 dependent) or specifically on Nrp1 (−Nrp1 dependent) or Flt1 (Flt1 dependent) or independent of Nrp1 or Flt1 expression (−Nrp1/Flt1 independent). (E) mRNA expression of up-regulated genes by RNA-seq in FACS-isolated basal keratinocytes (n = 2) (means ± SEM). (G) Enriched transcription TF motifs in the peaks up-regulated in an Nrp1/Flt1-dependent manner. (H) Percentage of total down-regulated genes and their distribution in the different categories. (I) mRNA expression of down-regulated genes by RNA-seq in FACS-isolated basal keratinocytes (n = 2) (means ± SEM). (J) Enriched TF motifs in the peaks down-regulated in an Nrp1/Flt1-dependent manner.
We then assessed which of these genes still present chromatin remodeling following Nrp1 or Flt1 deletion. The great majority of these genes (70%, 660 of 937, \(P = 10^{-8}\)) did not present significant change at these chromatin regions in the absence of Flt1 and Nrp1, and only 5% (\(P = 10^{-5}\)) of these genes (48/937) presented chromatin remodeling following Vegfa overexpression in the absence of Flt1 and Nrp1 (Fig. 5E), showing the key role of Flt1 and Nrp1 in epidermal cells to mediate the chromatin remodeling induced by Vegfa overexpression. GO analysis of the peaks up-regulated/genes up-regulated that are Nrp1/Flt1 dependent revealed that these genes were associated with the induction of initial phases of immune responses and type I interferon–induced genes (\(P = 10^{-12}\)) (Fig. 5F and table S3).

To get further insights into the gene regulatory network that controls psoriasis, we performed motif enrichment analysis of the chromatin regions that became more opened during Vegfa-mediated psoriasis development. Among the genes for which the ATAC-seq peaks in their regulatory regions were opened and accompanied by an increase in gene expression in an Flt1/Nrp1-dependent manner, we found that the most enriched TF motifs in these peaks were AP-1 TFs (32% of the peaks, \(P = 10^{-23}\)), NRF1 (18% of the peaks, \(P = 10^{-13}\)), GATA TFs (34% of the peaks, \(P = 10^{-11}\)), P63 (18% of the peaks, \(P = 10^{-11}\)), CCCTC-binding factor (CTCF) (9% of the peaks, \(P = 10^{-11}\)), TEAD (35%, \(P = 10^{-10}\)), bHLH TFs (23%, \(P = 10^{-10}\)), consistent with the motifs found on all peak (Fig. 5G).

We then assessed which of the down-regulated peaks in K14-Vegfa epidermis were associated with down-regulation of gene expression by more than twofold (peaks down/genes down) (Fig. 5, H and I). Among these 467 genes, the vast majority of them were no longer down-regulated following Flt1 or Nrp1 cKO (60%, 279 of 467 for both cKO, \(P = 10^{-8}\)), 26% (123 of 467, \(P = 10^{-7}\)) following Flt1 deletion, and 4% (\(P = 10^{-7}\)) following Nrp1 deletion, whereas only 10% of these genes presented chromatin remodeling upon Vegfa expression independently of Nrp1 and Flt1 (43 of 490, \(P = 10^{-6}\)) (Fig. 5H).

Motif discovery analysis in the down-regulated peaks associated with down-regulation of gene expression in an Flt1/Nrp1-dependent manner revealed that Klf TFs (26% of the peaks, \(P = 10^{-20}\)), AP-1 TFs (33% of the peaks, \(P = 10^{-14}\)), and p63 (23% of the peaks, \(P = 10 \times 10^{-13}\)) were the most statistically significant TF motifs enriched in these down-regulated peaks (Fig. 5I).

Together, these data demonstrate that most of the chromatin remodeling associated with gene activation or repression induced by Vegfa is mediated by the cell autonomous function of Flt1 and Nrp1 in epidermal cells, and identify the TFs associated with the chromatin remodeling–mediated Vegfa in an Flt1- and Nrp1-dependent manner.

**Fosl1 acts downstream of Vegfa/Flt1 signaling in promoting psoriasis**

AP-1 binding sites were among the most significantly enriched TF motifs found in the chromatin regions of genes that were up-regulated in an Flt1- and Nrp1-dependent manner during Vegfa-induced psoriasis-like disease. AP-1 represents a family of TFs composed of Jun and Fos that function as homo- or heterodimer, and epidermal deletion of c-Jun/JunB leads to a psoriasis-like disease in mice (16).

Many of the chromatin regions of these Vegfa-regulated genes presenting AP-1 binding sites were no longer remodeled when Flt1 or Nrp1 was deleted from the epidermis (32%, 211 of 660 of the genes up-regulated; 33%, 92 of 279 of the genes down-regulated), consistent with a key role for Flt1/Nrp1 in regulating the chromatin remodeling associated with AP-1 TFs. GO analysis performed on genes up-regulated/peaks up-regulated and containing AP-1 motifs showed enrichment in immunity-related genes (e.g., Tlr4, Art3, Ikzf2, Alox5ap, and Nlrc5) (fig. S5A). GO analysis performed on genes down-regulated/peaks down-regulated and containing AP-1 motifs showed enrichment in ECM-related genes (e.g., Tgfb2, Mmp13, Angiopo17, Ism1, and Tnfrsf19) (fig. S5B).

These genes were similarly up-regulated or down-regulated by Vegfa signaling in in vitro–cultured keratinocytes and in the skin epidermis in vivo (fig. S5, C to F), further supporting the importance of a cell autonomous Vegfa/Flt1/AP-1 signaling in keratinocytes that mediates psoriasis. We have confirmed the up-regulation of Toll-like receptor 4 (TLR4) and down-regulation of Ism1 at the protein level by immunofluorescence on tail skin sections (fig. S5G). To determine which AP-1 TFs relay Vegfa signaling to induce the chromatin remodeling and change in gene expression associated with psoriasis, we assessed which members of the AP-1 family are up- or down-regulated following Vegfa overexpression. We found that among AP-1 TFs, only Fosl1 was up-regulated by Vegfa overexpression (Fig. 6A). Fosl1 overexpression was no longer up-regulated following Flt1 deletion in K14-Vegfa/Flt1 cKO mice, showing the importance of cell autonomous Vegfa/Flt1 signaling in promoting Fosl1 overexpression. In addition, we also demonstrated the over-expression of Fosl1 in epidermal cells by immunohistochemistry on skin section in the K14-Vegfa and c-Jun/JunB cKO psoriasis models (fig. S6, B and C).

To directly and functionally assess the importance of Fosl1 in the cell autonomous Vegfa/Flt1 signaling that promotes psoriasis development, we performed short hairpin RNA (shRNA) knockdown of Fosl1 in mouse cultured keratinocytes in vitro and assessed the impact of Fosl1 down-regulation on chromatin remodeling and change in gene expression mediated by Vegfa signaling. Primary culture of keratinocytes from epidermis was infected with lentiviruses expressing shRNA against Fosl1, stimulated or not with Vegfa (50 ng/ml) (Fig. 6, D to F) and then assessed for change in gene expression and chromatin accessibility using real-time ATAC-PCR and qRT-PCR (Fig. 6, G to J). It is important to note that the addition of Vegfa to primary cultured keratinocytes is important, as the level of Vegfa by the Rosa promoter may not be strong enough to promote angiogenesis in the absence of secretion of other angiocrine factors by the keratinocytes in response to autocrine Vegfa signaling in those cells. In addition, Vegfa induced the phosphorylation of Flt1 in 293T cells transfected with Flt1-Flag expression plasmids and in keratinocytes in vitro (fig. S6, A to C), similarly to what has been reported in tumor cells (15, 29). Western blot analysis showed that Fosl1 shRNA down-regulated Fosl1 efficiently (Fig. 6, D and F). ATAC-PCR showed that Fosl1 knockdown blocked the chromatin remodeling associated with Vegfa/Flt1/AP-1 signaling in keratinocytes in vitro (Fig. 6, G and H), resulting in the absence of up- or down-regulation of these genes upon Vegfa signaling in vitro (Fig. 6, I and J). Together these results demonstrate the key role of Fosl1 in mediating chromatin remodeling and changes in gene expression associated with Vegfa/Flt1/AP-1 signaling during psoriasis development.

**DISCUSSION**

Our study uncovers the essential epidermal autonomous functions of Flt1 and Nrp1 in promoting Vegfa-induced psoriasis-like disease (fig. S7). Several case studies have previously reported the improvement of psoriatic disease in patients with cancer treated with anti-VEGFA.
Fig. 6. Fosl1 acts downstream of Vegfa/Flt1 signaling in the regulation of chromatin remodeling and transcriptional change associated with psoriasis. (A) mRNA expression of AP-1 members by RNA-seq in FACS-isolated basal keratinocytes (n = 2) (means ± SEM). (B) IHC (immunohistochemistry) of Fosl1 nuclear staining in tail epidermis. Scale bars, 10 μm. (C) IHC of Fosl1 in ear epidermis. Scale bars, 10 μm. (D) Fosl1 expression assessed by Western blot on primary cultured keratinocytes from K14-Vegfa after transduction with control (sh Ctrl) or Fosl1-specific shRNA (sh Fosl1). (E) Protein expression of Fosl1 (n = 3). Histogram represents means ± SEM. (F) Vegfa and Fosl1 mRNA expression measured par qRT-PCR on primary cultured keratinocytes from K14-Vegfa transduced with sh Ctrl or sh Fosl1 (n = 3) (means ± SEM, Mann-Whitney). (G) Relative chromatin accessibility measured by ATAC qPCR of chromatin regions presenting AP-1 binding sites in the regulatory regions of up- or down-regulated genes in primary cultured keratinocytes from K14-Vegfa transduced with sh Ctrl or sh Fosl1 (n = 3) (means ± SEM, Mann-Whitney). (H) mRNA expression by qRT-PCR of up- or down-regulated genes (J) presenting AP-1 binding sites in their regulatory regions in primary cultured keratinocytes from K14-Vegfa transduced with sh Ctrl or sh Fosl1 (n = 3) (means ± SEM, Mann-Whitney test).
antibodies or anti-VEGFA receptor small-molecule inhibitors (30, 31). Likewise, the use of anti-VEGFA inhibitors in different mouse models of psoriasis (K14-Vegfa, epidermal deletion of c-Jun/JunB) induced regression of the psoriasis phenotypes including epidermal hyperproliferation, thickness, altered differentiation, dermal immune infiltration, and increased angiogenesis (7, 8, 32, 33). In these studies, it was thought that anti-VEGFA therapy acts by targeting ECs and immune cells located in the dermis. In sharp contrast, our study shows that most of the psoriasis phenotypes mediated by Vegfa on keratinocytes, immune cells, and ECs are the consequences of Vegfa signaling in keratinocytes in an Flt1/Nrp1-dependent manner. The cell autonomous role of Flt1 and Nrp1 in Vegfa-induced psoriasis is reminiscent of the autocrine or paracrine role of Vegfa in promoting skin tumor progression and cancer stem cell function (14, 15).

The decrease in neoangiogenesis and immune cell infiltration in the absence of Nrp1 and Flt1 expression in the epidermis suggests that the keratinocytes are essential to orchestrate the vascular remodeling and immune cell infiltration. RNA-seq showed that upon Vegfa signaling, keratinocytes expressed chemotactants for immune cells (e.g., S100A8/A9) and proangiogenic molecules (e.g., Adm), which together with Vegfa regulate the neoangiogenesis and immune infiltration associated with the psoriasis phenotype.

By performing RNA-seq and ATAC-seq of FACS-isolated basal keratinocytes in the presence of Vegfa overexpression and in the presence or absence of Nrp1 or Flt1 expression, we defined the changes in the chromatin and transcriptional landscape associated with the cell autonomous signaling of Vegfa in mediating psoriatic-like disease. Our bioinformatics analysis of these molecular changes uncovers Fos1 in regulating gene expression mediated by Vegfa/Flt1 signaling in keratinocytes. Moreover, the Vegfa/Flt1/Nrp1 keratinocyte cell autonomous epigenetic and transcriptional signatures uncovered here may represent novel biomarkers to predict the response of antipsoriatic treatments. The rapid improvement of psoriatic-like disease following the administration of anti-Nrp1 blocking antibodies that specifically target the interaction between Nrp1 and Vegfa demonstrates the therapeutic potential of blocking the Nrp1/Vegfa interaction in psoriasis.

In conclusion, our study demonstrates a keratinocyte autonomous function of Nrp1 and Flt1 in mediating Vegfa signaling in the epidermis and the development of psoriatic-like disease in mice. These results have important implications for our understanding of the pathogenesis of psoriasis and open new avenues for psoriasis treatment.

MATERIAL AND METHODS

Study design

The study was conducted on mouse models as described in the “Experimental models” section to perform in vivo and in vitro laboratory experiments. The sample size was not predetermined. We used a sample size allowing statistical significance to be reached between the different groups. No animals were excluded from the analysis. No technical replicates were used to calculate statistics. No randomization and no blinding were used in this study. For each experiment, we used biological-independent replicates per condition, and the number of replicate has been reported in the legends.

Experimental models

K14-Cre (34) mice were mated with Rosa26-VEGF-164 (35), Nrp1fl/fl (36), and Flt1fl/fl (37) mice. All mice used in this study were composed of males and females with mixed genetic background. Mouse colonies were maintained in a certified animal facility in accordance with the European guidelines and with approved ethical protocol (no. 526N).

Primary cell culture

Tail skin was removed from the tail bone and incubated overnight at 4°C in Hanks’ balanced salt solution (HBSS) (Gibco) and 0.25% trypsin (Gibco). The epidermis was then separated from the dermis and incubated on a rocking plate (100 rpm) at room temperature for 5 min. Basal cells were mechanically separated from the epidermis by flushing 10 times under the epidermis. Trypsin was then neutralized by adding Dulbecco’s modified Eagle’s medium. (DMEM) (Gibco) supplemented with 5% Chelex fetal calf serum (FCS) and filtered on a 70-μm filter (Falcon). Cells were cultured in MEM supplemented with 10% FBS, hydrocortisone (0.4 μg/ml), epidermal growth factor (10 ng/ml), 2 × 10−7 MT3, 1% penicillin/streptomycin, 2 mM l-glutamine and incubated at 37°C with 20% O2 and 5% CO2. Twenty-four hours later, the cells were treated with recombinant vascular endothelial growth factor (VEGF) (50 ng/ml; R&D).

Viruses production, infection, and selection

Stable knockdown cell lines were generated using lentiviral pLKO/ PuroR vectors (Sigma-Aldrich) after puromycin selection (2.5 μg/ml). Knockdown was confirmed by qRT-PCR and Western blot. Three different shRNAs (NM_010235.1-664s1c1, NM_010235.1-1050s1c1, and NM_010235.1-851s1c1) were used to target the same gene.

For virus production, 5 × 106 human embryonic kidney (HEK) 293T cells were seeded into 10-cm dishes and transfected with the vector of interest and appropriate packaging plasmids psPax2 and pMD2.G (12260 and 12259, respectively; Addgene). Medium was changed 24 hours after transfection, and supernatants were collected at 48 hours and passed through a 0.45-μm filter. Keratinocytes were plated in six-well plate cells and incubated with viruses (40 μl/ml) when they reach 50% of confluence in the presence of polybrene (5 μg/ml). Medium was changed 24 hours later, and infected cells were selected by puromycin (5 μg/ml) for at least 1 week.

Antibodies

The following primary antibodies were used: anti-Ki67 (rabbit, 1:200, Abcam, catalog number ab15580), anti-Ki67 (rabbit, 1:1,000, Thermo Fisher Scientific, catalog number PA5-28002), anti-Nrp1 (goat, 1:100, R&D, catalog number AF 566), anti-CD31 (rat, 1:200, BD Biosciences, Clone MEC13.3, catalog number 550274), anti-CD45 (rat, 1:500, BD Biosciences, clone 30F11, catalog number 12-0451/55081), anti-Fos1 (mouse, 1:500, Santa Cruz, clone C20, catalog number sc-28310), CD4 (rat, 1:100, BD Biosciences, clone RM4-5, catalog number 565650), CD8 (rabbit, 1:100, Abcam, catalog number ab203035), CD19 (rat, 1:100, BD Biosciences, clone 1D3, catalog number 561737), Ly6G (rat, 1:100, BioLegend, clone 1A8, catalog number 177603), F4/80 (rat, 1:500, Serotec, clone A3-1, catalog number MCA497 GA), TLR4 (rabbit, 1:100, Abcam, ab22048), and ISM1 (rabbit, 1:100, Abcam ab103338). The following secondary antibodies were used: anti-rabbit, anti-rat, and anti-chicken conjugated to Alexa Fluor 488 (1:400, Molecular Probes), to Rhodamine Red-X, or to Cy5 (1:400, Jackson ImmunoResearch).

Western blot analysis

Keratinocytes were lysed in radioimmunoprecipitation assay (RIPA) buffer for 4 hours on ice and then centrifuged for 10 min at 14,000 rpm.
at 4°C. Forty micrograms of cell lysate was loaded in 4/12% bis/tris-acrylamide gel (Invitrogen) and separated by electrophoresis. Proteins were transferred on polyvinylidene difluoride (PVDF) membranes. The membranes were incubated overnight with anti-Nrp1 (goat, 1:200, R&D, catalog number AF 566) or anti-Flt1 (mouse, 1:1000, Santa Cruz, clone H-225, catalog number sc-9029) or anti-Fosl1 (mouse, 1:1000, Santa Cruz, clone C12, catalog number sc-28310) or Flt1 (rabbit, 1:1000, Abcam, ab3252) or anti–phospho-Flt1 Y1213 (rabbit, 1:1000, R&D, catalog number AF4170), (mouse, 1:1,000, Cell Signaling, catalog number 9106), anti–HA (human influenza hemagglutinin tag) (mouse, 1:1000, Sigma–Aldrich, catalog number: 11583816001), and anti–β-actin (1:3000, Abcam, catalog number ab8227). Anti-mouse or anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) (1:3000 or 1:10,000; Healthcare) was used as the secondary antibody.

**Immunoprecipitation assay**

hFlt1-HEK293 lysates were immunoprecipitated by incubation with anti-HA (1 µg of antibody per 1.5 mg of protein, rabbit, Abcam, ab9110) coupled to Protein G Dynabeads (Invitrogen, catalog number 10003D) overnight at 4°C. Proteins were eluted in sample buffer by heating to 70°C for 10 min. For Western blot analysis, equal amounts of total protein were loaded and resolved on a NuPAGE 4/12% bis/tris gel (Invitrogen) and transferred to a PVDF membrane. Blots were probed with specific antibodies for HA-tag (mouse, 1:1000, Sigma, catalog number 11583816001), Flt1 (rabbit, 1:1000, Abcam, ab3252), and anti–PF1 (rabbit, 1:1,000, R&D, Y1213). Enhanced chemiluminescence anti-mouse or anti-rabbit IgG conjugated with HRP (1:3000 or 1:10,000; Healthcare) was used as the secondary antibody.

**Phosphorylation assay for Flt1**

Cells were serum starved for 12 hours and stimulated either by rVEGFA (50 ng/ml; R&D, catalog number 293 CF) or by hPLGF (human placental growth factor) (50 ng/ml; R&D, catalog number 264-PGB). We used as control condition nonstimulated transfected hFlt1–HEK293 cells as previously described (13).

**Nrp1 antibody treatment**

Mice were treated by Nrp1-blocking antibodies: anti-Nrp1a, which blocks binding of semaphorins, and anti-Nrp1b, which blocks binding of Vegfa (Genentech, 10 mg/kg) by intraperitoneal injection, two times per week during 15 days as described previously for tumor models (14, 26). The antibodies were generated and provided by Genentech.

**Histology and immunostaining**

Skin was embedded in OCT (optimal cutting temperature) (Tissue-Tek). Samples were sectioned at 4- to 6-µm sections using CM3050S cryostat (Leica Microsystems GmbH). For the staining on frozen sections, tissues were fixed in 4% paraformaldehyde for 10 min at room temperature, and then washed in phosphate-buffered saline (PBS). Non-specific antibody binding was blocked with 5% horse serum, 1% bovine serum albumin, and 0.2% Triton X-100 during 1 hour at room temperature. Primary antibodies were incubated overnight at 4°C in blocking buffer. Sections were rinsed in PBS and incubated with secondary antibodies during 1 hour at room temperature. Nuclei were stained with Hoechst (4 µM). Slides were mounted using Glycergel (Dako) supplemented with 2.5% DABCO (Sigma–Aldrich). For the staining on paraffin sections, 4-µm paraffin sections were deparaffinized and rehydrated. Antigen unmasking was performed in citrate buffer (pH 6) at 98°C for 20 min using the PT (pre-treatment) module. Endogenous peroxidase was blocked using 3% H2O2 (Merck) in methanol for 20 min at room temperature. Endogenous avidin and biotin were blocked using the Endogenous Blocking kit (Invitrogen) for 20 min at room temperature. Primary antibodies were incubated overnight at 4°C. Anti-mouse biotinylated secondary antibodies, as well as Standard ABC kit, and ImmPACT DAB (Vector Laboratories) were used for the detection of HRP activity. Slides were mounted using SafeMount (Labonorad).

**Image acquisition**

Microscopic imaging was performed on a Zeiss Axio Imager M1 (Thornwood) fluorescence microscope with a Zeiss Axiocam MR3 camera and a Zeiss Axiocam MRC5 camera for bright-field microscopy using the Axiovision release 4.6 software. Brightness, contrast, and picture size were adjusted using Photoshop CS6 (Adobe). Macroscopic imaging were performed using a Leica DFC 420C camera with an objective Leica 10446261 0.63×.

**FACS isolation of epithelial cells for RNA sequencing**

Tail skin was removed from the tail bone and incubated overnight at 4°C in HBSS (Gibco) and 0.25% trypsin (Gibco). The epidermis was then separated from the dermis and incubated on a rocking plate (100 rpm) at room temperature for 5 min. Basal cells were mechanically separated from the epidermis by flushing 10 times under the epidermis. Tissues were then cut into small pieces with a scalpel and incubated again for 5 min on a rocking plate (100 rpm) at room temperature. Trypsin was then neutralized by adding DMEM (Gibco) supplemented with 5% Chelex-treated FCS, and the cells were mechanically separated by pipetting 10 times and filtered on a 70-µm filter (Falcon). Cells were incubated in 2% FCS/PBS with primary antibodies for 30 min on ice. Cells were washed with 10 ml of 2% FCS/PBS and then incubated for 30 min in APC (allophycocyanin)–conjugated streptavidin (BD Biosciences), and then washed again and resuspended in 200 µl of 2% FCS/PBS with Hoechst (10 mg/ml) diluted at 1:4000. Living epidermal cells were gated by forward scatter, side scatter, and negative for Hoechst. Basal interfollicular epidermis keratinocytes were stained using fluorescein isothiocyanate–conjugated anti–α6 integrin (clone GoH3, 1:200, eBioscience, catalog number MAB1378) and biotinylated CD34 (clone RAM34, 1:50, BD Biosciences, catalog number 13-0341), followed by avidin/APC-streptavidin used to stain and exclude hair follicle stem cells. Basal keratinocytes were isolated on the basis of α6 integrin expression with exclusion of CD34-positive cells. In Vegfa overexpression conditions, basal keratinocytes expressing the transgene are GFP positive, and we labeled basal keratinocytes using PE-conjugated anti–α6 integrin (clone GoH3, 1:200, eBioscience, catalog number MAB1378), and buge cells were labeled with biotinylated CD34 (clone RAM34, 1:50, BD Biosciences). We used PE-conjugated anti–CD45 (clone 30–f11, 1:200, eBioscience, catalog number 12–0451/553081), PE-conjugated anti–CD31 (clone MEC13.3, 1:100, BD PharMingen, catalog number 550274), and PE-conjugated anti–Pdgfra (clone APA–5, 1:200 eBioscience, catalog number 12–1401/624049) to exclude dermal cells and avoid dermal contamination, and anti–mouse/human CD11b (clone M1/70, BioLegend), anti-mouse CD16/32 (clone 93, BioLegend), anti-mouse Ly6C (HK1.4, BioLegend), and anti-mouse Ly6G (1A8, BioLegend) were used to gate for neutrophils, macrophages, and monocytes. FACS analysis was performed using FACSAria I at high pressure (70 psi) and FACSDiva software (BD Biosciences). Sorted cells were collected into lysis buffer for RNA extraction.
RNA extraction and real-time RT-PCR

RNA extraction from FACS-isolated cells was performed using the RNAeasy Micro Kit (QIAGEN) according to the manufacturer’s recommendations with DNase (deoxyribonuclease) treatment. After NanoDrop RNA quantification, the first-strand cDNA was synthesized using Superscript II (Invitrogen) and random hexamer (Roche) in 50 μl of final volume. Control of genomic contamination was measured for each sample by performing the same procedure with or without reverse transcriptase. qPCR assays were performed using 1 ng of cDNA as template, SYBR Green Mix (Applied Bioscience) and a LightCycler 96 (Roche) real-time PCR system. β-Actin housekeeping gene was used for normalization. Primers were designed using the Roche Universal Probe library assay design center: https://lifescience.roche.com/webapp/wcs/stores/servlet/CategoryDisplay?tab=Assay+Design+Center&identifier=Universal+Probe+Library&langId=-1. qPCR analysis was performed using LightCycler 96 (Roche) real-time PCR system and the DDCT (Delta Delta CT) method with β-actin as a reference.

List of primers

Primers used for RT-qPCR are listed in Table 1.

ATAC-seq and library preparation

ATAC followed by sequencing was performed as follows: 100,000 sorted cells were collected in 1 ml of PBS and 3% FBS at 4°C. Cells were centrifuged, and then cell pellets were resuspended in 100 μl of lysis buffer (10 mM tris-HCl, 10 mM NaCl, 3 mM MgCl2, and 1% Triton X-100) and centrifuged (5000 g) for 25 min at 4°C. Supernatant was discarded, and nuclei were resuspended in 50 μl of reaction buffer (2.5 μl of Tn5 transposase, 22.5 μl of tagmentation DNA buffer, and 25 μl of H2O-Nextera DNA Sample Preparation Kit, Illumina). The reaction was performed for 30 min at 37°C and then blocked by the addition of 5 μl of clean-up buffer (900 mM NaCl and 300 mM EDTA). DNA was purified using the minElute purification kit (QIAGEN) following the manufacturer’s protocol. DNA libraries were PCR amplified (Nextera DNA Sample Preparation Kit, Illumina) and size selected from 200 to 800 base pairs (bp) (BluePippin, Sage Sciences) following the manufacturer’s recommendations.

Samples for the ATAC-qPCR experiment were processed in the same manner, and primers were designed around the control region and peak region. Primers are listed in Table 2.

ATAC-seq analysis

Two samples of control, K14-Vegfa and K14-Vegfa/Nrp1 cKO, and one sample of K14-Vegfa/Flt1 cKO were sequenced. ATAC-seq paired-end reads of 50 bp were trimmed for adaptor sequences using Trimomatic. ATAC-seq paired-end reads were then aligned to the mouse GRCm38 genome using Bowtie2 (version 2.2.6) using options “-X 2000 --fr --very-sensitive --no-discordant --no-unal --non-mixed --non-deterministic.” More than 18 million reads were mapped to mouse genomic DNA in each condition (between 18 and 86 millions). Mitochondrial reads and reads aligned to scaffolds and undefined chromosomes were excluded from downstream analysis.

With a mapping quality lower than 20 were eliminated with samtools, and duplicated reads were removed by Picard tools (http://broadinstitute.github.io/picard/). Read start sites were adjusted to represent the center of the transposon binding event as described in (38). Peak calling was performed on each individual sample using Macs2 (version 2.1.0.20151222) with parameter setting of “callpeak -f BAMPE -g mm -q 0.05 --nomodel --call-summits -B -SPMR.” Peaks from all ATAC-seq samples were merged for downstream analysis using bedtools. Peaks were designed around the control region and primers were designed around the control region. Primers were designed using 1 ng of cDNA as template, SYBR Green Mix (Applied Bioscience) and a LightCycler 96 (Roche) real-time PCR system. β-Actin housekeeping gene was used for normalization. Primers were designed using the Roche Universal Probe library assay design center: https://lifescience.roche.com/webapp/wcs/stores/servlet/CategoryDisplay?tab=Assay+Design+Center&identifier=Universal+Probe+Library&langId=-1. qPCR analysis was performed using LightCycler 96 (Roche) real-time PCR system and the DDCT (Delta Delta CT) method with β-actin as a reference.

| Gene name | Forward (5′-3′) | Reverse (5′-3′) |
|------------|----------------|----------------|
| Nrp1       | CATCTCCGGTTACCTCATTTTC | GCGGCCGCTTCTACATCC |
| Vegfa      | TGGGCTCTTCTCGTCCGAGTAG | GCGGCCCTACCAGGCTCCAT |
| Fosl1      | CAAGTGTGTAGCTACCAGAAGA | CACAAAAGTGGAGCTCTGG |
| Flt1       | AAAGGCTGACATCATCCTCCC | CTACAGGGTAGAGGCGG |
| Flk1       | TGGTCTCTGTGTCGTCGGGG | CTTCAAAACCTGAGCTTCC |
| Art3       | CGGGACGCAGTTCTAAAGGG | CAGGATGTGGTCGCTAACAGC |
| Nlrc5      | AACAAATGCTGTCGCTCCCT | CGGCTTGCTCAAGAGG |
| Alox5ap    | CTAACCTTGGCTGCTGCTATCC | ATCCCTAGCCTTCTGGG |
| Ifk2       | GACGCGTGAAGTAGAGATCAG | CTCCTGCGCTGCTGAGT |
| Tlr4       | TCGACATGGAATCATTTATCGG | CCTGCTGCTGTTGCTTTAG |
| Tnfrsf19   | TCTGTGAGGGGAGACGATG | AGAATTTAGGCGAGATGG |
| B actin    | CAGGCTCTCTTTCTCCGGAATTTA | TGGTGCGATTAGAGCTTTCC |

Gene name Forward (5′-3′) Reverse (5′-3′)
Nrp1 CACTCTCCGGTTACCTCATTTTC GCGGCCGCTTCTACATCC
Vegfa TGGGCTCTTCTCGTCCGAGTAG GCGGCCCTACCAGGCTCCAT
Fosl1 CAAGTGTGTAGCTACCAGAAGA CACAAAAGTGGAGCTCTGG
Flt1 AAAGGCTGACATCATCCTCCC CTACAGGGTAGAGGCGG
Flk1 TGGTCTCTGTGTCGTCGGGG CTTCAAAACCTGAGCTTCC
Art3 CGGGACGCAGTTCTAAAGGG CAGGATGTGGTCGCTAACAGC
Nlrc5 AACAAATGCTGTCGCTCCCT CGGCTTGCTCAAGAGG
Alox5ap CTAACCTTGGCTGCTGCTATCC ATCCCTAGCCTTCTGGG
Ifk2 GACGCGTGAAGTAGAGATCAG CTCCTGCGCTGCTGAGT
Tlr4 TCGACATGGAATCATTTATCGG CCTGCTGCTGTTGCTTTAG
Tnfrsf19 TCTGTGAGGGGAGACGATG AGAATTTAGGCGAGATGG
B actin CAGGCTCTCTTTCTCCGGAATTTA TGGTGCGATTAGAGCTTTCC
pairwise comparisons on the means of read counts (for control, K14-Vegfa, and K14-Vegfa/Nrp1 cKO) or on the read count (for K14-Vegfa/Flt1 cKO). Peaks up-regulated were defined as those having at least a twofold change, and merged peaks nonintersecting a peak called with a q value of 0.05 in the up-regulated condition were removed. Peaks were associated to genes with the GREAT software with the following association rules: “basal plus extension” with parameters 5.0 kb in proximal upstream, 1.0 kb in proximal downstream, and 100.0 kb in distal.

Table 2. List of ATAC qPCR primers.

| Control region | Forward (5′-3′) | Reverse (5′-3′) |
|----------------|-----------------|-----------------|
| **Nlrc5**      | AAGGAACGGGAGCACAGCC   | AAAGGAGCAAGACCAACCTTG   |
| **Alox5ap**    | GTACGGGCGAGGGTTTCA   | AATGCTTCTGTGCGCCCTTG   |
| **Ikkδ**       | CTCGACCTCACACCTCTCTG | CTTCACAGTGACCTTTCTG   |
| **Tlr4**       | TCTCTGGGAACACTTGTTGGAAT | GCTGAGAAGACGATTCTGCTT   |
| **Ism1**       | GTACAACGGCTGGGAGAGTTC | ATCTGAGGCCCAACTGGAATGA |
| **Mmp13**      | AAGCTGTCAGCTCTTGTTTTT | GTAGGGGAGAACAGAAGGGA   |
| **Angpt17**    | AGGAGGCTAGTCCGATAGGGG | AAACCGAGTTACAGGCGA   |
| **Tgfb2**      | GCAACTGCACTTTTTCGGGCC | GGATGGGACAGCCATCTCTT |
| **Tnfrsf19**   | CCAATCTTCAAGGGCAAGGTTT | AGAATCTGTCGACACCCACT |

| Peak region | Forward (5′-3′) | Reverse (5′-3′) |
|-------------|-----------------|-----------------|
| **Art3**    | TCCCAGGGAAACCTCAAAGG | GCCTTCACCTTCAGGCT |
| **Nlrc5**   | GGTGGGAGGGGGGTATATGTGAATTATCTAAGGACATGGGCG | ATGTAATTACTAAGGACATGGGCG |
| **Alox5ap** | TAGACTCAGGCCAAACCCCT | GCCGACTGTCATCACAGGCT |
| **Ikkδ**    | TCTGACGTACGGCCATCCCA | CAGGACCTGGCTTTGAGGCTT |
| **Tlr4**    | ACTGACCTAGCTCTCTCTTG | AATGCTGACCTCTCCCAAC |
| **Ism1**    | TTCCTGACTAGCCCCATCCA | CAGGACCTGGTCTTCTGCGC |
| **Mmp13**   | GAGGAGGCTGTGGGTATATGTGAATTATCTAAGGACATGGGCG | ATGTAATTACTAAGGACATGGGCG |
| **Angpt17** | GGAGGCGCTTCAATTTTTCGG | CCATTGATGGGATTTGTTTGT |
| **Tgfb2**   | AGAATCTGTCGACACCTCCTC | TGGATGCAATGAGATGGGCC |
| **Tnfrsf19**| AAACGAATGTTTGCTGACAG | TGTCGGCAATGAGATGGGCC |

Table 3. Statistical analysis performed for each experiment.

| Figures | Normal distribution | Equality of variances | Comparison tests |
|---------|---------------------|-----------------------|------------------|
|         | Yes | No | Yes | No | Student’s t test | Mann-Whitney |
| 1 (B and D) | ✓ | ✓ | | ✓ | |
| 1 (G, I, K, and M) | ✓ | ✓ | ✓ | ✓ | |
| 2 (B and D) | ✓ | ✓ | | ✓ | |
| 2 (G, I, K, and M) | ✓ | ✓ | ✓ | ✓ | |
| 3 (E, I, J, and K) | ✓ | ✓ | | ✓ | |
| 6 (F to K) | ✓ | ✓ | | ✓ | |
| S1 (D, F, H, and J) | ✓ | ✓ | ✓ | ✓ |
| S1K | ✓ | | | |
| S2B | ✓ | | | |
| S2D | ✓ | | | |
| S3 (F to H) | ✓ | | | |
| S4 (D to F and I) | ✓ | ✓ | | ✓ | |
| S5 (C to F) | ✓ | | | |
| S6C | ✓ | | | |

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Motif analysis

De novo motif search was performed using the findMotifsGenome.pl program in the HOMER package with parameter setting of “-size -250,250 -S 15 -len 6,8,10,12,16.” Incidences of specific motif were examined by the program of annotatePeaks.pl in the HOMER package with default parameters.

GO analysis

Genes up- or down-regulated between were tested for enrichment in each GO class using the DAVID web server. Statistically significant enrichments correspond to those presenting a Benjamini-corrected P value less than or equal to 0.05.

Quantification and statistical analysis

All the statistical analyses were based on biological replicates (n is indicated in the text, figures, or figure legends). The statistical tests used for each experiment have been added to the corresponding figure legends. Statistical analysis was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software). Before performing the statistical analysis, we checked the distribution of the data by performing the Shapiro–Wilkinson test, and we checked the equality of variances between two comparisons by performing the Fisher-Snedecor test. Comparisons between two groups were carried out using an unpaired two-tailed Student’s t test. Comparisons between two groups were carried out using the Welsh test if the data were normally distributed and if the variances were equal between the two comparisons. If the data were not normally distributed, Mann-Whitney test was performed. Data are presented as the arithmetic means ± SEM. The normality of the distribution of the data and the equality of the variances between the groups were verified. Based on this, the means of the groups were compared using the most appropriate comparison tests as summarized in Table 3.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/2/eaax5849/DC1.

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