Chemogenetic modulation of sensory neurons reveals their regulating role in melanoma progression

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Abstract
Sensory neurons have recently emerged as components of the tumor microenvironment. Nevertheless, whether sensory neuronal activity is important for tumor progression remains unknown. Here we used Designer Receptors Exclusively Activated by a Designer Drug (DREADD) technology to inhibit or activate sensory neurons’ firing within the melanoma tumor. Melanoma growth and angiogenesis were accelerated following inhibition of sensory neurons’ activity and were reduced following overstimulation of these neurons. Sensory neuron-specific overactivation also induced a boost in the immune surveillance by increasing tumor-infiltrating anti-tumor lymphocytes, while reducing immune-suppressor cells. In humans, a retrospective in silico analysis of melanoma biopsies revealed that increased expression of sensory neurons-related genes within melanoma was associated with improved survival. These findings suggest that sensory innervations regulate melanoma progression, indicating that manipulation of sensory neurons’ activity may provide a valuable tool to improve melanoma patients’ outcomes.

Keywords: Sensory neurons, Tumor microenvironment, Melanoma, Neuronal activity, Chemogenetics

Introduction
Melanoma represents one of the leading causes of cancer-related deaths, being the most aggressive skin cancer type worldwide [124]. It emerges from molecularly altered melanocytes, which are the producers of melanin in the skin [48]. These cancer cells are embedded within the cutaneous microenvironment where they reside and interact dynamically with its constituents during disease progression [15, 54]. Understanding the interplay between the different components within the tumor microenvironment is crucial for the success of therapeutic applications, since each component can be influenced by the others, resulting in impacts on the cancer cells [9, 10, 45, 52, 88, 106]. The presence of individual nerve fibers within the tumor microenvironment was ignored for many years as they are difficult to detect in classical histology. For a long time, only large nerve trunks were detected within tumors, and they were always associated with perineural invasion of cancer cells, a process in which these cells grow and migrate along native passive tissue nerves [84]. Recently, a different phenomenon was described, by which the tumor itself is infiltrated pro-actively by newly developed peripheral nerve projections [32, 36, 71, 89, 108, 115, 116, 121, 154, 158].

To understand how peripheral innervations behave within the tumors, functional studies, in which...
intra-tumoral nerves were eliminated, have relied on the surgical or pharmacological manipulation of nerves. Each such strategy, however, has its disadvantages. Peripheral nerves contain mixtures of different nerve fiber types [41, 82], and therefore, surgical denervation of a peripheral nerve leads to the disruption of all the nerve fibers present within that specific nerve [101]. Consequently, the role of particular nerve projections in the tumor cannot be isolated, as other nerve fibers are also affected. On the other hand, pharmacological drugs cause systemic reactions in several organs and indirect effects on unexpected targets. Thus, achieving the neuronal type-specificity that is needed to understand the role that specific nerve fibers perform in the tumor microenvironment is difficult with these methods, and the observed outcomes could be due to the unwanted effects on other innervations in addition to the targeted neurons. Wherefore, conclusions drawn from studies based on surgical or pharmacological denervation may be imprecise. These are some of the reasons, in addition to tumor tissue specificity, for some of the ambiguity about the roles of specific nerve fibers in cancer behavior. Accordingly, contradictory reports have been published: while some studies have claimed that certain neuronal types promote cancer progression [57, 158], others concluded that they suppress tumorigenesis [32, 116].

Therefore, to study the role of specific innervations, these should be directly manipulated in a nerve-fiber-type-specific manner. Recently, this approach became possible with the advent of powerful genetically-based tools, that precisely allow the targeting and elimination of specific peripheral nerve fibers for studying their functions in vivo [13]. Our group showed that specific genetic depletion of sensory neurons promotes melanoma growth [108]. Nevertheless, genetic ablation of these innervations may result in the generation of a pro-inflammatory microenvironment, secondary to cell death in the site where the neurons were ablated (Männ et al. 2016; Christiaansen, Boggiatto, and Varga 2014; Bennett et al. 2005), which itself is strongly tumor growth promoting [49, 67], and can affect cancer cells’ behavior [50]. Thus, it remains unclear which facets of the sensory neuron-ablated tumor phenotype are due to the loss of sensory innervations, rather than indirect effects due to the local inflammation caused by the death of these neurons. To circumvent this issue, in the present study, we used chemogenetics, an experimental strategy that has empowered neuroscience studies [131, 147], to determine the precise role of sensory neurons in the regulation of melanoma progression. Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) enable the silencing or overactivation of genetically defined neuronal populations upon binding to small-molecule designer drugs [119]. This approach allowed for highly selective and non-invasive modulation of sensory neurons’ activity in the tumor. Here, we revealed that silencing of sensory neurons’ activity, without ablating them, is sufficient to trigger increase in melanoma growth and in intra-tumoral new blood vessel formation. In contrast, chemogenetic stimulation of sensory neurons counteracted melanoma progression, by regulating tumoral growth, angiogenesis and immunosurveillance. Our results provide unequivocal evidence of the influence of sensory neurons in cancer progression.

**Materials and methods**

**Animals**

Generation of Nav1.8-Cre mice, in which Nav1.8 + sensory neurons express Cre recombinase, have been previously described. These animals were obtained from Infrafrontier (EMMA ID: 04 582). R26-LSL-hM4Di-DREADD (hM4Di) and CAG-LSL-hM3Dq-DREADD (hM3Dq) mice were purchased from the Jackson Laboratory (jax) (Bar Harbor, ME).

To silence neuronal activity in sensory innervations in vivo, Nav1.8-Cre mice were crossed with R26-LSL-hM4Di-DREADD (hM4Di), a mouse line conditionally expressing a Gi-coupled engineered human muscarinic 4 receptor (hM4Di) [159]. hM4Di is a mutant G protein-coupled receptor which induces the canonical Gi pathway following binding to the pharmacologically inert drug clozapine-N-oxide (CNO). In Nav1.8-Cre+/hM4Di + mice, upon removal of the loxP-stop-loxP cassette by Cre recombination, the Gi-coupled hM4Di is expressed only in Nav1.8 + sensory neurons. Thus, sensory neuronal activity can be silenced by the administration of CNO. Nav1.8-Cre-/hM4Di + mice were used as controls.

To promote sensory neuron overactivation in vivo, Nav1.8-Cre mice were crossed with CAG-LSL-hM3Dq-DREADD (hM3Dq) animals, a mouse line conditionally expressing an evolved Gq protein-coupled receptor (hM3Dq), to generate Nav1.8-Cre+/hM3Dq + mice. In Nav1.8-Cre+/hM3Dq + animals, upon removal of loxP-stop-loxP cassette by Cre recombination, the Gq-coupled hM3Dq is expressed specifically in Nav1.8-sensory neurons. hM3Dq is a mutant G protein-coupled receptor which induces the canonical Gq pathway following the binding to CNO. Thus, sensory neuron firing can be chemically induced by administration of CNO. Nav1.8-Cre-/hM3Dq + animals were used as controls.

All animal care and experimental procedures were approved by the Ethics Animal Care and Use Committee (CEUA), in accordance with the Guide for the Care and Use of Laboratory Animals from the Federal University of Minas Gerais. All colonies were housed in
a pathogen-free animal facility of the Department of Pathology, UFMG, under controlled light cycle (12:12-h light/dark cycle) and fed ad libitum. Age-matched 8- to 12-week-old mice were used for all experiments. All experiments used mice heterozygous for both NaV1.8-Cre and DREADD receptors.

**Cell culture**

Murine B16-F10 melanoma is a common cell line that naturally originated in melanin-producing epithelia of C57BL6 mice. These cells were originally obtained from ATCC (USA), and were used to study melanoma development in vivo. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum/2 mM L-glutamine/100 U/ml penicillin/100 μg/ml streptomycin. The cells have been tested and found negative for mycoplasma. Cells were cultured in a humidified atmosphere of 95% air and 5% (v/v) CO2 at 37°C.

**Melanoma tumor implantation**

B16-F10 cells were suspended in PBS and examined for viability using trypan blue staining. B16-F10 cells were used for transplantation only when more than 90% of cells were viable. For subcutaneous injection, the skin of all mice at an age of 8-12 weeks was shaved at the place of application. 10^5 cells in 100 μL were injected subcutaneously into the right flank of each mouse and the growth of the tumors was monitored until sacrifice. Growth of the tumors was assessed over time with a caliper as previously reported [12]. For determination of tumor volume, tumor-bearing animals were anesthetized with isoflurane in O2 by manually restraining the mice and placing their heads in in-house-built nose cones. Tumors were removed 16 days after transplantation and weighted. Length (L) and width (W) were calculated to measure tumor volume (V) using the formula V = 0.5 × (L × W)2 [40]. Tumor area was determined using calibrated photographs of each tumor using Fiji software®, version 1.53 (National Institute of Health, Bethesda, MD).

**CNO treatment**

The DREADD ligand clozapine-N-oxide (CNO) (1 mg/kg in saline) (Sigma-Aldrich, St Louis, MO, USA) [7] was administered intra-peritoneally using a 25-gauge needle daily to test the effect of neuronal inhibition or activation on melanoma progression in Nav1.8-Cre+/hM4Di+ and Nav1.8-Cre+/hM3Dq+ animals, respectively. Control Nav1.8-Cre-/hM4Di+ and Nav1.8-Cre-/hM3Dq+ mice were similarly injected with CNO.

**Capsaicin-induced spontaneous behavior**

To confirm sensory neurons inhibition efficiency, following acclimation, Nav1.8-Cre-/hM4Di+ and Nav1.8-Cre+/hM4Di+ mice were injected with an intra-plantar subcutaneous dose of 10 μl of capsaicin (1 μg/10 μl; Sigma-Aldrich). A video recording was taken for 5 min post-capsaicin injection. The time that the animals spent performing spontaneous behaviors of licking, lifting, and flicking the paw were measured for 5 min after injection of capsaicin from these videos.

**Immunohistochemistry and microscopy**

Adult mice were deeply anesthetized with isoflurane and transcardially perfused with saline followed by 4% buffered paraformaldehyde (PFA, pH = 7.4). After dissection, B16F10 tumors were fixed overnight at 4°C in 4% buffered paraformaldehyde, incubated overnight at 4°C with 30% sucrose diluted in PBS, embedded and frozen in optimal cutting temperature compound (OCT; Tissue-Tek). Embedded tumors were stored at −80°C. 20 μm cryosections were cut and blocked for 2 h in 3% BSA in PBS + 0.5% Triton and immunostained with the following antibodies: CD31-PE (dilution 1:100) (BioLegend), Ki67 (dilution 1:100) (BD Biosciences), and anti-Guinea pig-AlexaFluor-647 (1:1000) (Life Technologies) [10, 23]. After this, the sections were washed with PBS containing 4'/6-diamidino-2-phenylindole (DAPI, 5 μg/ml, Invitrogen) and mounted using Dako fluorescence mounting medium (Dako, Santa Clara, CA). Stained tumor sections were imaged and analyzed by confocal microscopy using an inverted Zeiss LSM 880 confocal microscope (Oberkochen, Germany). CD31 area and the number of Ki67+ cells were quantified using Fiji software®, version 1.53 (National Institute of Health). Multiple random fields of each section were used for quantification.

**Tumor-infiltrating leukocytes immunophenotyping and intracellular cytokine measurement**

Tumors, their draining lymph nodes and non-tumor draining lymph nodes were harvested. Tissues were macerated and filtrated trough cell strainers of 40 μm (Falcon) to isolate the cells used for immunophenotyping. Cells were washed in phosphate-buffered saline (PBS), incubated with Live/Dead solution (Invitrogen), for dead cell exclusion, and with monoclonal antibodies, washed, fixed, and permeabilized (FoxP3 staining buffer set, eBioscience) according to manufacturer’s instructions. Antibodies are listed in Table 1. Acquisition was realized on a LSR-FORTESSA. For analyses, to exclude debris, combinations of fluorochromes was done, to remove doublets a forward scatter area (FSC-A) versus forward scatter height (FSC-H) gate was used, and then cells were gated in function of time versus FSC-A to
Table 1 Antibodies used in flow cytometry

| Antigen      | Fluorochrome | Clone           | Company          |
|--------------|--------------|-----------------|------------------|
| CD3          | eFluor450    | 145-2C11        | Thermofisher     |
| CD8a         | eFluor 450   | S3–6.7          | Thermofisher     |
| CD11c        | eFluor 450   | N418            | Thermofisher     |
| LIVE/DEAD    | Acqua        |                 | Thermofisher     |
| Streptavidin | Pacific Orange |               | Thermofisher     |
| CD45         | Super Bright 600 | 30-F11 | Thermofisher     |
| TCR gamma/delta | Super Bright 645 | eBioGL3   | Thermofisher     |
| CD4          | Alexa Fluor 488 | GK1.5       | Thermofisher     |
| F4/80        | FITC         | BM8             | Hyclut           |
| NK1.1        | PE-eFluor 610 | PK136           | Thermofisher     |
| CD8a         | PerCP-Cyanine5.5 | S3–6.7       | Thermofisher     |
| Ly-6G        | PerCP-eFluor 710 | 1A8-Ly6g   | Thermofisher     |
| IL-17A       | PerCP-Cyanine5.5 | eBio17B7     | Thermofisher     |
| CTLA-4       | PE-Cyanine7   | UC10-4B9       | Thermofisher     |
| FoxP3        | Alexa Fluor 647 | 3G3         | Thermofisher     |
| CD3e         | Cyanine5     | 500A2           | Thermofisher     |
| Ki67         | AlexaFluor 700 | SolA15       | Thermofisher     |
| PD-1         | APC-eFluor 780 | J43         | Thermofisher     |
| Ly-6C        | APC-eFluor 780 | HK1.4        | Thermofisher     |
| IFN-γ        | APC-eFluor 780 | XMG1.2       | Thermofisher     |
| CD11b        | Biotin       | M1/70           | Biolegend        |

avoid a possible interference of flux interruptions. Only live leukocytes were used using a Live/Dead gate versus CD45. We assessed different immune cell subpopulations based on molecular markers of each cell subset: CD4 + T cells (CD4+/CD3+), CD8 + T cells (CD8+/CD3+), γδ T cells (CD3+/CD4−/CD8−/TCRγδ+), NK cells (CD3−/NK+), regulatory T cells (Foxp3+/CD4+/CD3+), NK cells (CD3−/NK+), neutrophils (CD11b+/CD11c−/Ly6C−/Ly6G+), PMN/MDSCs (CD11b+Ly6C−/Ly6G+) and dendritic cells (CD11b−/CD11c+/Ly6C+/Ly6G−). In each T-cell subset, frequencies of cells expressing checkpoint inhibitors CTLA-4 and PD1 were evaluated. Cytokine analyses in lymphocytes from the tumor microenvironment and lymph nodes were done using intracellular staining. Briefly, cells were isolated from tumor samples and lymph nodes and cultivated for 4 h at 37 °C in 10% FBS RPMI supplemented with 2 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin, in the presence of Brefeldin A (ThermoFisher) and Monensin (ThermoFisher). Following this, cells were washed in FACS buffer and stained for cell surface markers. Cells were then fixed for 35 min at 4 °C with eBiosciences Cytofix/Cytoperm buffer and, subsequently, washed once in eBioscience Perm/Wash buffer. Then, cells were stained for 45 min at 4 °C with anti-IFN-γ and anti-IL-17 (Table 1) antibodies diluted in eBioscience Perm/Wash [78]. Cells were washed twice and the data was acquired. Ki67 is a nuclear factor transcript in the late G1, S, G2, and M of cell cycle, therefore marks proliferating cells [44, 128]. Thus, we evaluated proliferation in viable CD45 negative cells, suggesting tumoral proliferation. GraphPad Prism V7.0 (GraphPad software) and FlowJo V10.4.11 (TreeStar) were used for data analysis and graphic presentation.

Quantification of CGRP within tumors

Tumor samples from Nav1.8-Cre+/hM4Di+ and Nav1.8-Cre+/hM3Dq+ animals, as well as from their respective controls (Nav1.8-Cre-/hM4Di+ and Nav1.8-Cre-/hM3Dq+) were analyzed to measure the amount of CGRP using commercially available Sandwich-CGRP ELISA kit purchased from Elabscience (Catalog # E-EL-M2744). Briefly, tumor pieces were weighed and then homogenized in PBS (0.01 M, pH = 7.4) with a glass homogenizer on ice. The homogenates were centrifuged for 5 min at 5000 × g at 4 °C to get the supernatant. ELISA of CGRP were performed according to manufacturer’s instructions. After ELISA, Optical Density (OD) was measured using Varioskan Flash (Thermo) set at 450 nm.

In silico analysis

RNA sequencing count data of 103 Skin Cutaneous Melanoma (SKCM) patients was downloaded from The Cancer Genome Atlas (TCGA) repository (https://portal.gdc.cancer.gov/). Differential gene expression analyses were performed between samples of alive and dead patients (considering a 5-year interval) using DESeq2 [83]. We stratified patients in these two groups, alive or dead, based on their vital status in a 5-year interval of their tumor diagnosis (clinical data available at TCGA and curated by Liu et al. (2018) [85]. Genes with absolute log2(Fold-change) ≥ 1 and False Discovery Rate (FDR) adjusted P-value < 0.05 were considered differentially expressed. To identify biological processes associated with genes differentially expressed, we performed a Gene Ontology (GO) enrichment analysis using ShinyGO [42]. We used the STRING database [132] (parameters: full STRING network, considering only text-mining, databases and experiments interactions with score > 0.400, and only genes with 3 or more interactions) and Cytoscape (https://cytoscape.org/) to construct protein–protein interactions (PPIs) among our manually curated list of 34 gene related to sensory neurons selected based on the literature [29, 37, 51, 114, 141, 144]. The set of 18 genes showing at least two PPI interactions are shown. For the remaining analyses, RNA sequencing counts were first Transcripts Per Million (TPM)-normalized using a local R script. To identify a gene signature associated with SKCM cancer patient survival we used Reboot [31] with parameters “-B 100 -G 5 -P 0.3 -V 0.01.”
Briefly, Reboot finds genes associated with cancer patient prognosis using multivariate penalized Cox regression combined with a bootstrap approach. In the first step of Reboot, it produces regression coefficients (numerical values) that determine the contribution of each submitted gene to patients’ survival. These coefficients may be positive or negative values indicating that high expression of a particular gene potentially contributes to worse or better prognosis, respectively. Once these coefficients are produced, Reboot then calculates the score of each patient (sample) as the sum of each gene coefficient multiplied by the corresponding gene expression level in that patient. Finally, when all patients’ scores are calculated, we then stratify them into groups with high/low scores based on the median score of all patients to create the survival curve (Kaplan–Meier). For further information, see [31]. SCN10A box plots and survival curves were created using R (https://www.r-project.org/) scripts.

**Statistical analysis**

Graphs were plotted using GraphPad Prism 7 (San Diego, CA). Shapiro-Wilk normality test was performed, and unpaired t test was used to determine statistical significance.

**Results**

**Chemogenetic inhibition of Nav1.8 + neurons accelerates melanoma progression**

We have previously demonstrated that melanomas are infiltrated by Nav1.8 + sensory innervations, and that those tumors grow slower when these neurons are pharmacologically or genetically ablated [108]. However, these investigations were performed using Nav1.8-Cre+/DTA + mice, in which a diphtheria toxin fragment A is constitutively activated in Nav1.8 + sensory neurons, resulting in the toxin induced-death of these cells. Therefore, this technique lacks temporal control of neuronal ablation, and enables compensatory effects during the development of these animals. Importantly, the approach by which specific neurons are ablated from the tissue microenvironment is also limited because of the secondary consequences, such as inflammation, caused in the tissue where sensory neurons are eliminated. Thus, it remains unclear whether these damage-induced changes in the tissue may influence the observed cancer outcomes. Here, we applied a chemogenetic approach to specifically inhibit the activity of Nav1.8-expressing sensory neurons without killing these cells. We used DREADDs to specifically control sensory neuron activity. DREADDs are derived from different types of mutated muscarinic receptors that have been engineered to lose affinity to their endogenous ligand acetylcholine [5], but to gain responsiveness to a synthetic ligand, clozapine-N-oxide (CNO). Inhibitory DREADDs (hM4Di) elicit an intracellular cascade that results in the silencing of neuronal activity [113], without changing the number of innervations as previously reported [68]. This method allows for the selective silencing of specific types of neurons in vivo without physical manipulation or destruction in the tissue. DREADDs were expressed specifically in sensory neurons, using a transgenic murine approach: mice carrying the construct encoding for Cre-dependent expression of hM4Di were crossed to Nav1.8-Cre animals. In the resulting mice, Nav1.8-Cre+/hM4Di +, only Nav1.8 + sensory neurons expressed inhibitory DREADDs. As controls in this study, we used littermate mice carrying Cre-dependent hM4Di, but lacking the Cre gene (Nav1.8-Cre-/hM4Di +) (Fig. 1A). This allowed us to control for any potential side effects from CNO administration.

In order to ascertain that the expression of DREADD receptors was driven to intra-tumoral Nav1.8-expressing neurons in Nav1.8-Cre+/hM3Di + mice, we used the solid tumor model B16F10. We assessed tumor sections from melanoma grown in Nav1.8-Cre+/TdTomato + mice and detected Nav1.8 + neurons expressing TdTomato present within the tumor microenvironment (Fig. 1B). To validate sensory neuronal inhibition following daily CNO injection, we used a behavioral test to evaluate the sensitivity to capsaicin, confirming the silencing of sensory neurons, as previously described [2]. Indeed, Nav1.8-Cre+/hM4Di + animals spent less time (25.53 ± 2.27 s) licking their paws after intra-plantar injection of capsaicin, compared to control animals (56.53 ± 3.92 s) (Fig. 1C). To analyze the effect of sensory neurons silencing on tumor growth, we subcutaneously transplanted B16F10 cells to the lower right flank of both inhibitory DREADD-expressing mice (Nav1.8-Cre+/hM4Di +) and their controls (Nav1.8-Cre-/hM4Di +). Following cancer cell injection, we treated the animals daily with CNO to induce sensory neuronal activity inhibition (controls were also treated with CNO) (Fig. 1D). After 14 days of continuous sensory inhibition, tumor volume was significantly enhanced in the sensory neuron-silenced mice when compared to the controls (tumor volume was increased from 82.1 ± 29.6 to 319.6 ± 72.8 mm³; Fig. 1E). After 16 days of repeated sensory inhibition, tumor weight was also significantly enhanced in the sensory neuron-silenced mice when compared to the controls (tumor weight was increased from 0.50 ± 0.04 to 0.98 ± 0.23 g; Fig. 1F, G). Animal weights were not affected by sensory inhibition (data not shown).

Increase in neoangiogenesis within melanoma tumors is correlated with worse outcomes in these patients [111]. We detected, in melanoma-bearing animals with silenced sensory neurons, an enhancement in the
Chemogenetic inhibition of neuronal activity in sensory Nav1.8 + nerve fibers triggers melanoma growth. A Schematic diagram of the Nav1.8-Cre+/hM4Di+ experimental mouse model. Cre recombinase directs the expression of hM4Di specifically to sensory neurons in those mice. After the administration of CNO to those mice, neuronal activity in sensory neurons is inhibited. B Tumor-infiltrating sensory neurons are targeted in Nav1.8-Cre mice. 1 x 10^5 B16F10 melanoma cells were subcutaneously injected into Nav1.8-Cre/TdTomato mice, and tumor tissues were surgically removed 16 days later. Representative image of a Nav1.8-Cre/TdTomato mouse tumoral section with sensory nerve fibers infiltrating the tumor labelled with TdTomato fluorescence (red) and nuclei with DAPI (blue). C Capsaicin-induced spontaneous behavior test corroborates chemogenetic inhibition of sensory Nav1.8 + nerve fibers in Nav1.8-Cre+/hM4Di+ mice after CNO treatment. Column charts show the licking time after capsaicin application of Nav1.8-Cre−/hM4Di+ (n=5) and Nav1.8-Cre+/hM4Di+ (n=5) animals. D Representation of the protocol for subcutaneous allograft melanoma growth. 1 x 10^5 B16F10 melanoma cells were subcutaneously injected into Nav1.8-Cre−/hM4Di+ (n=5) and Nav1.8-Cre+/hM4Di+ (n=5) mice, followed by tumors removal for analysis after 16 days. CNO was daily intra-peritoneal injected at 1 mg/kg. E Development curve of tumor growth from Nav1.8-Cre−/hM3Dq+ and Nav1.8-Cre+/hM3Dq+. Tumor volumes were assessed over time with a caliper. F Representative macroscopic image of B16F10 melanoma after dissection, left panel (Nav1.8-Cre−/hM4Di+) and right panel (Nav1.8-Cre+/hM4Di+). G Tumor weight. (Nav1.8-Cre−/hM4Di+: 0.50 ± 0.04 g; Nav1.8-Cre+/hM4Di+: 0.98 ± 0.23 g). Data are shown as mean ± SEM. Unpaired t test (ns P > 0.05; *P < 0.05; **P < 0.01)
intra-tumoral blood vessels’ area (from 0.02±0.00 to 0.03±0.01 µm²) (Fig. 2A, B). Expression of Ki67 is used to determine the proliferation rate of malignant cancer cells [139], which is also associated with melanoma aggressiveness [76]. We found that genetic silencing of sensory innervations led to an increase in the proliferation rate within the melanoma (from 2074±55.32 to 2454±168.4 Ki67+ cells per µm²) (Fig. 2C, D). We also observed after inhibition of sensory neurons firing a decrease in tumor-infiltrating CD4+ T cells (from 4.47×10⁷ ± 1.15×10⁷ to 1.73×10⁷ ± 7.92×10⁶ cells per mg of tumor) (Fig. 2E), in special, in IL-17-producing CD4+ T cells (from 1.63×10⁷ ± 1.30×10⁶ to 3.77×10⁶ ± 3.27×10⁶ cells per mg of tumor) (Fig. 2F),

**Fig. 2**  Chemogenetic inhibition of neuronal activity in sensory Nav1.8+ innervations increases intra-tumoral proliferation and angiogenesis, and blocks anti-tumoral immune response. 1×10⁵ B16F10 melanoma cells were subcutaneously injected into Nav1.8-Cre−/hM4Di+ (n=5) and Nav1.8-Cre+/hM4Di+ (n=5) mice, followed by tumors removal for analysis after 16 days. A Representative immunofluorescence images of tumors labelled for endothelial cells (CD31; red) to identify blood vessels and nuclei (DAPI; blue). B Quantification of angiogenesis in melanomas by blood vessel area. C Representative immunofluorescence images of tumors labelled for Ki67 (Ki67; green) to identify cell proliferation and nuclei (DAPI; blue). D Quantification of proliferation in melanomas by the counting of Ki67+ cells per µm². Absolute number of CD4+ E and CD8+ G T cells from the melanomas of B16F10–inoculated mice. F Graph shows absolute numbers of CD4+ T cells producers of IL-17. IL-17 levels were measured in cells isolated from tumors of B16F10–inoculated Nav1.8-Cre+/hM4Di+ and Nav1.8-Cre−/hM4Di+ animals. Data are shown as mean ± SEM. Unpaired t test (ns P>0.05; *P<0.05)
and a decrease in melanoma-infiltrating CD8+ T cells (from $3.27 \times 10^6 \pm 5.22 \times 10^5$ to $7.62 \times 10^5 \pm 6.78 \times 10^5$ cells per mg of tumor) (Fig. 2G). Our results indicate that inhibition of neuronal activity in sensory neurons promotes melanoma tumor advancement.

**Chemogenetic activation of hM3Dq excitatory DREADD receptors in Nav1.8-+ neurons promotes melanoma regression**

As we found that inhibition of sensory neuron activity promotes melanoma advancement, we hypothesized that increasing sensory excitability would result in the reverse: blockage of melanoma progression. To test this hypothesis, we used again chemogenetics, by which we induced the expression of excitatory hM3Dq DREADDs [127] only in Nav1.8+ sensory neurons. We crossed Nav1.8-Cre mice to a mouse line with a Cre-dependent evolved Gq protein-coupled receptor (hM3Dq) expression. In the resulting Nav1.8-Cre+/hM3Dq+ mice, upon removal of loxP-stop-loxP cassette by Cre recombination, the Gq-coupled hM3Dq is expressed specifically in Nav1.8-sensory nerve fibers. Sensory neurons in those mice can thus be overactivated by the administration of CNO. It has been shown previously that Gq-DREADD activation by CNO increases neuronal activity in the targeted neurons, including sensory neurons [68, 90], without changing the number of neurons [110].

To evaluate the role of sensory stimulation on tumor growth, we transplanted subcutaneously B16F10 melanoma cells to the lower right flank of both stimulatory DREADD-expressing mice (Nav1.8-Cre+/hM3Dq+) and their controls (Nav1.8-Cre-/hM3Dq+). Following the cancer cell implantation, we treated mice daily with CNO to induce Nav1.8+ sensory neuron activation (controls were also treated with CNO) (Fig. 3A, B). After repeated sensory neuron activation, melanoma development was decreased in the sensory neuron-over-activated mice when compared to the controls (at day 14, tumor volume per body weight was reduced from $3.51 \pm 0.89$ to $0.71 \pm 0.20 \text{ mm}^3$; at day 16, tumor weight was reduced from $0.38 \pm 0.07$ to $0.17 \pm 0.03 \text{ g}$; Fig. 3C–F). Animal weights were not affected by genetic stimulation of sensory neurons in melanoma-bearing mice (data not shown). Moreover, genetic overactivation of sensory neurons led to a decrease in proliferating cells within the tumor (from $3050 \pm 203$ to $1292 \pm 367$ Ki67+ cells per $\mu\text{m}^2$, analyzed by immunohistochemistry) (Fig. 3G, H), corroborated by flow cytometry analysis of CD45- cells for Ki67 expression (the was a decrease from $8.13 \pm 1.00$ to $5.07 \pm 0.70\%$ of CD45-/Ki67+ cells within the population of CD45- cells) (Fig. 3I). Additionally, there was a decrease in the intra-tumor blood vessels' area (from $0.010 \pm 0.001$ to $0.006 \pm 0.001 \mu\text{m}^2$ of CD31+area / $\mu\text{m}^2$ of tumor area) (Fig. 3J, K). Our data suggest that increase in neuronal activity in sensory neurons counteracts melanoma development.

**Increase in sensory neuron activity affects melanoma immunosurveillance**

Functional studies in combination with histological analysis have demonstrated that tumor-infiltrating immune cells modulate melanoma cells' behavior, altering cancer outcomes [38, 72, 79, 83, 112, 130, 133, 134, 152, 153]. Given that sensory neurons may influence immune responses in non-cancer contexts, we sought to probe whether sensory neurons stimulation alters immune surveillance within the tumor.

Accumulating evidence has demonstrated that tumor-infiltrating neutrophils and PMN-MDSCs promote tumor development and progression [21, 39, 65, 107, 136, 138, 140, 150]. Thus, we evaluated whether these cells are affected by sensory neurons' overactivation. We found that the number of melanoma-infiltrating neutrophils and PMN-MDSCs was significantly decreased in the sensory neuron-overactivated mice (Nav1.8-Cre+/hM3Dq+) when compared to the controls (Nav1.8-Cre-/hM3Dq+) (from $12.02 \times 10^6 \pm 3.45 \times 10^6$ to $4.69 \times 10^7 \pm 7.10 \times 10^6$ PMN-MDSCs per mg of tumor; and from $10.45 \times 10^7 \pm 3.70 \times 10^7$ to $2.78 \times 10^7 \pm 5.65 \times 10^6$ neutrophils per mg of tumor).
(Fig. 4A, B). On the other hand, we found that the number of tumor-infiltrating dendritic cells, which counteract the proliferation of melanoma cells [137], was significantly increased (from $5.53 \times 10^7 \pm 8.80 \times 10^6$ to $1.07 \times 10^8 \pm 2.27 \times 10^7$ dendritic cells per mg of tumor) (Fig. 4C).

Recent breakthroughs in cancer immunotherapy have revealed the remarkable ability of the immune system...
to fight different types of cancers, including melanoma. The phenotypes and numbers of prevalent tumor-infiltrating lymphocytes are predictive of response to immunotherapy and key modulators of disease progression. Thus, we examined how tumor-infiltrating lymphocytes are affected by sensory neurons’ overstimulation. We detected an increase in tumor-infiltrating CD4+ T cells (from $2.91 \times 10^6 \pm 1.04 \times 10^6$ to $1.09 \times 10^7 \pm 2.92 \times 10^6$ cells per mg of tumor), CD8+ T cells (from $8.94 \times 10^6 \pm 1.60 \times 10^6$ to $1.96 \times 10^7 \pm 5.20 \times 10^6$ cells per mg of tumor), γδ T cells (from $31.76 \times 10^7 \pm 7.32 \times 10^7$ to $74.14 \times 10^7 \pm 20.40 \times 10^7$ cells per mg of tumor), NKT cells (from $16.34 \times 10^7 \pm 4.6 \times 10^7$ to $34.92 \times 10^7 \pm 10.42 \times 10^7$ cells per mg of tumor) and NK cells (from $1.72 \times 10^7 \pm 2.90 \times 10^6$ to $3.47 \times 10^7 \pm 4.40 \times 10^6$ cells per mg of tumor) (Fig. 4D–H), while regulatory T cells, which mediate immunosuppression in the tumor microenvironment [66], were not altered (Fig. 4I). Immune checkpoint molecules, such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death 1 (PD-1), act fine-tuning the intense immune responses that might kill healthy cells [27, 55, 122]. Their expression in cytotoxic T cells may lead to dysfunction of these cells, affecting their effector function [11, 146]. We found that increase in the firing of sensory neurons prevented the increase of immune checkpoint markers of tumor infiltrating CD8+ T cells and CD4+ T cells (Fig. 5 and Additional file 1: Figure 1). The percentage of CTLA-4-expressing CD4+ tumor-infiltrating lymphocytes decreased from 29.43±4.04% in Nav1.8-Cre−/hM3Dq+ (n = 14) to 19.08±2.80% in Nav1.8-Cre+/hM3Dq+ (n = 13) animals (Fig. 5A); similarly, the percentage of PD-1-expressing CD4+ tumor-infiltrating lymphocytes decreased from 15.02±2.62% in Nav1.8-Cre−/hM3Dq+ to 7.85±1.43% in Nav1.8-Cre+/hM3Dq+ mice (Fig. 5B, C). The percentage of PD-1-expressing CD4+ tumor-infiltrating cytotoxic lymphocytes also decreased from 22.03±2.66% in Nav1.8-Cre−/hM3Dq+ to 12.99±3.85% in Nav1.8-Cre+/hM3Dq+ animals (Fig. 5E), while the expression of CTLA-4 did not vary in these cells (Fig. 5D, F). In addition, no differences were found in CTLA-4 and PD-1 expression on γδ T cells (Fig. 5G, H and I), NKT...
cells (Fig. 5, K and L), NK cells (Fig. 5M, N and O) and Treg cells (Fig. 5P, Q and R). Overall, our data suggest that sensory neurons overactivation induces improvement of T cells effector functions within the tumor microenvironment.

It has been reported that CD4+ and CD8+ lymphocytes secreting IL-17 promote melanoma regression [91, 99]. Here, we detected in response to sensory neuron firing an increase in melanoma-infiltrating IL-17-producing CD4+ T cells (from $2.45 \times 10^7 \pm 6.05 \times 10^6$ to
30.78 × 10^7 ± 9.20 × 10^7 cells per mg of tumor) as well as in melanoma-infiltrating IL-17-producing CD8+ T cells (from 5.02 × 10^7 ± 0.90 × 10^7 to 20.08 × 10^7 ± 5.92 × 10^7 cells per mg of tumor) (Fig. 6A). In parallel, we did not detect significant changes in the number of other tumor-infiltrating lymphocytes producing IL-17 or in IFN-γ-producing lymphocytes after sensory neuron overactivation (Fig. 6B). Altogether, our results suggest that sensory neurons induce a Th17-immune response in the melanoma microenvironment.

Lymph nodes are an integral part of the adaptive immune system in our organism and are essential for the effective immune responses. Melanoma draining lymph nodes are influenced by the primary tumor, but may also prime the immune suppressive microenvironment, playing critical roles in promoting cancer immune escape [33, 86, 87, 97]. It is completely unknown whether sensory neuron overactivation may affect the immune cells also within the tumor draining lymph nodes. Herein, we analyzed immune cells from tumor draining and non-tumor-draining lymph nodes from CNO-treated stimulatory DREADD-expressing animals (Nav1.8-Cre+/hM3Dq+) and their controls (Nav1.8-Cre-/hM3Dq+). Tumor draining and non-tumor-draining lymph nodes were isolated from the ipsilateral and contralateral side, respectively, of the implanted melanoma (Fig. 7A, B). We found that the effect of sensory neurons stimulation in tumor draining lymph nodes mimics the immune response within the primary tumor, but not in non-tumor-draining lymph nodes. In the tumor-draining lymph nodes, we found an increase in the number of CD8+ cytotoxic T cells after sensory neurons’ overstimulation (from 13.68 × 10^7 ± 3.50 × 10^7 to 22.41 × 10^7 ± 2.17 × 10^7 cells per mg of tumor) (Fig. 7C); while we did not detect any differences in the numbers of T cells in the tumor non-draining lymph nodes (Fig. 7D). These data indicate a possible priming effect of tumor on the adjacent draining lymph nodes.

We also observed, in the tumor-draining lymph nodes, increases in IFN-γ-producing CD4+ T cells (from 8.31 × 10^6 ± 2.65 × 10^6 to 1.94 × 10^7 ± 3.36 × 10^6 cells per lymph node), in IFN-γ-producing NK cells (from 4.72 × 10^4 ± 2.46 × 10^4 to 8.44 × 10^5 ± 2.22 × 10^5 cells per lymph node), and in IFN-γ-producing NKT cells (from 2.38 × 10^6 ± 4.27 × 10^5 to 1.28 × 10^7 ± 1.64 × 10^6 cells per lymph node), and in IL-17-producing CD8+ T cells (from 1.36 × 10^5 ± 5.57 × 10^4 to 4.77 × 10^5 ± 1.19 × 10^6 cells per lymph node), in IFN-γ-producing γδ T cells (from 2.27 × 10^6 ± 8.55 × 10^5 to 1.03 × 10^7 ± 3.22 × 10^6 cells per lymph node), and in IFN-γ-producing NK cells (from 1.80 × 10^5 ± 4.70 × 10^4 to 6.82 × 10^5 ± 1.53 × 10^5 cells per lymph node) (Fig. 7E). In tumor non-draining lymph nodes, we detected increases in IFN-γ-producing CD4+ T cells (from 5.73 × 10^6 ± 2.17 × 10^6 to 1.92 × 10^7 ± 4.23 × 10^6 cells per lymph node), in IFN-γ-producing CD8+ T cells (from 1.36 × 10^5 ± 5.57 × 10^4 to 4.77 × 10^6 ± 4.23 × 10^5 cells per lymph node), in IFN-γ-producing γδ T cells (from 2.27 × 10^6 ± 8.55 × 10^5 to 1.03 × 10^7 ± 3.22 × 10^6 cells per lymph node), and in IFN-γ-producing NK cells (from 1.80 × 10^5 ± 4.70 × 10^4 to 6.82 × 10^5 ± 1.53 × 10^5 cells per lymph node) (Fig. 7F). Our data suggest that lymphocytes at the lymph nodes may be contributing to the response against the melanoma observed within the tumor microenvironment after sensory neurons’ overactivation as both, IFN-γ and IL-17, may contribute to enhance the anti-tumoral response in the context of melanoma [92, 109, 151]. Altogether, our data suggest that
High expression of genes related to sensory neurons correlates with best prognosis in human melanoma patients

In order to investigate our findings also in human tumors, we analyzed The Cancer Genome Atlas (TCGA) samples

sensory neurons stimulation alters immune surveillance that impacts melanoma development.
from 103 Skin Cutaneous Melanoma (SKCM) patients. First, we stratified SKCM patients in two groups, alive or dead based on a 5-year interval. Next, we searched for differentially expressed genes between these groups. We found 112 up-regulated and 195 down-regulated genes (|log2(Fold change)| ≥ 1 and adjusted P-value < 0.05; Table 2). Next, we performed a Gene Ontology (GO) analysis of Biological Processes (BP) in which these genes are involved. The up-regulated genes set are enriched for three biological processes, while the down-regulated genes are enriched to a wide variety of processes (Fig. 8A; Table 3). Curiously, for the former (up-regulated genes), two out of three Biological Processes represent nervous system development (Fig. 8A), indicating the importance of neuronal networks in melanoma outcomes. Next, we investigated the interactions (and putative regulation) of 34 sensory neurons-related genes which were selected from the literature [29, 37, 51, 114, 141, 144] (Table 4).

Figure 8B shows a strong connection among 18 of these genes, suggesting that they work on the same cellular pathways or cell types. Next, using the expression levels of these 34 gene markers for sensory neurons we investigated their potential to be “a signature” associated with SKCM cancer patient survival. Figure 8C shows that high expression of these genes (lower patient scores) are associated with a better overall survival of SKCM patients. Next, we investigated the expression of these genes in the two sample sets (alive and dead patients). We found that SCN10A, which codifies Nav1.8, a key gene based on which our mouse models target sensory neurons, is more expressed in alive than in dead patients (Fig. 8D). Finally, we investigated the impact of SCN10A expression on SKCM patients’ survival. Figure 8E shows that high expression of SCN10A trends to be associated with a better overall survival of SKCM patients, even without statistical support (P-value = 0.26). Taken together, these results confirm that genes related to nervous system development are enriched in samples from live Skin Cutaneous Melanoma patients. Focusing on gene markers for sensory neurons, we confirmed that these genes are strongly connected, suggesting a synergistic activity, and that the higher expression of some of these genes are associated with a higher overall survival. Strikingly, the high expression of SCN10A is potentially associated with better SKCM patient survival, indicating that the presence of sensory neurons within melanoma counteracts cancer progression. We also found that TCGA samples from tumors with a worst prognosis (dead patients) have an enrichment of genes promoting angiogenesis (Tables 3 and 5; 15 genes related to angiogenesis). We focused on this set of 15 genes related to angiogenesis and we confirmed that they are strongly connected (Additional file 2: Fig. 2), indicating a synergistic function in promoting angiogenesis. Our results indicate that there is an increase in genes related with angiogenesis in tumors with worst prognosis (from dead patients) and a decrease in their expression in tumors from alive patients which show an increased expression of SCN10a, a sensory neuron marker used in this study (Fig. 8C, D, E). By using the CIBERSORT tool [102], we investigated immune infiltrated cells in the same TCGA cohort of alive vs. dead patients (Additional file 3: Fig. 3). CIBERSORT uses gene expression (RNA sequencing data) and support vector regression combined with prior knowledge of expression profiles from purified leukocyte subsets (gene signatures) to produce an estimation of the abundances of immune infiltrated cells subpopulations in a sample. In line with our data presented in this manuscript, we have checked the enrichment of immune infiltrated cells in the tumors of patients alive vs. dead (Additional file 4: Fig. 4). We found an increase of CD4+ T cells, CD8+ T cells, NK cells and dendritic cells in patients with better prognosis (alive). Thus, tumors showing a better prognosis (alive) have an increased infiltration of some key immune cells. Additionally, microarray data evidenced a down-regulation of genes related to the Th17 immune response in melanoma patients (Additional file 5: Fig. 5). These analyses are consistent with the data generated in our mouse models: that the overactivation of sensory innervations in the tumor microenvironment was associated with suppressed melanoma progression. Albeit gene expression in tumor biopsies from human cancer patients is used as a tool to define novel biomarkers and to contribute to prognosis, the obtained data should be also validated by the quantification of sensory neuron-related proteins in human melanoma biopsies and correlation with clinical outcomes in future research.

**Discussion**

In the present study, we examined how melanoma progression is affected by sensory neurons activity. Our chemogenetic approach revealed that inhibition of sensory activity promotes tumor growth and intra-tumoral angiogenesis. In contrast, excitation of sensory neurons induces melanoma regression with decrease in tumor growth and in new blood vessel formation, as well as a boost in the anti-tumor immune surveillance (Fig. 9). This work indicates that induction of hyperactivation in Nav1.8-expressing sensory neurons represents a potential new therapeutic path in the battle against melanoma.

Just as the role of particular genes in a specific biological process can be examined by evaluating examining the assessable consequences that result from their removal (e.g. using knockout animals), the role of neurons in the tumor microenvironment was previously assessed by eliminating them [32, 36, 71, 89, 108, 115,
### Table 2
Analyzes of genes from The Cancer Genome Atlas (TCGA) samples from 103 Skin Cutaneous Melanoma (SKCM) patients

| Gene symbol | log2FC | FDR   |
|-------------|--------|-------|
| SLCSA4      | 4.35910| 1.29E-09 |
| VGF         | 3.26059| 1.06E-05 |
| NPPC        | 3.24826| 2.60E-05 |
| LINCO0698   | 3.76414| 3.90E-05 |
| SPACA3      | 2.67598| 1.97E-04 |
| VFOX3A      | 4.95714| 2.21E-04 |
| PRSS16      | 4.13411| 4.15E-04 |
| ARHGAP8     | 2.75929| 4.15E-04 |
| VCX         | 3.63292| 4.28E-04 |
| LINC01287   | 3.48781| 7.19E-04 |
| NGFR        | 1.99998| 1.16E-03 |
| NAT16       | 2.23940| 1.16E-03 |
| RP13-143G15.4| 2.26728| 1.34E-03 |
| HLA-J       | 1.67323| 1.38E-03 |
| LHPL4       | 2.80159| 2.04E-03 |
| RP11-376N17.4| 2.27953| 2.10E-03 |
| ZNF689      | 1.08305| 2.33E-03 |
| DCD         | 6.91950| 2.44E-03 |
| SLITRK5     | 2.38821| 2.59E-03 |
| ARPP21      | 3.84108| 2.67E-03 |
| TFAP2B      | 2.73943| 2.68E-03 |
| VIT         | 2.42509| 3.21E-03 |
| HPCAL4      | 1.70772| 3.30E-03 |
| LINN0645    | 3.54408| 4.05E-03 |
| KLHL32      | 1.29974| 4.09E-03 |
| TRIML2      | 2.32595| 4.80E-03 |
| GFAP        | 2.04141| 5.21E-03 |
| MYOZ2       | 2.33172| 6.94E-03 |
| PPY         | 2.13167| 7.88E-03 |
| ARX         | 2.02055| 8.39E-03 |
| LRRTM2      | 1.75474| 8.39E-03 |
| C20o0203    | 1.95612| 8.43E-03 |
| LSMEM2      | 1.36377| 8.43E-03 |
| RNTN        | 1.74226| 8.58E-03 |
| RP11-800C18.3| 1.54681| 8.89E-03 |
| FOSB        | 1.08702| 9.10E-03 |
| PASD1       | 4.57206| 9.14E-03 |
| UNCO3B8     | 1.84987| 9.14E-03 |
| RP11-469H8.6| 2.35144| 9.14E-03 |
| BCO1        | 2.17260| 9.08E-03 |
| XKR7        | 1.93913| 1.00E-02 |
| RDH5        | 1.36688| 1.04E-02 |
| PAG1        | 1.36926| 1.10E-02 |
| RPS-907D15.4| 1.73346| 1.10E-02 |
| AC003092.1  | 2.31155| 1.20E-02 |
| IGH-V-58    | 3.09550| 1.20E-02 |
| FOXG1       | 3.56195| 1.36E-02 |
| GBA3        | 2.67229| 1.39E-02 |
| FOSB        | 1.08702| 9.10E-03 |

### Table 2 (continued)

| Gene symbol | log2FC | FDR   |
|-------------|--------|-------|
| FX5G51      | 1.89383| 1.39E-02 |
| BTN1L8      | 1.87248| 1.46E-02 |
| ACH1        | 1.18749| 1.52E-02 |
| KCNJ11      | 1.81031| 1.52E-02 |
| ENPP3P2     | 1.45231| 1.64E-02 |
| CCKBR       | 2.06353| 1.73E-02 |
| PCSK1N      | 1.87853| 1.76E-02 |
| RP1-114G11.5| 3.04186| 1.76E-02 |
| EFTUD1P1    | 2.13819| 1.77E-02 |
| OR2N1P      | 5.08848| 1.83E-02 |
| CA10        | 2.21148| 1.85E-02 |
| SLCSO5A1    | 1.55420| 1.88E-02 |
| IBSP        | 1.81676| 1.91E-02 |
| RP1-18821.1 | 3.56590| 1.94E-02 |
| MAGEA9      | 5.10603| 1.96E-02 |
| SYP         | 1.03514| 2.15E-02 |
| NBEAP1      | 1.55393| 2.17E-02 |
| RPS-965G21.4| 1.14894| 2.18E-02 |
| MPZ         | 1.95997| 2.22E-02 |
| RP1-299H22.3| 2.95030| 2.22E-02 |
| FBXO2       | 1.34819| 2.31E-02 |
| LGSN        | 2.41190| 2.31E-02 |
| RDH8        | 2.14341| 2.32E-02 |
| AC073325.2  | 1.90901| 2.38E-02 |
| GAGE1       | 3.92238| 2.44E-02 |
| MYB         | 1.16344| 2.55E-02 |
| AATK        | 1.23997| 2.63E-02 |
| DOK7        | 1.48390| 2.72E-02 |
| AC068580.7  | 2.45533| 2.72E-02 |
| RP1-360D19.9| 1.99056| 2.72E-02 |
| HAPLN2      | 1.51979| 2.73E-02 |
| TDRD12      | 2.24362| 3.09E-02 |
| RP1-159H10.3| 1.83751| 3.18E-02 |
| CHGB        | 1.62908| 3.32E-02 |
| RCN3        | 1.25033| 3.32E-02 |
| RPS-1171110.5| 1.33948| 3.32E-02 |
| NMRK2       | 2.15724| 3.33E-02 |
| TNN13       | 1.50305| 3.33E-02 |
| DPEP3       | 1.81124| 3.33E-02 |
| DYSL5       | 2.12990| 3.33E-02 |
| ZNF365      | 1.53513| 3.39E-02 |
| KCNQ2       | 1.98246| 3.51E-02 |
| PMP2        | 2.27248| 3.53E-02 |
| HAVCR1      | 2.17185| 3.56E-02 |
| RP1-140K8.5 | 1.72113| 3.72E-02 |
| ROR1-A51    | 2.03339| 3.75E-02 |
| C1QTNF1-A51 | 1.82857| 3.75E-02 |
| WFDCC1      | 1.74139| 3.99E-02 |
| DEFBI126    | 2.40171| 3.99E-02 |
| LBP         | 1.71514| 3.99E-02 |
### Table 2 (continued)

**Up-regulated genes in alive x dead**

| Gene symbol | log2FC | FDR       |
|-------------|--------|-----------|
| CDH12       | 2.39970| 3.99E-02  |
| FABP7       | 2.11963| 3.99E-02  |
| MAGEB17     | 1.73309| 3.99E-02  |
| MYBPC1      | 1.99301| 3.99E-02  |
| RP4-764D2.1 | 1.03824| 4.07E-02  |
| CDSL        | 1.77129| 4.21E-02  |
| CPN2        | 1.81842| 4.21E-02  |
| ZNF727      | 2.20430| 4.21E-02  |
| CST1        | 2.16587| 4.35E-02  |
| RP11-9G1.3  | 2.55148| 4.48E-02  |
| LL22NC03-22D1.1 | 2.49624 | 4.50E-02 |
| NPFRR1      | 1.60868| 4.54E-02  |
| MYRIP       | 1.45769| 4.54E-02  |
| RP11-369C8.1| 2.61906| 4.95E-02  |

**Down-regulated genes in alive x dead**

| Gene symbol | log2FC | FDR       |
|-------------|--------|-----------|
| AVPR1A      | -2.06066| 8.65E-08  |
| KRT16P2     | -5.08308| 1.00E-07  |
| CHST8       | -3.38063| 7.38E-07  |
| ST6GAL2     | -2.68516| 1.74E-06  |
| PRSS35      | -3.33395| 4.71E-06  |
| SLC8A3      | -3.18840| 9.15E-06  |
| CRABP1      | -3.07142| 9.15E-06  |
| HSPB3       | -3.24770| 2.15E-05  |
| TREGX2      | -2.43750| 2.15E-05  |
| B3GNT4      | -1.77784| 2.26E-05  |
| NPTX1       | -2.61603| 1.97E-04  |
| PI3         | -3.60121| 2.70E-04  |
| HEYL        | -1.67534| 2.99E-04  |
| ID3         | -1.32736| 3.67E-04  |
| ADRB2       | -1.81924| 3.91E-04  |
| C6orf223    | -2.13191| 4.15E-04  |
| AJAP1       | -1.92423| 4.56E-04  |
| CHRNA4      | -2.95110| 4.69E-04  |
| RHCG        | -2.92093| 5.10E-04  |
| PART1       | -2.14901| 5.50E-04  |
| ALOX12      | -1.23574| 5.50E-04  |
| RSPD3       | -1.48997| 6.13E-04  |
| GDPD3       | -1.74062| 7.38E-04  |
| CNFN        | -2.84236| 8.27E-04  |
| ANO2        | -1.70534| 9.14E-04  |
| OVOL1       | -2.75293| 9.14E-04  |
| CLDN4       | -2.52180| 9.56E-04  |
| GREB1L      | -2.33513| 1.12E-03  |
| IGLV9-40    | -3.84182| 1.20E-03  |
| TGM1        | -2.85878| 1.28E-03  |
| CYSTR1      | -2.10472| 1.34E-03  |
| LYPD5       | -2.27815| 1.34E-03  |

### Table 2 (continued)

**Down-regulated genes in alive x dead**

| Gene symbol | log2FC | FDR       |
|-------------|--------|-----------|
| CYP19A1     | -1.84923| 1.41E-03  |
| ACTG2       | -1.63424| 1.41E-03  |
| ABCC4       | -2.12582| 1.41E-03  |
| FAM3D       | -2.12972| 1.41E-03  |
| PAPPS2      | -1.50601| 1.41E-03  |
| AC124789.1  | -2.07689| 1.41E-03  |
| RASL11B     | -1.54784| 1.43E-03  |
| MAFB        | -1.28214| 1.43E-03  |
| NGF         | -1.73693| 2.01E-03  |
| COO06116.20 | -1.47472| 2.01E-03  |
| S100A12     | -2.64402| 2.10E-03  |
| KLK14       | -2.29647| 2.39E-03  |
| IL1RN       | -2.09008| 2.39E-03  |
| B3GNT8      | -1.78028| 2.39E-03  |
| GPX3        | -1.88978| 2.39E-03  |
| CDA         | -2.22423| 2.68E-03  |
| CHN1        | -1.30585| 2.68E-03  |
| PRSS27      | -1.71351| 2.68E-03  |
| TMEM79      | -1.43089| 3.52E-03  |
| NOTCH3      | -1.22297| 3.57E-03  |
| FGF18       | -1.35348| 3.80E-03  |
| HGF         | -1.19158| 4.28E-03  |
| SH3RF3      | -1.23159| 4.49E-03  |
| KRT37       | -4.22818| 4.57E-03  |
| SH3RF3-AS1  | -1.31293| 4.57E-03  |
| ZC3H12A     | -1.35742| 4.67E-03  |
| TDX4        | -1.84817| 5.39E-03  |
| CLIC3       | -2.31078| 5.58E-03  |
| LRRCA4      | -1.34142| 5.69E-03  |
| NRARP       | -1.42862| 5.97E-03  |
| B3GNT3      | -1.97566| 6.61E-03  |
| ELF3        | -2.07351| 6.73E-03  |
| LRRN2       | -1.90993| 6.80E-03  |
| NFE4        | -2.76901| 6.80E-03  |
| KLK10       | -2.72909| 6.94E-03  |
| LINCO1121   | -1.68460| 7.15E-03  |
| SDCBP2      | -1.48707| 7.88E-03  |
| FAM83G      | -1.50253| 7.88E-03  |
| ZNF469      | -1.06394| 7.88E-03  |
| ROR2        | -1.15831| 7.92E-03  |
| LYNX1       | -1.75074| 7.92E-03  |
| VSIG10L     | -1.54237| 7.92E-03  |
| PITX1       | -2.50531| 8.39E-03  |
| PNMA5       | -2.07397| 8.43E-03  |
| LTB4R2      | -1.38009| 8.43E-03  |
| GTSF1       | -1.95576| 8.89E-03  |
| RP3-449H6.1 | -2.80745| 8.89E-03  |
| PKDCC       | -1.47224| 9.03E-03  |
| Gene symbol | log2FC | FDR |
|-------------|-------|-----|
| C11orf87    | -1.86276 | 9.10E-03 |
| C9orf47     | -1.63063 | 9.10E-03 |
| KLK12       | -3.43996 | 9.10E-03 |
| SPNS2       | -1.33949 | 9.43E-03 |
| TCHH        | -1.56077 | 1.00E-02 |
| ADRA1D      | -1.45232 | 1.01E-02 |
| LINC00675   | -2.28274 | 1.01E-02 |
| GLP1R       | -1.93142 | 1.04E-02 |
| DLX5        | -1.61403 | 1.05E-02 |
| JUP         | -1.81687 | 1.10E-02 |
| GREM1       | -1.36182 | 1.10E-02 |
| FUT2        | -1.65558 | 1.10E-02 |
| FLJ43879    | -1.73000 | 1.10E-02 |
| RP4-530I15.9| -1.37950 | 1.10E-02 |
| ADAMTS14    | -1.15965 | 1.10E-02 |
| LGALS9C     | -1.74166 | 1.11E-02 |
| RP11-145A3.1| -1.28593 | 1.20E-02 |
| LINC00689   | -2.44100 | 1.20E-02 |
| RP11-57C13.6| -2.13756 | 1.20E-02 |
| WNT11       | -1.67218 | 1.23E-02 |
| Sox11       | -1.31510 | 1.27E-02 |
| SMCO2       | -1.86262 | 1.29E-02 |
| ACO10465.4  | -1.54674 | 1.34E-02 |
| RP11-715H19.2| -2.54830 | 1.45E-02 |
| MALL        | -1.65870 | 1.50E-02 |
| SLP1        | -2.56276 | 1.50E-02 |
| ZNF385B     | -1.82165 | 1.52E-02 |
| AC079305.10 | -1.07337 | 1.52E-02 |
| RARRE1      | -1.09119 | 1.56E-02 |
| C1orf177    | -1.80915 | 1.64E-02 |
| MIR4635     | -1.81095 | 1.68E-02 |
| KCNK12      | -1.74033 | 1.70E-02 |
| CTC-252D6.2 | -3.65998 | 1.75E-02 |
| FGF7        | -1.14265 | 1.76E-02 |
| KRT82       | -2.71344 | 1.76E-02 |
| CTD-2554C21.3| -2.09957 | 1.76E-02 |
| VNN3        | -1.87348 | 1.94E-02 |
| KRT17P6     | -2.54509 | 1.94E-02 |
| CD36        | -1.47332 | 1.98E-02 |
| ALDH1J1     | -1.84277 | 1.99E-02 |
| KRT25       | -3.64328 | 2.04E-02 |
| GLI5        | -1.24797 | 2.06E-02 |
| SPRR2D      | -2.65861 | 2.06E-02 |
| CEACAM19     | -1.48405 | 2.11E-02 |
| ZNF154      | -1.19730 | 2.12E-02 |
| FBLM1       | -1.00682 | 2.15E-02 |
| OR7E11P     | -3.14060 | 2.15E-02 |
| ZNF185      | -1.43741 | 2.27E-02 |

| Gene symbol | log2FC | FDR |
|-------------|-------|-----|
| MYOC        | -3.14651 | 2.30E-02 |
| SULT2B1     | -2.16618 | 2.31E-02 |
| PLA2G4E-AS1 | -1.49669 | 2.32E-02 |
| IL36G       | -2.54342 | 2.39E-02 |
| IGKV2-29    | -3.58127 | 2.43E-02 |
| PAD3        | -1.60173 | 2.45E-02 |
| WDR87       | -2.67496 | 2.45E-02 |
| CTD-2555C10.3| -1.62741 | 2.45E-02 |
| SMPD3       | -1.51129 | 2.63E-02 |
| LYPD3       | -2.15151 | 2.63E-02 |
| SPINK9      | -2.18753 | 2.63E-02 |
| RF3-4OSJ10.2| -1.89008 | 2.63E-02 |
| KRT17       | -2.51044 | 2.65E-02 |
| RP11-845M18.6| -2.34005 | 2.70E-02 |
| CPXM2       | -1.33321 | 2.72E-02 |
| GDP2D       | -1.69177 | 2.79E-02 |
| LIN01482    | -1.58713 | 2.84E-02 |
| KRT8B13     | -2.24864 | 3.07E-02 |
| ANGPT2      | -1.42767 | 3.23E-02 |
| TMEM45B     | -1.81149 | 3.23E-02 |
| KLF13       | -2.41601 | 3.23E-02 |
| IGHE        | -2.55145 | 3.23E-02 |
| SPPR2A      | -2.94281 | 3.23E-02 |
| RP11-91J3.3 | -1.87453 | 3.23E-02 |
| CTC-490G23.2| -2.34066 | 3.32E-02 |
| ADAMTS15    | -1.20759 | 3.33E-02 |
| RHOD        | -1.51767 | 3.33E-02 |
| COL28A1     | -1.56903 | 3.33E-02 |
| RP11-752L20.3| -1.07531 | 3.33E-02 |
| AC133785.1  | -2.02852 | 3.47E-02 |
| GNA15       | -1.37100 | 3.49E-02 |
| FAM468      | -1.22702 | 3.53E-02 |
| KRT80       | -2.07094 | 3.53E-02 |
| TWIST2      | -1.11079 | 3.53E-02 |
| KRT42P      | -2.45179 | 3.53E-2 |
| PRSS50      | -1.86776 | 3.64E-02 |
| TBPX1       | -1.26122 | 3.69E-2 |
| KRT81       | -1.69382 | 3.75E-02 |
| ALOX12B     | -2.41352 | 3.75E-02 |
| KCNMA1-AS1  | -1.82562 | 3.79E-02 |
| HS3ST3A1    | -1.28721 | 3.84E-02 |
| USP2        | -1.07928 | 3.99E-02 |
| BMP4        | -1.23615 | 3.99E-02 |
| G6PC2       | -2.36196 | 3.99E-02 |
| RP11-169K16.4| -1.98903 | 3.99E-02 |
| COLBA1      | -1.22669 | 4.09E-02 |
| SIX2        | -1.02697 | 4.09E-02 |
| KRT17P2     | -2.21877 | 4.21E-02 |
Our findings suggest that sensory neurons’ overactivation affects the immune response to melanoma. Melanoma progression is influenced by the complex interplay between cancer cells and different components of the immune system [8]. Melanoma cells may cause disruption of the organism’s immunity to overrun and escape the immune system control [96, 104]. The role of sensory innervations in these interactions remains completely unknown. Lymphocytes are the dominant immune elements found infiltrating the melanoma microenvironment. Their composition correlates with patients’ survival [38]. While regulatory T cells play pro-tumorigenic roles; CD8+ T cells, CD4+ T cells, γδ T cells, and NK cells have been shown to act against the transformed cells, [38, 43, 46, 47, 58, 64, 73, 118]. Conversely neutrophils and myeloid-derived suppressor cells have been associated with poor prognosis and are largely pro-tumorigenic [22, 28, 69, 126, 129, 145, 155]. Our data shows that sensory overactivation induce an increase in the number of tumor-infiltrating anti-cancer lymphocytes (CD8+ T cells, CD4+ T cells, γδ T cells, and NK cells), while we did not detect changes in the number of tumor-infiltrating regulatory T cells. We also found that there is a decrease in the number of neutrophils and myeloid-derived suppressor cells within the tumor. We found that these changes were tumor-specific, as we did not detect any alterations in the number of lymphocytes in the non-draining lymph nodes. Tumor-draining lymph nodes presented an increase in some of the anti-tumor lymphocytes probably because of the tumor-priming effect previously reported [135]. Signals transmitted to T cells via PD-1 or CTLA-4 (considered markers for T cells “exhaustion”) promote T cell dysfunction, thereby turning off the immune response [59, 98, 149]. We found that the tumor-infiltrating lymphocytes decrease their expression of PD1 and CTLA-4, possibly indicating that these cells are “less exhausted” within the melanoma microenvironment after sensory hyperactivation. The more active phenotypes of lymphocytes have been associated to the increase in the production of cytokines. A variety of lymphocytes are capable of producing IL-17 [14, 20, 24, 53, 56, 77, 94, 103] which has presented anti-tumorigenic effects in melanoma [3, 63, 74, 75, 91, 99, 100]. We found an increase in tumor-infiltrating lymphocytes producing IL-17 after sensory stimulation. Thus, in light of our overall findings, we suggest that induced increase in firing of sensory innervations contributes to boosting

### Table 2 (continued)

| Gene symbol | log2FC  | FDR     |
|-------------|--------|---------|
| BDKRB1      | -1.22248 | 4.35E-02 |
| LINC00857   | -1.30729 | 4.35E-02 |
| CREBL1      | -1.04632 | 4.44E-02 |
| CCB1        | -1.04094 | 4.44E-02 |
| PRR15L      | -1.31182 | 4.48E-02 |
| STB5I2A     | -1.70296 | 4.50E-02 |
| SI00A9      | -2.02615 | 4.55E-02 |
| SIK1        | -1.83293 | 4.60E-02 |
| EPN3        | -1.93269 | 4.65E-02 |
| ZBTB16      | -1.22227 | 4.67E-02 |
| ATP8B5P     | -1.00252 | 4.76E-02 |
| DUOX1       | -1.72011 | 4.78E-02 |
| TRIMS3CP    | -2.79481 | 4.80E-02 |
| C6orf132    | -1.81165 | 4.87E-02 |
| MDFI        | -1.36942 | 4.91E-02 |
| CATSPERB    | -1.85527 | 4.94E-02 |
| HSP1P5      | -1.47888 | 4.94E-02 |
| DEF8A4      | -3.10613 | 4.95E-02 |
| SDC1        | -1.36628 | 4.96E-02 |

Nevertheless, although biomed-ical research has gained some insights into the function of intra-tumoral neurons using loss-of-function studies with surgical or pharmacologic denervation, these strategies are mostly not specific to a given neuronal type. Importantly, a disadvantage of all these studies is that neuronal killing may generate secondary undesirable indirect side effects caused by the inflammatory tis-sular response which may influence the observed phenotypes (Männ et al. 2016; Christiaansen, Boggiatto, and Varga 2014; Bennett et al. 2005). To circumvent these limitations, novel powerful technologies have been created in the field of modern neuroscience which allow to manipulate the firing of specific neurons without killing them: optogenetics and chemogenetics. These methods use genetic strategies to deliver the expression of light-sensitive proteins or designer receptors exclusively activated by designer drugs, respectively, to the membrane of defined neuronal populations. Therefore, by using these techniques, manipulation of neurons by exposure to light or to designer drugs, without killing them, became feasible. As melanoma is a chronic disease, the use of optogenetics for longer periods, may not be the best approach, as the unavoidable surgical preparation with the chronic implantation of hardware for stimulation and prolonged exposure to highly energetic laser light will eventually culminate in confounding regional inflammation and tissue degradation. Therefore, we chose to use chemogenetics to examine the participation of sensory neurons in melanoma development, as it is more suitable to evaluate the long-term effects of sensory stimulations with less side-effects. Future studies may use similar approaches to explore the role of sensory neurons and other innervations in other cancers.
of the immune response against melanoma. Future studies will need to explore the exact molecular mechanisms involved in the interactions of sensory neurons and immune cells in the melanoma microenvironment. A variety of cellular and molecular mechanisms may be involved in the effect of sensory neurons’ modulation on melanoma behavior. For instance, it has been documented that the same drug that is used to denervate sensory neurons, resiniferatoxin (RTX), an analogue of capsaicin, also induces stress by causing hyperactivation of the sympathetic nervous system [16, 62, 157]. These studies also revealed that sensory nerves may tune down sympathetic nerve activity [16, 62, 105]. Sympathetic neurons release norepinephrine [70, 123], which has been shown to strongly induce tumorigenesis [1, 60, 154]. It remains open the important question whether the effect of sensory innervations in the tumor microenvironment depend also on the modulation of the sympathetic tone.

**Future perspectives**

The present study reveals the short-term impact of chemogenetic modulation of sensory neurons on melanoma behavior. It remains to be examined what are the long-term effects of this manipulations. In the current study, the sensory neurons’ activity is being continuously inhibited or overactivated. Are changes in sensory neurons’ activity at specific time points sufficient to influence cancer outcomes? Also, it remains to be determined what are the changes within the tumor microenvironment at different stages of cancer progression. Are some stages more sensible to changes in the activity of sensory neurons? Moreover, this study focuses on melanoma tumors. Future studies should explore what is the role of sensory neurons in the development of other solid tumors. A variety of factors secreted from sensory neurons may be implicated in the regulation of the melanoma microenvironment described here [18]. The overactivation of sensory nerve fibers may induce the release of neuropeptides, such as substance P, CGRP, VIP, GRP, neurokinin A, neurokinin B, neuropeptide Y (NPY) and adrenomedullin, which have been shown to interact
Table 3  Gene Ontology (GO) and analysis of Biological Processes (BP) in which specific genes are involved

| Functional category                                   | Genes in list | Total genes | FDR            | Genes                                                                 |
|-------------------------------------------------------|---------------|-------------|----------------|----------------------------------------------------------------------|
| Up-regulated genes in alive × dead                   |               |             |                |                                                                      |
| Nervous system development                            | 22            | 2474        | 0.004805005   | ARX HAPLN2 SLITRKS VCX3A VCX ZNF365 NRTN TFAFP28 NGFR MYB GFAP LRRTM2  |
|                                                       |               |             |                | LHPFL4 DFPYSL5 FABP7 FOX1 VIT MPZ CA10 KCNQ2 ACHE HPICAL4             |
| Central nervous system development                    | 12            | 1054        | 0.029644095   | HAPLN2 VCX3A VCX ARX GFAP ZNF365 FABP7 SLITRKS FOX1 VIT CA10 HPICAL4 |
| Regulation of hormone levels                          | 9             | 583         | 0.029644095   | ACHE PCSX1N MYB VGF RDHS BCO1 TFAFP2 BCN1J11 MYRIP                   |
| Down-regulated genes in alive × dead                  |               |             |                |                                                                      |
| Cornification                                         | 17            | 125         | 8.55E−15      | TME79 TGM1 KRT37 P13 KRT12 TCHH KRT82 SPRR2D KLK13 KRT80 JUP KKL12 KRT82 |
|                                                       |               |             |                | KRT25 SPINK9 KRT81 SPRR2A                                            |
| Epithelium development                                | 42            | 1386        | 9.40E−15      | KLK14 SPRR2D OVOL1 NARP R SPRR2A HGF TGM1 BMIP4 FGF7 AJAP1 WNT11 CNFN DLX5 ALOX12 SDC1 ID3 TBX4 KRT17 GRE81 ADAMTS14 RSP03 TCHH ELF3 TME79 HEYL GREM1 ROR2 SOKX11 KRT25 SULT2B1 TX1 RHCG KRT37 P13 KRT82 KKL13 KRT80 JUP KKL12 SPINK9 KRT81 |
| Tissue development                                    | 51            | 2168        | 2.40E−14      | KLK14 SPRR2D OVOL1 NARP R SPRR2A HGF TGM1 BMIP4 FGF7 AJAP1 PFX1 WNT11 SMPO3 CNFN DLX5 ALOX12 SDC1 ID3 TBX4 KRT17 GRE81 ADAMTS14 RSP03 TCHH PKDCC ELF3 TME79 HEYL GREM1 ROR2 ADRB2 S2OX12 S11 KRT25 COL8A1 SULT2B1 TX1 RHCG KRT37 P13 KRL82 KKL13 KRT80 JUP KKL12 SPINK9 KRT81 |
| Skin development                                      | 23            | 464         | 1.11E−11      | SPRR2D SPRR2A TGM1 CNFN ALOX12 KRT12 TCHH FGF7 TCHH TME79 OVO1 JUP ALOX12 B2 TME82 CLDN4 KRT37 P13 KRL82 KKL13 KRT80 JUP KKL12 SPINK9 KRT81 |
| Keratinization                                        | 18            | 268         | 4.20E−11      | TGM1 CNFN KRT17 TCHH SPRR2D TME799 SPRR2A KRT37 P13 KRL82 KKL13 KRT80 JUP KKL12 SPINK9 KRT81 |
| Animal organ development                              | 62            | 3779        | 4.72E−11      | SPRR2D HEYL S2OX12 BMIP4 FGF7 GRE81 AJAP1 MAFB MYOC USP2 PFX1 NOTCH3 WNT11 ANGPT2 SLCA83 SMPO3 CNFN DLX5 ALOX12 MDF1 SDC1 ID3 TBX4 KRT17 CYP19A1 COL8A1 RSP03 FGF18 TCHH PKDCC ELF3 TME79 HEYL GREM1 ROR2 ADRB2 S2OX12 S11 KRT25 COL8A1 SULT2B1 TX1 RHCG KRT37 P13 KRL82 KKL13 KRT80 JUP KKL12 SPINK9 KRT81 |
| Epidermal cell differentiation                        | 21            | 410         | 5.29E−11      | SPRR2D OVOL1 SPRR2A TGM1 BMIP4 CNFN KRT17 TCHH TME799 SULT2B1 TX1 RHCG KRT37 P13 KRL82 KKL13 KRT80 JUP KKL12 SPINK9 KRT81 |
| Epidermis development                                 | 23            | 521         | 6.96E−11      | KLK14 SPRR2D OVOL1 SPRR2A TGM1 BMIP4 CNFN KRT17 TCHH TME799 SULT2B1 TX1 RHCG KRT37 P13 KRL82 KKL13 KRT80 JUP KKL12 SPINK9 KRT81 |
| Keratinocyte differentiation                          | 18            | 346         | 1.87E−09      | SPRR2D SPRR2A TGM1 CNFN KRT17 TCHH TME799 KRT37 P13 KRL14 KRT82 KKL13 KRT80 JUP KKL12 SPINK9 KRT81 |
| Ossification                                          | 18            | 396         | 1.56E−08      | MYOC ZBT16 BMIP4 TWIST2 WNT11 SMPO3 DLX5 ID3 GPD2D2 FGF18 PKDCC ROR2 ADRB2 S2OX12 S11 GREM1 CREB3L1 HGF |
| Cellular developmental process                        | 64            | 6471        | 3.21E−08      | WNT11 DLX5 ID3 BMIP4 NGF SPRR2D ELF3 HEYL OVOL1 RHOD SOX11 SPRR2A MYOC TGM1 ZC3H12A GREM1 AJAP1 MABF TWIST2 HGF PFX1 NOTCH3 ANGPT2 SLCA83 SMPO3 CNFN ZBT16 MDF1 SDC1 KRT17 CHN1 GPD2 CATSPE8 CD36 SIK1 ADAMTS4 LGF TCHH FBLUM1 PKDCC TME799 AVPR1A ROR2 ADRB2 S2OX12 GTSF1 NARP COL8A1 SULT2B1 CREB3L1 S100A9 TX1 RHCG KRT37 P13 KRL14 KRT82 KKL13 KRT80 JUP KKL12 KRT25 SPINK9 KRT81 |
| Cell differentiation                                  | 62            | 4459        | 3.79E−08      | WNT11 DLX5 ID3 BMIP4 NGF SPRR2D HEYL OVOL1 SOX11 SPRR2A MYOC TGM1 ZC3H12A AJAP1 MABF TWIST2 HGF PFX1 NOTCH3 ANGPT2 SLCA83 SMPO3 CNFN ZBT16 MDF1 SDC1 KRT17 CHN1 GPD2 CATSPE8 CD36 SIK1 ADAMTS4 LGF TCHH PKDCC TME799 AVPR1A GREM1 ROR2 ADRB2 S2OX12 GTSF1 NARP COL8A1 SULT2B1 CREB3L1 S100A9 TX1 RHCG KRT37 P13 KRL14 KRT82 KKL13 KRT80 JUP KKL12 KRT25 SPINK9 KRT81 |
### Table 3 (continued)

#### Down-regulated genes in alive x dead

| Functional category                                      | Genes in list | Total genes | FDR    | Genes                                                                 |
|----------------------------------------------------------|---------------|-------------|--------|-----------------------------------------------------------------------|
| **Anatomical structure morphogenesis**                   | 46            | 2785        | 1.00E−07 | PITX1 DLX5 KLK14 NGF HEYL RHOD NRARP HGF MYOC BMP4 IL1RN FGF7 ZC3H12A BMP4 SMAD3 ALOX12 ZBTB16 MDFI SDC1 ID3 Tbx4 Krt17 Chn1 CD36 GREB1L COLA1 RSPD3 FGF18 FBPLM1 PKDCC ELF3 TME79 ROR2 SIX2 SOX11 CCB1 MABF KRT25 CREB3L1 JUP TXB1 ACTG2 |
| **Tissue morphogenesis**                                | 21            | 719         | 8.87E−07 | KLK14 NRARP HGF BMP4 A1AP1 Wnt11 ALOX12 Tbx4 Krt17 Fgf7 GREB1L RSPD3 TME79 HEYL GREM1 ROR2 SIX2 SOX11 KRT25 ACTG2 TXB1 |
| **Regulation of cartilage development**                 | 8             | 68          | 1.39E−06 | ZBTB16 BMP4 SMAD3 FGF18 PKDCC SIX2 GREM1 Wnt11 |
| **Tube development**                                    | 25            | 1062        | 1.93E−06 | NRARP BMP4 FGF7 ZC3H12A HGF NOTCH3 Wnt11 ANGPT2 SMAD3 ALOX12 SDC1 Tbx4 GREB1L COLA1 RSPD3 FGF18 PKDCC HEYL GREM1 SIX2 SOX11 CCB1 CREB3L1 JUP TXB1 |
| **Morphogenesis of an epithelium**                       | 18            | 581         | 3.91E−06 | KLK14 NRARP HGF BMP4 A1AP1 Wnt11 ALOX12 Tbx4 Krt17 Fgf7 GREB1L RSPD3 TME79 GREM1 ROR2 SIX2 SOX11 KRT25 |
| **Regulation of anatomical structure morphogenesis**     | 25            | 1125        | 4.57E−06 | NGF RHOD HGF MYOC BMP4 IL1RN FGF7 ZC3H12A BMP4 A1AP1 Wnt11 ANGPT2 SMAD3 ALOX12 SDC1 Tbx4 GREB1L COLA1 RSPD3 FGF18 PKDCC HEYL GREM1 SIX2 SOX11 CCB1 CREB3L1 JUP TXB1 |
| **Regulation of ossification**                           | 11            | 193         | 4.57E−06 | ZBTB16 BMP4 TWIST2 SDC3 GDPPD2 PKDCC ADRB2 SIX2 SOX11 GREM1 HEYL |
| **Skeletal system development**                          | 17            | 541         | 6.10E−06 | ZBTB16 BMP4 MYOC PITX1 SMD3 DLX5 MDFI FGF18 PKDCC ROR2 SIX2 SOX11 PAPSS2 Wnt11 Tbx4 GREM1 TXB1 |
| **Renal system development**                             | 13            | 307         | 7.35E−06 | SIX2 BMP4 S AGE1L NOTCH3 Wnt11 ANGPT2 ZBTB16 SDC1 ID3 HEYL GREM1 OVO1 SSOX1 |
| **Animal organ morphogenesis**                           | 24            | 1099        | 1.06E−05 | HEYL BMP4 FGF7 A1AP1 Wnt11 SMD3 DLX5 MDFI SDC1 HEYL BMP4 COLA1 FGF18 ELF3 GREM1 ROR2 SIX2 SOX11 MABF TBBX4 TXB1 ACTG2 RSPD3 |
| **Osteoblast differentiation**                           | 11            | 218         | 1.18E−05 | MYOC BMP4 TWIST2 WNT11 DLX5 ID3 GDPPD2 GREM1 SDC1 CREB3L1 HEYL |
| **Regulation of developmental process**                  | 41            | 2763        | 1.21E−05 | ID3 NGF HEYL RHOD HGF MYOC ZBTB16 BMP4 IL1RN FGF7 ZC3H12A A1AP1 MABF TWIST2 NOTCH3 Wnt11 ANGPT2 SMAD3 ALOX12 SDC1 Tbx4 GREM1 SDC3 GDPPD2 CD36 FGF18 FBPLM1 PKDCC TME79 GREM1 ROR2 ADRB2 SIX2 SOX11 CCB1 CREB3L1 JUP ULT2B1 SSK1 S100A9 TXB1 RSPD3 |
| **Anatomical structure formation involved in morphogenesis** | 24            | 1164        | 2.67E−05 | DLX5 NRARP BMP4 ZC3H12A HGF NOTCH3 Wnt11 ANGPT2 SMAD3 Tbx4 CD36 COLA1 RSPD3 FGF18 HEYL GREM1 ROR2 SIX2 SOX11 CCB1 CREB3L1 JUP TXB1 |
| **Skeletal system morphogenesis**                        | 11            | 245         | 3.39E−05 | SMD3 DLX5 MDFI BMP4 FGF18 ROR2 SIX2 SOX11 Tbx4 GREM1 TXB1 |
| **Regulation of chondrocyte differentiation**            | 6             | 49          | 3.91E−05 | BMP4 FGF18 PKDCC SIX2 ZBTB16 GREM1 |
| **Appendage morphogenesis**                             | 9             | 155         | 3.91E−05 | PITX1 DLX5 ZBTB16 Tbx4 BMP4 PKDCC GREM1 ROR2 SOX11 |
| **Limb morphogenesis**                                  | 9             | 155         | 3.91E−05 | PITX1 DLX5 ZBTB16 Tbx4 BMP4 PKDCC GREM1 ROR2 SOX11 |
| **Tube morphogenesis**                                  | 20            | 860         | 3.91E−05 | NRARP BMP4 ZC3H12A HGF NOTCH3 Wnt11 ANGPT2 ALOX12 Tbx4 GREM1 COLA1 RSPD3 FGF18 HEYL GREM1 SDOX11 CCB1 CREB3L1 JUP TXB1 |
| **Regulation of morphogenesis of an epithelium**         | 10            | 204         | 4.32E−05 | HGF BMP4 A1AP1 Wnt11 ALOX12 FGF18 GREM1 ROR2 SIX2 RSPD3 |
| **Epidermis morphogenesis**                             | 5             | 29          | 5.24E−05 | KLK14 KRT17 FGF7 TME79 KRT25 |
| **Angiogenesis**                                         | 15            | 511         | 5.42E−05 | NRARP BMP4 ZC3H12A HGF NOTCH3 ANGPT2 Tbx4 COLA1 RSPD3 FGF18 GREM1 CCB1 CREB3L1 JUP TXB1 |
| **Cell migration**                                       | 27            | 1506        | 5.42E−05 | HGF SDC1 ILL6G ILRN DFB4A RHOD MYOC ANGPT2 BMP4 FGF7 FGF18 S100A9 ZC3H12A ROR2 SIX2 Wnt11 BDBR1 SMD3 ALOX12 CYPI9A1 GREM1 CCB1 CREB3L1 JUP TWST2 Tbx1 S100A12 |
| **Cell death**                                            | 36            | 2415        | 5.45E−05 | HGF BMP4 NGF ADAM5L4 ZNF385B S100A9 ZC3H12A Wnt11 ALOX12 ZBTB16 ID3 CREB3L1 TME79 GREM1 SDOX11 TWST2 CD36 SSK1 TXB1 PINAS TG41 KRT37 R3 KRT7 KLK14 TCHH KRT25 SPARO KLK13 KRT10 JUP KLK12 KRT25 SPIN9 KRT81 SPARO |
| **Respiratory system development**                       | 10            | 215         | 6.07E−05 | BMP4 FGF SMAD3 DLX5 Tbx4 FGF18 PKDCC SOX1 CCB1 Wnt11 |
| **Cartilage development**                               | 10            | 216         | 6.17E−05 | ZBTB16 BMP4 PITX1 SMD3 FGF18 PKDCC ROR2 SIX2 GREM1 Wnt11 |
### Table 3 (continued)

| Functional category                                      | Genes in list | Total genes | FDR       | Genes                                                                 |
|----------------------------------------------------------|---------------|-------------|-----------|----------------------------------------------------------------------|
| Positive regulation of ossification                      | 7             | 88          | 6.78E-05  | ZBTB16 BMP4 GDPD2 PKDCC ADRB2 SOX11 HGF                               |
| Regulation of multicellular organismal development       | 33            | 2138        | 7.25E-05  | ID3 NGF HEYL HGF ZBTB16 BMP4 IL1RN FGF7 ZC3H12A AIPAP1 MAPB NOTCH3 WNT11 ANGPT2 SMPD3 ALOX12 KRT17 CHN1 FGF18 PKDCC GREM1 ROR2 ADRB2 SX2 SOX11 CEB1 NRARP CREB3L1 JUP SULT2B1 S100A9 TBX1 RSP03 |
| Blood vessel morphogenesis                               | 16            | 603         | 7.46E-05  | NRARP BMP4 ZC3H12A HGF NOTCH3 WNT11 ANGPT2 TX4 COLA1 RSP03 FGF18 GREM1 CEB1 CREB3L1 JUP TBX1 |
| Programmed cell death                                    | 34            | 2257        | 8.00E-05  | HGF BMP4 NGF ADAMTS4 ZNF358B S100A9 WNT11 ALOX12 ZBTB16 ID3 CREB3L1 TME79 ZC3H12A GREM1 TWIST2 SX11 TNAP TGM1 KRT37 P13 KRT17 KLK14 TCHN KRT82 SPRR2D KLK13 KRT80 JUP KLK12 KRT25 SPINK9 KRT81 SPRR2A |
| Blood vessel development                                 | 17            | 687         | 8.58E-05  | HEYL NRARP BMP4 ZC3H12A HGF NOTCH3 WNT11 ANGPT2 TX4 COLA1 RSP03 FGF18 GREM1 CEB1 CREB3L1 JUP TBX1 |
| Embryonic morphogenesis                                  | 16            | 615         | 8.98E-05  | BMP4 IL1RN PITX1 WNT11 Dlx5 ZBTB16 MDFI TX4 RSP03 GREM1 ROR2 SX2 SOX11 MAFB COLA1 TX1 |
| Cell motility                                            | 28            | 1670        | 9.30E-05  | HGF SDC1 IL36G IL1RN DEFB4A RHOD MYOC ANGPT2 BMP4 FGF7 FGF18 S100A9 ZC3H12A ROR2 SX2 WNT11 BOKR1 SMPD3 ALOX12 CYP19A1 GREM1 CEB1 LTB4R2 DUOX1 JUP TWIST2 TX1 S100A12 |
| Localization of cell                                     | 28            | 1670        | 9.30E-05  | HGF SDC1 IL36G IL1RN DEFB4A RHOD MYOC ANGPT2 BMP4 FGF7 FGF18 S100A9 ZC3H12A ROR2 SX2 WNT11 BOKR1 SMPD3 ALOX12 CYP19A1 GREM1 CEB1 LTB4R2 DUOX1 JUP TWIST2 TX1 S100A12 |
| Cellular response to growth factor stimulus              | 17            | 694         | 9.30E-05  | HGF BMP4 NGF FGF18 CREB3L1 GREM1 CEB1 FAM83G ANGPT2 SMPD3 DLX5 RAS1L1B FGF7 HEYL ROR2 SOX11 TX1 |
| Cell–cell signaling                                      | 29            | 1774        | 9.65E-05  | WNT11 CHRNA4 NGF RSP03 NRARP JUP HGF CYP19A1 GREM1 FM3D SLC8A3 SMPD3 Dlx5 Dcl1 BMP4 IL1RN FGF7 ROR2 ADRB2 SOX11 LYNX1 MYOC G6PC2 MDFI GLR1 IL36G FGF18 S100A9 ADR31D |
| Tissue migration                                          | 11            | 293         | 1.10E-04  | ANGPT2 BMP4 FGF7 FGF18 ZC3H12A ALOX12 GREM1 CEB1 LTBR4 JUP ACTG2 |
| Appendage development                                    | 9             | 186         | 1.10E-04  | PITX1 DLX5 ZBTB16 TX4 BMP4 PKDCC GREM1 ROR2 SOX11 |
| Limb development                                          | 9             | 186         | 1.10E-04  | PITX1 DLX5 ZBTB16 TX4 BMP4 PKDCC GREM1 ROR2 SOX11 |
| Lung development                                          | 9             | 188         | 1.18E-04  | BMP4 FGF7 SMPD3 TX4 FGF18 PKDCC SOX11 CEB1 WNT11 |
| Vasculature development                                  | 17            | 715         | 1.20E-04  | HEYL NRARP BMP4 ZC3H12A HGF NOTCH3 WNT11 ANGPT2 TX4 COLA1 RSP03 FGF18 GREM1 CEB1 CREB3L1 JUP TBX1 |
| Respiratory tube development                              | 9             | 192         | 1.34E-04  | BMP4 FGF7 SMPD3 TX4 FGF18 PKDCC SOX11 CEB1 WNT11 |
| Response to growth factor                                 | 17            | 723         | 1.34E-04  | HGF BMP4 NGF FGF18 CREB3L1 GREM1 CEB1 FAM83G ANGPT2 SMPD3 DLX5 RAS1L1B FGF7 HEYL ROR2 SOX11 TX1 |
| Cardiovascular system development                         | 17            | 724         | 1.34E-04  | HEYL NRARP BMP4 ZC3H12A HGF NOTCH3 WNT11 ANGPT2 TX4 COLA1 RSP03 FGF18 GREM1 CEB1 CREB3L1 JUP TBX1 |
| Locomotion                                               | 30            | 1921        | 1.34E-04  | HGF SDC1 IL36G IL1RN DEFB4A RHOD MYOC ANGPT2 BMP4 FGF7 FGF18 S100A9 ZC3H12A ROR2 SX2 WNT11 BOKR1 SMPD3 DLX5 ALOX12 C1H7 CYP19A1 GREM1 CEB1 LTB4R2 DUOX1 JUP TWIST2 TX1 S100A12 |
| Mesonephros development                                  | 7             | 104         | 1.40E-04  | BMP4 ZBTB16 SDC1 CREB1 GREM1 SX2 WNT11 |
| Positive regulation of developmental process             | 25            | 1433        | 1.40E-04  | BMP4 ZBTB16 BMP4 FGF7 ZC3H12A HGF ANGPT2 ALOX12 KRT17 GDPD2 CD36 FGF18 PKDCC ADRB2 SOX11 HEYL GREM1 ROR2 ADRB2 SOX11 CEB1 JUP SULT2B1 S100A9 TX1 |
| Nephron development                                      | 8             | 147         | 1.41E-04  | BMP4 NOTCH3 ANGPT2 GREL1 HEYL GREM1 SX2 WNT11 |
| Positive regulation of cell communication                | 30            | 1937        | 1.48E-04  | BMP4 IL36G IL1RN RSP03 NRARP JUP HGF MYOC KL14 CYP19A1 FGF18 S100A9 S100A12 ZC3H12A ADRB2 ALOX12 CEB1 SLC8A3 DLX5 CHN1 CD36 GREM1 ROR2 SOX11 WNT11 SHRP3 NGF TBX1 FGF7 ELF3 |
| Positive regulation of signaling                         | 30            | 1945        | 1.56E-04  | BMP4 IL36G IL1RN RSP03 NRARP JUP HGF MYOC KL14 CYP19A1 FGF18 S100A9 S100A12 ZC3H12A ADRB2 ALOX12 CEB1 SLC8A3 DLX5 CHN1 CD36 GREM1 ROR2 SOX11 WNT11 SHRP3 NGF TBX1 FGF7 ELF3 |
| Circulatory system development                           | 21            | 1077        | 1.56E-04  | HEYL NRARP BMP4 ZC3H12A HGF NOTCH3 WNT11 ANGPT2 ID3 TX4 GREL1 COLA1 RSP03 FGF18 GREM1 SOX11 CEB1 CREB3L1 JUP TBX1 SKT1 |
| Positive regulation of signal transduction               | 28            | 1762        | 1.91E-04  | BMP4 IL36G IL1RN RSP03 NRARP JUP HGF MYOC KL14 FGF18 S100A9 S100A12 ZC3H12A ADRB2 ALOX12 CEB1 DLX5 CHN1 CD36 GREM1 ROR2 SOX11 WNT11 SHRP3 NGF TBX1 FGF7 ELF3 |
Table 3 (continued)

| Functional category                                      | Genes in list | Total genes | FDR   | Genes                                                                 |
|----------------------------------------------------------|---------------|-------------|-------|----------------------------------------------------------------------|
| Ameboidal-type cell migration                             | 12            | 392         | 2.39E−04 | ANGPT2 BMP4 FGFr7 FGFr8 ZC3H12A WNT11 ALOX12 GREM1 CCB1 LTBR2 JUP TBX1 |
| Regulation of osteoblast differentiation                 | 7             | 115         | 2.44E−04 | BMP4 TWIST2 ID3 GDF20 GREM1 SOX11 HGF                               |
| Bone mineralization                                       | 7             | 116         | 2.55E−04 | BMP4 WNT11 SMPD3 PKDCC ROR2 ADRB2 GREM1                             |
| Establishment of skin barrier                             | 4             | 22          | 2.65E−04 | ALOX12 TMEM79 ALOX12 CLDN4                                         |
| Regulation of cell motility                              | 19            | 946         | 2.69E−04 | HGF RHOD MYOC ANGPT2 BMP4 FGFr7 FGFr8 ZC3H12A ROR2 BDKRB1 SMPD3 ALOX12 CYP19A1 GREM1 CCB1 DUOX1 JUP TWIST2 WNT11 |
| Mesenchymal cell proliferation                            | 5             | 46          | 2.69E−04 | BMP4 FGFr7 WNT11 SOX1 TBX1                                          |
| Positive regulation of cell motility                    | 14            | 544         | 2.73E−04 | HGF MYOC BMP4 FGFr7 FGFr8 ZC3H12A ROR2 BDKRB1 ALOX12 CCB1 DUOX1 TWIST2 WNT11 RHOD |
| Response to BMP                                           | 8             | 165         | 2.73E−04 | BMP4 GREM1 FABM3 SMPD3 DLX5 HEYL ROR2 SOX11                         |
| Cellular response to BMP stimulus                        | 8             | 165         | 2.73E−04 | BMP4 GREM1 FABM3 SMPD3 DLX5 HEYL ROR2 SOX11                         |
| Biomineral tissue development                             | 8             | 167         | 2.93E−04 | BMP4 WNT11 SMPD3 PKDCC ROR2 ADRB2 GREM1 TBX1                       |
| Connective tissue development                             | 10            | 280         | 3.18E−04 | ZBTB16 BMP4 PITX1 SMPD3 FGFr8 PKDCC ROR2 SOX2 GREM1 WNT11           |
| Regulation of animal organ morphogenesis                 | 10            | 281         | 3.22E−04 | HGF BMP4 FGFr7 ADRB2 BMP4 WNT11 ROR2 SOX2 TBX1 RSPD3 PKDCC ELF3 GREM1 ROR2 SOX11 NRARP MAFB COLB1 TBX1 |
| Embryo development                                        | 20            | 1054        | 3.22E−04 | BMP4 IL1RN PITX1 WNT11 DLX5 ZBTB16 MDF1 DT3 TBX4 RSPD3 PKDCC ELF3 GREM1 ROR2 SOX11 NRARP MAFB COLB1 TBX1 |
| Chondrocyte differentiation                              | 7             | 123         | 3.22E−04 | SMPD3 BMP4 FGFr8 PKDCC SOX1 ZBTB16 GREM1                           |
| Epithelial cell migration                                 | 10            | 284         | 3.33E−04 | SMPD3 BMP4 FGFr8 PKDCC SMPD3 ZBTB16 GREM1 LTBR2 JUP                |
| Regulation of cell migration                              | 18            | 883         | 3.33E−04 | HGF RHOD MYOC ANGPT2 BMP4 FGFr7 FGFr8 ZC3H12A ROR2 BDKRB1 SMPD3 ALOX12 CYP19A1 GREM1 CCB1 DUOX1 TWIST2 WNT11 |
| Positive regulation of cellular component movement       | 14            | 558         | 3.33E−04 | HGF MYOC BMP4 FGFr7 FGFr8 ZC3H12A ROR2 BDKRB1 ALOX12 CCB1 DUOX1 TWIST2 WNT11 RHOD |
| Epithelium migration                                      | 10            | 287         | 3.60E−04 | ANGPT2 BMP4 FGFr7 FGFr8 ZC3H12A ALOX12 GREM1 CCB1 LTBR2 JUP        |
| Hair follicle morphogenesis                               | 4             | 25          | 3.68E−04 | KRT17 FGFr7 TMEM79 KRT25                                            |
| Regulation of water loss via skin                        | 4             | 25          | 3.68E−04 | ALOX12 TMEM79 ALOX12 CLDN4                                         |
| Embryonic limb morphogenesis                              | 7             | 130         | 4.20E−04 | PITX1 DLX5 ZBTB16 TBX4 BMP4 GREM1 ROR2                              |
| Embryonic appendage morphogenesis                         | 7             | 130         | 4.20E−04 | PITX1 DLX5 ZBTB16 TBX4 BMP4 GREM1 ROR2                              |
| Positive regulation of locomotion                        | 14            | 576         | 4.34E−04 | HGF MYOC BMP4 FGFr7 FGFr8 ZC3H12A ROR2 BDKRB1 ALOX12 CCB1 DUOX1 TWIST2 WNT11 RHOD |
| Endochondral ossification                                 | 4             | 27          | 4.78E−04 | SMPD3 DLX5 BMP4 FGFr8                                               |
| Replacement ossification                                  | 4             | 27          | 4.78E−04 | SMPD3 DLX5 BMP4 FGFr8                                               |
| Positive regulation of MAPK cascade                       | 14            | 583         | 4.79E−04 | IL36G IL1RN BMP4 FGFr8 ZC3H12A ADRB2 ALOX12 CBF CD36 ROR2 SHFR3 TBX1 GREM1 S100A12 |
| Poly-N-acetyllactosamine biosynthetic process             | 3             | 10          | 5.48E−04 | B3GNT4 B3GNT8 B3GNT3                                               |
| Regulation of multacellular organismal process            | 41            | 3382        | 5.95E−04 | ID3 NGF IL36G IL1RN HEYL AVPR1 ADRB2 HGF ANGPT2 ZBTB16 BMP4 FGFr7 FGFr8 ZC3H12A CCB1 ADRB2 BMP4 WNT11 SMPD3 ALOX12 CYP19A1 GREM1 CCB1 DUOX1 TWIST2 WNT11 |
| Response to endogenous stimulus                          | 26            | 1704        | 5.97E−04 | BMP4 NGF AVPR1 IL1RN FGFr8 CREB1L1 HEYL GREM1 JUP FABM3 SLCA8 B3GNT4 SLC8A3 CHRNA4 SMPD3 DLX5 GLP1R SD1 RASL11B CD36 FGFr7 ROR2 ADRB2 SOX21 CLDN4 TBX1 GNA15 CATS8R8 |
| Positive regulation of cell migration                    | 13            | 521         | 6.09E−04 | HGF MYOC BMP4 FGFr7 FGFr8 ZC3H12A ROR2 BDKRB1 ALOX12 CCB1 DUOX1 TWIST2 WNT11 RHOD |
| Chemotaxis                                                | 15            | 680         | 6.18E−04 | IL36G IL1RN DEFB4A HGF ANGPT2 FGFr7 FGFr8 S100A9 DLX5 BMP4 CHN1 CYP19A1 GREM1 S100A12 LTBR2 JUP |
| Regulation of cellular component movement                | 19            | 1028        | 6.21E−04 | HGF RHOD MYOC ANGPT2 BMP4 FGFr7 FGFr8 ZC3H12A ROR2 BDKRB1 SMPD3 ALOX12 CYP19A1 GREM1 CCB1 DUOX1 JUP TWIST2 WNT11 RHOD |
| Movement of cell or subcellular component                 | 30            | 2139        | 6.25E−04 | HGF RHOD MYOC ANGPT2 BMP4 FGFr7 FGFr8 S100A9 ZC3H12A ROR2 SOX2 WNT11 BDKRB1 SMPD3 DLX5 ALOX12 CHN1 CYP19A1 GREM1 CCB1 LTBR2 DUOX1 JUP TWIST2 TBX1 S100A12 |
| Taxis                                                    | 15            | 683         | 6.25E−04 | IL36G IL1RN DEFB4A HGF ANGPT2 FGFr7 FGFr8 S100A9 DLX5 BMP4 CHN1 CYP19A1 GREM1 S100A12 LTBR2 JUP |
Table 3 (continued)

| Down-regulated genes in alive x dead | Functional category | Genes in list | Total genes | FDR  | Genes                                                                 |
|-------------------------------------|---------------------|---------------|-------------|------|----------------------------------------------------------------------|
| Nephron tubule development           | 6                   | 96            | 6.25E−04    | BMP4 GREB1L HEYL GREM1 SIX2 WNT11 |
| Embryonic organ development          | 12                  | 452           | 6.34E−04    | WNT11 DLX5 MDF1 I3D BMP4 RPSO3 PKDCC ROR2 SIK2 SOX11 MAFB TBX1 |
| Morphogenesis of a branching epithelium | 8                   | 194           | 6.34E−04    | NRARP HGF BMP4 FGF7 GREB1L RSP03 GREM1 SIX2 |
| Kidney epithelium development        | 7                   | 143           | 6.55E−04    | BMP4 SDC1 GREB1L HEYL GREM1 SIX2 WNT11 |
| Poly-N-acetyllactosamine metabolic process | 3                   | 11            | 6.64E−04    | B3GNT4 B3GNT8 B3GNT3 |
| Positive regulation of intracellular signal transduction | 20                  | 1133          | 6.72E−04    | IL36G IL1RN HGF MYOC BMP4 FGF18 S100A9 S100A12 ZC3H12A ADRB2 ALOX12B CD36 GREM1 ROR2 SIK2 WNT11 SH3RF3 NGF TGFB1 FGF7 |
| Regulation of locomotion             | 19                  | 1041          | 6.74E−04    | HGF RHOD MYOC ANGPT2 BMP4 FGF7 FGF18 ZC3H12A ROR2 BDKRB1 |
| Ureteric bud development             | 6                   | 99            | 6.85E−04    | BMP4 SDC1 GREB1L GREM1 SIX2 WNT11 |
| Renal tubule development             | 6                   | 99            | 6.85E−04    | BMP4 GREB1L HEYL GREM1 SIX2 WNT11 |
| Positive regulation of cell differentiation | 4                   | 31            | 6.94E−04    | ZBTB16 BMP4 FGF7 PKDCC |
| Mesonephric epithelium development   | 6                   | 100           | 6.94E−04    | BMP4 SDC1 GREB1L GREM1 SIX2 WNT11 |
| Mesonephric tubule development       | 6                   | 100           | 6.94E−04    | BMP4 SDC1 GREB1L GREM1 SIX2 WNT11 |
| Positive regulation of gene expression | 29                  | 2060          | 7.22E−04    | DLX5 ZBTB16 HEYL JUP SIK11 BMP4 ELF3 ZC3H12A CCBE1 P1CP1 BMP4 FGF7 CREB1L GREM1 ROR2 ADRB2 SIX2 OXC1 MAFF WNT11 NGF ALOX12B GL35 ACTC2 TBX1 HGF |
| Regulation of morphogenesis of a branching structure | 5                   | 63            | 7.98E−04    | HGF BMP4 FGF7 GREM1 SIX2 |
| BMP signaling pathway                | 7                   | 152           | 8.66E−04    | BMP4 GREM1 FAM83G SMPD3 DLX5 ROR2 SIK11 |
| Endothelial cell migration           | 8                   | 206           | 8.66E−04    | ANGPT2 BMP4 FGF18 ZC3H12A ALOX12 GREM1 CCBE1 JUP |
| Morphogenesis of a branching structure | 8                   | 208           | 9.13E−04    | NRARP HGF BMP4 FGF7 GREB1L RSP03 GREM1 SIX2 |
| Mesenchymal to epithelial transition involved in metanephros morphogenesis | 3                   | 13            | 1.01E−03    | BMP4 GREM1 SIX2 |
| Mesenchymal cell differentiation     | 8                   | 212           | 1.01E−03    | WNT11 BMP4 HEYL SIK2 SOX11 GREM1 TBX1 HGF |
| Mesenchyme development               | 9                   | 273           | 1.01E−03    | WNT11 BMP4 HEYL SIK2 SOX11 GREM1 ACTG2 TBX1 HGF |
| Ureteric bud morphogenesis           | 5                   | 67            | 1.01E−03    | BMP4 GREB1L GREM1 SIX2 WNT11 |
| Mesonephric tubule morphogenesis     | 5                   | 68            | 1.08E−03    | BMP4 GREB1L GREM1 SIX2 WNT11 |
| Regulation of cell-substrate adhesion | 8                   | 216           | 1.13E−03    | MYOC CD36 AJAP1 ANGPT2 COLBA1 GREM1 RHOD JUP |
| Regulation of endothelial cell migration | 7                   | 161           | 1.16E−03    | ANGPT2 BMP4 FGF18 ZC3H12A ALOX12 GREM1 CCBE1 JUP |
| Positive regulation of multicellular organismal process | 27                  | 1911          | 1.17E−03    | NGF IL36G IL1RN AIPRT1A ZBTB16 BMP4 FGF7 FGF18 ZC3H12A CCBE1 |
| Nephron epithelium development       | 6                   | 112           | 1.18E−03    | BMP4 GREB1L HEYL GREM1 SIX2 WNT11 |
| Regulation of epithelial cell migration | 8                   | 223           | 1.36E−03    | ANGPT2 BMP4 FGF7 FGF18 ZC3H12A ALOX12 GREM1 CCBE1 JUP |
| Positive regulation of epithelium development | 4                   | 38            | 1.36E−03    | BMP4 KRT17 TMEM379 SULT2B1 |
| Aminoglycan biosynthetic process     | 6                   | 116           | 1.39E−03    | B3GNT4 B3GNT8 B3GNT3 SMPD3 SDC1 HS3ST3A1 |
| Positive regulation of angiogenesis  | 7                   | 167           | 1.39E−03    | ZC3H12A HGF ANGPT2 GREM1 CCBE1 FGF18 JUP |
| Sprouting angiogenesis               | 6                   | 117           | 1.45E−03    | NRARP BMP4 RPS03 GREM1 CREB3L1 CCBE1 |
| Cell-substrate adhesion              | 10                  | 358           | 1.46E−03    | LYPD3 LYPD5 MYOC CD36 AJAP1 ANGPT2 COLBA1 GREM1 RHOD JUP |
| Sulfate assimilation                 | 2                   | 3             | 1.52E−03    | PAPS2 SULT2B1 |
| Cuticle development                  | 2                   | 3             | 1.52E−03    | TMEM379 DLX31 |
| Cloacal septation                    | 2                   | 3             | 1.52E−03    | BMP4 WNT11 |
| Aminoglycan metabolic process        | 7                   | 171           | 1.53E−03    | B3GNT4 B3GNT8 B3GNT3 HGF SMPD3 SDC1 HS3ST3A1 |
| Cardiac septum morphogenesis         | 5                   | 75            | 1.53E−03    | HEYL WNT11 BMP4 SIK2 WNT11 |
| Cellular response to endogenous stimulus | 22                  | 1432          | 1.61E−03    | BMP4 NGF AIPRT1A FGF18 CREB3L1 HEYL GREM1 JUP FAM83G SLC8A3 CHRNA4 SMPD3 DLX5 GLP1 RASL11B FGF7 ROR2 ADRB2 SIK2 CD36 TBX1 GNA15 |
| Nephron tubule morphogenesis         | 5                   | 76            | 1.61E−03    | BMP4 GREB1L GREM1 SIX2 WNT11 |
| Metanephric renal vesicle morphogenesis | 3                   | 16            | 1.65E−03    | BMP4 GREM1 SIX2 |
| Regulation of cell–matrix adhesion   | 6                   | 122           | 1.68E−03    | MYOC CD36 AJAP1 GREM1 RHOD JUP |
| Outflow tract morphogenesis          | 5                   | 77            | 1.68E−03    | WNT11 BMP4 HEYL SIK2 WNT11 |
| Nephron epithelium morphogenesis     | 5                   | 78            | 1.77E−03    | BMP4 GREB1L GREM1 SIX2 WNT11 |
### Table 3 (continued)

#### Down-regulated genes in alive x dead

| Functional category | Genes in list | Total genes | FDR       | Genes                                      |
|---------------------|---------------|-------------|-----------|--------------------------------------------|
| Ventricular septum morphogenesis | 4 42 | 1.79E−03 | HEYL WNT11 BMP4 SOX11 |
| Cell–matrix adhesion | 8 239 | 1.91E−03 | LYPD3 LYPD5 MYOC CD36 AIAPI GREM1 RHOD JUP |
| Embryonic organ morphogenesis | 9 305 | 1.91E−03 | WNT11 DLX5 MDFI BMP4 ROR2 SOX2 SOX11 MAFB TBX1 |
| Renal tubule morphogenesis | 5 80 | 1.91E−03 | BMP4 GREB1L GREM1 SX2 WNT11 |
| Neprhen morphogenesis | 5 80 | 1.91E−03 | BMP4 GREB1L GREM1 SX2 WNT11 |
| Nephric duct development | 3 17 | 1.91E−03 | BMP4 GREB1L WNT11 |
| Regulation of cell communication | 43 3903 | 2.15E−03 | BMP4 NGF IL36G IL1RN SK1 RSP03 NRRAP JUP HGF MYOC KLK14 CYP19A1 FGF18 CREB3L1 S100A9 S100A12 ZC3H12A HEYL GREM1 ADRB2 ALOX12B LYNX1 CCBE1 FAM83D WNT11 SLC8A3 CHRNA4 DLX5 RASL11B CHN1 CD36 FGF7 AVPR1A ROR2 SOX11 G6PC2 SH3RF3 MDFI TBX1 NOTCH3 GLP1R ELF3 RHOD |
| Enzyme linked receptor protein signaling pathway | 18 1072 | 2.17E−03 | HGF BMP4 NGF ROR2 ANGPT2 CREB3L1 GREM1 CBE1 FAM83G MYOC SMAD3 DLX5 RASL11B FGF7 FGF18 SX11 CHN1 ADRB2 |
| Regulation of MAPK cascade | 15 793 | 2.17E−03 | BMP4 IL36G IL1RN MYOC FGF18 ZC3H12A ADRB2 ALOX12B HGF CD36 ROR2 SH3RF3 TBX1 NGF S100A12 |
| Embryonic skeletal system development | 6 130 | 2.21E−03 | MDFI BMP4 SX2 SX11 WNT11 TBX1 |
| Positive regulation of vasculature development | 7 186 | 2.29E−03 | ZC3H12A HGF ANGPT2 GREM1 SX2 FGF18 JUP |
| Response to organic substance | 40 3547 | 2.30E−03 | BMP4 NGF IL36G IL1RN AVPR1A CD36 DLX5 FGF18 CREB3L1 ZC3H12A HEYL GREM1 JUP CBE1 FAM83G ANGPT2 SLC8A3 CHRNA4 DLX5 RASL11B CHN1 CD36 FGF7 AVPR1A ROR2 SOX11 G6PC2 SH3RF3 MDFI TBX1 NOTCH3 GLP1R ELF3 RHOD |
| Positive chemotaxis | 5 84 | 2.30E−03 | ANGPT2 FGF7 HGF BMP4 DEFB4A |
| Response to psychosocial stress | 2 4 | 2.56E−03 | GLP1R ADRB2 |
| Cloaca development | 2 4 | 2.56E−03 | BMP4 WNT11 |
| Positive regulation of epithelial cell proliferation | 7 190 | 2.56E−03 | NRRAP BMP4 FGF7 DLX5 SX11 TGM1 TBX1 |
| Regulation of branching involved in salivary gland morphogenesis by mesenchymal-epithelial signaling | 2 4 | 2.56E−03 | HGF FGF7 |
| Myoblast differentiation | 5 87 | 2.62E−03 | BMP4 PITX1 SDC1 ID3 GREM1 |
| Regulation of signaling | 43 3952 | 2.64E−03 | BMP4 Ngf IL36G IL1RN SK1 RSP03 NRRAP JUP HGF MYOC KLK14 CYP19A1 FGF18 CREB3L1 S100A9 S100A12 ZC3H12A HEYL GREM1 ADRB2 ALOX12B LYNX1 CCBE1 FAM83D WNT11 SLC8A3 CHRNA4 DLX5 RASL11B CHN1 CD36 FGF7 AVPR1A ROR2 SOX11 G6PC2 SH3RF3 MDFI TBX1 NOTCH3 GLP1R ELF3 RHOD |
| Negative regulation of transcription, DNA-templated | 20 1298 | 2.73E−03 | ZBTB16 BMP4 SX2 OVOL1 SX11 CNIP1 ID3 CREB3L1 ELF3 HEYL PITX1 NOTCH3 CD36 GREM1 NRRAP USP2 WNT11 G6PC2 MDFI TWIST2 |
| Positive regulation of chondrocyte differentiation | 3 20 | 2.78E−03 | FGF18 PKDCC ZBTB16 |
| Mesenchymal to epithelial transition | 3 20 | 2.78E−03 | BMP4 GREM1 SX2 |
| Renal vesicle morphogenesis | 3 20 | 2.78E−03 | BMP4 GREM1 SX2 |
| Response to oxygen-containing compound | 24 1725 | 2.88E−03 | IL36G IL1RN HGF DUX5 ZC3H12A JUP ANGPT2 SLC8A3 BDKR1 CHRNA4 SMAD3 GLP1R SDC1 ID3 SLPI CD36 AVPR1A ROR2 CD36 FGF7 AVPR1A ROR2 SOX11 G6PC2 SH3RF3 MDFI TBX1 NOTCH3 GLP1R ELF3 RHOD |
| Regulation of vasculature development | 9 328 | 2.89E−03 | BMP4 ZC3H12A HGF ANGPT2 GREM1 CBE1 FGF18 CREB3L1 JUP |
| Digestive tract morphogenesis | 4 50 | 3.01E−03 | BMP4 SX2 SX11 WNT11 |
| Metanephros development | 5 91 | 3.04E−03 | BMP4 ID3 GREG1L GREM1 SX2 |
| Positive regulation of epithelial cell migration | 6 141 | 3.04E−03 | BMP4 FGF7 FGF18 ZC3H12A ALOX12 CCEB1 |
| Regulation of cell differentiation | 26 1954 | 3.04E−03 | ID3 NGF HEYL MYOC BMP4 ZC3H12A ADRB2 CD36 FGF18 CREB3L1 JUP |
| Copulation | 3 21 | 3.06E−03 | KLK14 P13 AVPR1A |
| Positive regulation of cell differentiation | 17 1018 | 3.06E−03 | NGF MYOC BMP4 ZC3H12A HGF ZBTTB16 GDPP2 CD36 FGF18 PKDCC HEYL GREM1 ROR2 SX11 SULT2B1 S100A9 TBX1 |
| Renal vesicle development | 3 21 | 3.06E−03 | BMP4 GREM1 SX2 |
| Cell surface receptor signaling pathway involved in cell–cell signaling | 13 654 | 3.16E−03 | WNT11 RSP03 NRRAP JUP GREM1 CHRNA4 DLX5 SDC1 ROR2 ADRB2 MYOC BMP4 MDFI |
Table 3 (continued)

| Functional category                              | Genes in list | Total genes | FDR    | Genes                                      |
|--------------------------------------------------|---------------|-------------|--------|--------------------------------------------|
| Roof of mouth development                        | 5             | 93          | 3.25E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Leukocyte migration                               | 11            | 491         | 3.36E−03 | IL36G IL1RN S100A9 BXBD8 SMPD3 CYP19A1 GREM1 ROR2 ANGPT2 SDC1 S100A12 |
| Cell chemotaxis                                   | 9             | 339         | 3.45E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Sensory organ morphogenesis                       | 8             | 270         | 3.53E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Negative regulation of cellular biosynthetic process | 23            | 1658        | 3.67E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Hepoxilin metabolic process                       | 2             | 5           | 3.67E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Glomerulus vasculature morphogenesis              | 2             | 5           | 3.67E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Negative regulation of RNA metabolic process      | 21            | 1446        | 3.68E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Embryonic skeletal system morphogenesis           | 5             | 97          | 3.70E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Kidney morphogenesis                              | 5             | 97          | 3.70E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Cellular response to chemical stimulus            | 39            | 3536        | 3.70E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Positive regulation of endothelial cell migration | 5             | 98          | 3.86E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Regulation of cell adhesion                       | 14            | 765         | 3.94E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Negative regulation of nucleic acid-templated transcription | 20            | 1353        | 3.96E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Cellular response to organic substance            | 34            | 2938        | 3.99E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Negative regulation of RNA biosynthetic process   | 20            | 1355        | 3.99E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Regulation of branching involved in ureteric bud morphogenesis | 3             | 24          | 4.06E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Intermediate filament organization                | 3             | 24          | 4.06E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Positive regulation of transcription by RNA polymerase II | 19            | 1255        | 4.06E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Glomerulus vasculature development                | 3             | 24          | 4.06E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Negative regulation of biosynthetic process       | 23            | 1681        | 4.10E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Carbohydrate biosynthetic process                 | 7             | 214         | 4.10E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Positive regulation of protein phosphorylation    | 17            | 1057        | 4.10E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Negative regulation of nucleobase-containing compound metabolic process | 22            | 1575        | 4.15E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Regionalization                                   | 9             | 355         | 4.24E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Regulation of protein import into nucleus         | 4             | 58          | 4.41E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Negative regulation of macromolecule biosynthetic process | 22            | 1597        | 4.86E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Positive regulation of osteoblast differentiation  | 4             | 60          | 4.86E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Positive regulation of transcription, DNA-templated | 4             | 60          | 4.86E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Renal system vasculature development              | 3             | 26          | 4.86E−03 | WNT11 DLX5 PKDCC SOX11 TXB1                |
| Functional category                                      | Genes in list | Total genes | FDR       | Genes                                                                 |
|---------------------------------------------------------|---------------|-------------|-----------|-----------------------------------------------------------------------|
| Kidney vasculature development                          | 3             | 26          | 4.86E−03  | BMP4 NOTCH3 ANGPT2                                                    |
| Cell differentiation involved in metanephros development | 3             | 26          | 4.86E−03  | BMP4 GREM1 SIX2                                                       |
| Metanephric nephron morphogenesis                       | 3             | 26          | 4.86E−03  | BMP4 GREM1 SIX2                                                       |
| Glycosaminoglycan metabolic process                    | 6             | 161         | 4.99E−03  | HGF S1P3 DCD1 HS3ST3A1 B3GNT4 B3GNT3                                 |
| Regulation of protein import                            | 4             | 61          | 5.07E−03  | BMP4 ZC3H12A JUP CD36                                                 |
| Branching involved in ureteric bud morphogenesis        | 4             | 61          | 5.07E−03  | BMP4 GREB1 L GREM1 SIX2                                              |
| Glomerulus development                                 | 4             | 61          | 5.07E−03  | BMP4 NOTCH3 ANGPT2 HEYL                                              |
| Negative regulation of cartilage development           | 3             | 27          | 5.28E−03  | BMP4 GREM1 WNT11                                                     |
| Regulation of mesonephros development                   | 3             | 27          | 5.28E−03  | BMP4 GREM1 SIX2                                                       |
| Bone development                                        | 7             | 228         | 4.99E−03  | MYOC SM3D DLX5 BMP4 FGF18 PAPPS2 GREM1                                |
| Negative regulation of cellular macromolecule biosynthetic process | 21            | 1511        | 5.53E−03  | ZBTB16 BMP4 SIX2 OVQ1 SOX11 ZNF154 D3 CREB3L1 ELF3 ZC3H12A HEYL PITX1 NOTCH3 CD36 GREM1 NARPUS2 WNT11 GLIS3 MDFI TWIST2 |
| Regulation of angiogenesis                              | 8             | 298         | 5.53E−03  | ZC3H12A G HF ANGPT2 GREM1 CCB1 F G18 C CB3L1 JUP                    |
| Peptide cross-linking                                   | 4             | 63          | 5.57E−03  | SPRR2D SPRR2A TGM1 P3                                                 |
| Embryonic hindlimb morphogenesis                        | 3             | 28          | 5.73E−03  | PTX1 ZBTB16 BMP4                                                      |
| Secondary palate development                            | 3             | 28          | 5.73E−03  | SOX11 WNT11 TBX1                                                      |
| Glycosaminoglycan biosynthetic process                  | 5             | 111         | 5.78E−03  | SMP3 DCD1 HS3ST3A1 B3GNT4 B3GNT3                                     |
| Artery morphogenesis                                    | 4             | 64          | 5.81E−03  | BMP4 NOTCH3 WNT11 TBX1                                               |
| Regulation of localization                              | 33            | 2905        | 5.84E−03  | HGF RHOD MYOC ANGPT2 BMP4 CYP19A1 FGF7 FGF18 ZC3H12A ROR2 JUP FAM3D BDKB1 CHRNA4 SM3D ALX12 CD36 PKDCC AVPR1A GREM1 ADRB2 CLIC3 SOX11 CCB1 KKN2 DCX11 TWIST2 WNT11 SDC1 G6PC2 ALX12B SK1 GLP1R |
| Cardiac septum development                              | 5             | 112         | 5.93E−03  | HEYL WNT11 BMP4 SOX11 TBX1                                           |
| Response to lipid                                       | 16            | 2082        | 5.95E−03  | DLX5 ZBTB16 HEYL JUP SOX11 HGF BMP4 ELF3 ZC3H12A GREM1 PITX1 NOTCH3 KRT17 CD36 FGF CREB3L1 AVPR1A ROR2 ADRB2 SIX2 OVO1 MAF8 WNT11 GLIS3 MDFI SPK3D |
| MAPK cascade                                            | 16            | 1007        | 5.97E−03  | BMP4 IL36G IL1RN MYOC FGF18 ZC3H12A ADRB2 ALOX12 HGF CD36 ROR2 SH3RF3 TBX1 NSG FGF 7 5100A12 |
| MAPK cascade                                            | 16            | 1007        | 5.97E−03  | BMP4 IL36G IL1RN MYOC FGF18 ZC3H12A ADRB2 ALOX12 HGF CD36 ROR2 SH3RF3 TBX1 NSG FGF 7 5100A12 |
| Regulation of cell adhesion                             | 8             | 304         | 5.99E−03  | MYOC IL1RN AJP1 ANGPT2 ALX12 ZC3H12A NARP USP4 BMP4                  |
| Renal system vasculature morphogenesis                  | 2             | 7           | 6.07E−03  | NOTCH3 BMP4                                                          |
| Kidney vasculature morphogenesis                        | 2             | 7           | 6.07E−03  | NOTCH3 BMP4                                                          |
| Positive regulation of phosphorylation                  | 17            | 1111        | 6.10E−03  | BMP4 BMP4 IL36G IL1RN FGF7 FGF7 FGF18 ZC3H12A ADRB2 ALOX12 WNT11 CD36 GREM1 ROR2 SH3RF3 TBX1 NSG FGF 7 5100A12 |
| Cardiac chamber development                              | 6             | 172         | 6.32E−03  | HEYL WNT11 BMP4 GREB1L SOX11 TBX1                                    |
| Regulation of cell proliferation                        | 23            | 1756        | 6.32E−03  | NARP BMP USP BMP4 FGF7 GREM1 NOTCH3 WNT11 SMP3 DCD1 DLX5 ALOX12 ZBTB16 RARRES1 FGF18 AVPR1A SIX2 OVO1 SIX1 NULTB1 ADRB2 GREM1 BMP4 GREB1L SIX2 OVO1 CD36 |
| Negative regulation of nitrogen compound metabolic process | 30            | 2564        | 6.32E−03  | ZBTB16 BMP4 SIX2 OVO1 SIX1 ZNF154 HGF D3 CREB3L1 CD4 ELF3 ZC3H12A HEYL GREM1 PITX1 NOTCH3 BDKB1 SM3D P3 SLPI NSG CD36 NARP SPINK9 COL28A1 NSP WNT11 GLIS3 MDFI TWIST2 |
| Cardiac chamber development                              | 6             | 172         | 6.32E−03  | HEYL WNT11 BMP4 GREB1L SIX11 TBX1                                    |
| Negative regulation of developmental process            | 16            | 1017        | 6.40E−03  | ID3 BMP4 MAFB TWIST2 NOTCH3 ANGPT2 ZBTB16 ZC3H12A ADRB2 SIX2 NOTCH3 NARP CREB3L1 GREM1 WNT11 TBX1 |
| Signal transduction by protein phosphorylation          | 16            | 1018        | 6.44E−03  | BMP4 IL36G IL1RN MYOC FGF18 ZC3H12A ADRB2 ALOX12 HGF CD36 ROR2 SH3RF3 TBX1 NSG FGF 7 5100A12 |
| Transmembrane receptor protein tyrosine kinase signaling pathway | 13            | 730         | 6.45E−03  | HGF NSG ROR2 ANGPT2 CREB3L1 CCB1 MYOC SM3D FGF7 FGF18 GREM1 CHN1 ADRB2 |
| Cell adhesion                                           | 21            | 1541        | 6.46E−03  | LYPD3 LYPD5 MYOC CD36 L1RN S100A9 JUP AJP1 ANGPT2 ALX12 ZBTB16 COLB1A FBUM1 ZC3H12A GREM1 RHOD NARP COL28A1 BMP4 CLDN4 LIRN2 |
| Epithelial cell proliferation                           | 9             | 387         | 6.56E−03  | NARP BMP4 FGF7 HGF DLX5 COLB1A SOX11 TGM1 TBX1                       |
### Table 3 (continued)

| Functional category                                                                 | Genes in list | Total genes | FDR     | Genes                                                                 |
|-------------------------------------------------------------------------------------|---------------|-------------|---------|----------------------------------------------------------------------|
| Biological adhesion                                                                  | 21            | 1548        | 6.78E−03| LYPD3 LYPD5 MYOC CD36 IL1RN S100A9 JUP AJP1 ANGPT2 ALOX12 ZBTB16 COL8A1 FBDM1 ZC3H12A GREM1 RHOD NRARP COL28A1 BMP4 CLDN4 LRRN2 |
| Myeloid leukocyte migration                                                           | 7             | 241         | 6.79E−03| IL36G IL1RN S100A9 CYP19A1 GREM1 ROR2 S100A12                        |
| Positive regulation of biosynthetic process                                          | 26            | 2113        | 6.91E−03| DLXS ZBTB16 HEYL JUP SOX11 HGF BMP4 CREB3L1 ELF3 ZC3H12A GREM1 PITX1 NOTCH3 KRT17 CD36 FG7 AVPR1A ROR2 ADRB2 SIX2 OVOL1 MAFB WNT11 GLI53 TBX1 SMPD3 |
| Blood vessel endothelial cell migration                                               | 5             | 119         | 7.19E−03| ANGPT2 ALOX12 GREM1 FG18 JUP                                        |
| Mesenchymal cell differentiation involved in renal system development                | 2             | 8           | 7.48E−03| BMP4 SIX2                                                             |
| Mesenchymal-epithelial cell signaling                                                | 2             | 8           | 7.48E−03| NOTCH3 BMP4                                                           |
| Mesenchymal cell differentiation involved in kidney development                      | 2             | 8           | 7.48E−03| BMP4 SIX2                                                             |
| Negative regulation of mesenchymal cell proliferation                                | 2             | 8           | 7.48E−03| BMP4 WNT11                                                           |
| Negative regulation of cellular metabolic process                                    | 31            | 2724        | 7.95E−03| ZBTB16 BMP4 SIX2 OVOL1 SOX11 ZNF154 HGF ID3 CREB3L1 CDA ELF3 ZC3H12A HEYL GREM1 PITX1 NOTCH3 EDNB1 SMAPD3 P3 SLPI NGF BMP4 BMP4 BMP4 BMP4 BMP4 |
| Multicellular organismal water homeostasis                                           | 4             | 71          | 7.63E−03| ALOX12 TMEM79 ALOX12 CLDN4                                           |
| Endochondral bone morphogenesis                                                      | 4             | 72          | 8.01E−03| SMPD3 DLXS BMP4 FG18                                                 |
| Metanephros morphogenesis                                                            | 3             | 33          | 8.15E−03| BMP4 GREM1 SIX2                                                      |
| Positive regulation of nucleic acid-templated transcription                          | 22            | 1694        | 8.52E−03| DLXS ZBTB16 HEYL JUP SOX11 BMP4 ELF3 ZC3H12A PITX1 NOTCH3 FG7 CREB3L1 GREM1 ROR2 ADRB2 SIX2 OVOL1 MAFB WNT11 GLI53 TBX1 BMP4 |
| Positive regulation of RNA biosynthetic process                                      | 22            | 1695        | 8.52E−03| DLXS ZBTB16 HEYL JUP SOX11 BMP4 ELF3 ZC3H12A PITX1 NOTCH3 FG7 CREB3L1 GREM1 ROR2 ADRB2 SIX2 OVOL1 MAFB WNT11 GLI53 TBX1 BMP4 |
| Positive regulation of morphogenesis of an epithelium                                | 3             | 34          | 8.72E−03| ALOX12 BMP4 GREM1                                                   |
| Regulation of mesenchymal cell proliferation                                         | 3             | 34          | 8.72E−03| BMP4 WNT11 TBX1                                                     |
| Ventricular septum development                                                       | 4             | 74          | 8.72E−03| HEYL WNT11 BMP4 SOX11                                               |
| Ear morphogenesis                                                                    | 5             | 127         | 9.06E−03| DLXS ROR2 SIX2 MAFB TBX1                                             |
| Cell proliferation                                                                   | 26            | 2165        | 9.07E−03| NRARP DLXS BMP4 BMP4 FG7 GREM1 SIX2 JUP HGF NOTCH3 WNT11 SMPD3 BMP4 BMP4 BMP4 |
| Soft palate development                                                               | 2             | 9           | 9.07E−03| SOX11 TXB1                                                          |
| Regulation of branching involved in salivary gland morphogenesis                     | 2             | 9           | 9.07E−03| HGF FGF7                                                           |
| Mesonephric duct development                                                         | 2             | 9           | 9.07E−03| GREB1L WNT11                                                       |
| Regulation of DNA-binding transcription factor activity                               | 10            | 495         | 9.09E−03| ID3 ZC3H12A HEYL JUP GREM1 CD36 SIK1 MDF1 S100A9 S100A12           |
| Regulation of hormone levels                                                         | 11            | 583         | 9.12E−03| CYP19A1 FAM3D SMPD3 IL1RN DUOX1 CRABP1 SOX11 CHST8 G6PC2 GLP1R NGF |
| Water homeostasis                                                                    | 4             | 76          | 9.28E−03| ALOX12 TMEM79 ALOX12 CLDN4                                           |
| Cellular response to toxic substance                                                 | 7             | 259         | 9.32E−03| HGF SMPD3 CD36 DUOX1 S100A9 ZC3H12A GPX3                             |
| Cardiac chamber morphogenesis                                                        | 5             | 129         | 9.38E−03| HEYL WNT11 BMP4 SOX11 TXB1                                          |
| Cardiac ventricle development                                                        | 5             | 130         | 9.67E−03| HEYL WNT11 BMP4 GREB1L SOX11                                         |
| Regulation of signal transduction                                                    | 37            | 3529        | 9.74E−03| BMP4 BMP4 BMP4 BMP4 BMP4 BMP4 BMP4 BMP4 BMP4 BMP4 BMP4 BMP4 BMP4 BMP4 |
| Hindlimb morphogenesis                                                               | 3             | 36          | 9.82E−03| PITX1 ZBTB16 BMP4                                                   |
| Odontogenesis                                                                        | 5             | 131         | 9.89E−03| SMPD3 SDC1 ID3 BMP4 TXB1                                             |
| Regulation of bone mineralization                                                    | 4             | 78          | 9.90E−03| BMP4 PDKCC ADRB2 GREM1                                              |
| Polysaccharide biosynthetic process                                                  | 4             | 78          | 9.90E−03| B3GNT4 B3GNT8 B3GNT3 SMPD3                                        |
### Table 3 (continued)

| Functional category                                           | Genes in list | Total genes | FDR     | Genes                                                                 |
|---------------------------------------------------------------|---------------|-------------|---------|----------------------------------------------------------------------|
| Negative regulation of multicellular organismal process      | 18            | 1286        | 9.90E−03| ID3 ADRB2 ANGPT2 BMP4 ZC3H12A MAFB TWIST2 HGF NOTCH3 WNT11 ALOX12 BTRB16 AVPR1A SOX11 NRARP CREB3L1 JUP GREM1 |
| Regulation of protein phosphorylation                         | 20            | 1503        | 9.92E−03| HGF BMP4 IL36G IL1RN MYOC FGF7 FGF18 ZC3H12A GREM1 ADRB2 ALOX12B WNT11 BDKRB1 SMPD3 CD36 ROR2 SH3RF3 TBX1 NGF S100A12 |
| Cell surface receptor signaling pathway                       | 35            | 3287        | 1.00E−02| HGF WNT11 BMP4 NGF IL36G IL1RN RSPO3 HEYL ROR2 NRARP JUP ANGPT2 DUX1 CREB3L1 GREM1 CCB1 FAM83G MYOC NOTCH3 CHRNA4 SMPD3 DLX5 GLP1R SDC1 RASL11B CD36 FGF7 FGF18 ZC3H12A ADRB2 SOX11 MDFI CHN1 CDA ELF3 |
| Positive regulation of phosphorus metabolic process           | 17            | 1183        | 1.00E−02| HGF BMP4 IL36G IL1RN FGF7 FGF18 ZC3H12A ADRB2 ALOX12B WNT11 CD36 GREM1 ROR2 SH3RF3 TBX1 NGF S100A12 |
| Positive regulation of phosphate metabolic process            | 17            | 1183        | 1.00E−02| HGF BMP4 IL36G IL1RN FGF7 FGF18 ZC3H12A ADRB2 ALOX12B WNT11 CD36 GREM1 ROR2 SH3RF3 TBX1 NGF S100A12 |
| Cellular response to oxidised low-density lipoprotein particle stimulus | 2            | 10          | 1.05E−02| SMPD3 CD36 |
| Positive regulation of keratinocyte proliferation             | 2             | 10          | 1.05E−02| FGF7 TG1M |
| Positive regulation of mitochondrial depolarization           | 2             | 10          | 1.05E−02| MYOC ALOX12 |
| Epithelial cell proliferation involved in lung morphogenesis  | 2             | 10          | 1.05E−02| BMP4 FGF7 |
| Epithelial tube morphogenesis                                  | 8             | 344         | 1.06E−02| NRARP BMP4 ALOX12B GREB1L GREM1 SIX2 SOX11 WNT11 |
| Epithelial to mesenchymal transition                          | 5             | 135         | 1.08E−02| WNT11 BMP4 HEYL GREM1 HGF |
| Negative regulation of growth                                  | 7             | 269         | 1.08E−02| BMP4 CDA BDKRB1 CD36 GREM1 ADRB2 WNT11 |
| Mesoderm development                                           | 5             | 136         | 1.11E−02| BMP4 WNT11 SIX2 SOX11 OVO1 TBX1 |
| Canonical Wnt signaling pathway                                | 8             | 350         | 1.16E−02| RSPO3 NRARP JUP GREM1 WNT11 DLXS SDC1 ROR2 |
| Digestive tract development                                    | 5             | 139         | 1.21E−02| BMP4 PKDCC SIX2 SOX11 WNT11 |
| Mesenchymal cell development                                   | 4             | 84          | 1.23E−02| BMP4 HEYL SOX11 TBX1 |
| Positive regulation of bone mineralization                     | 3             | 40          | 1.24E−02| BMP4 PKDCC ADRB2 |
| Embryonic digestive tract development                          | 3             | 40          | 1.24E−02| PKDCC SIX2 SOX11 |
| Regulation of cellular response to growth factor stimulus      | 7             | 277         | 1.24E−02| BMP4 WNT11 SIX2 SOX11 OVO1 |
| Cell fate commitment                                           | 7             | 277         | 1.24E−02| BMP4 WNT11 SIX2 SOX11 OVO1 |
| Glycoprotein biosynthetic process                              | 8             | 357         | 1.28E−02| HS3ST3A1 B3GNT4 FUT2 B3GNT8 B3GNT3 CHST8 STASSA2 ST6GA2 |
| Transmembrane receptor protein serine/threonine kinase signaling pathway | 8             | 358         | 1.30E−02| BMP4 GREM1 FAM83G SMPD3 DLX5 SDC1 ROR2 |
| Axis specification                                             | 4             | 86          | 1.30E−02| BMP4 SIX2 NRARP MDF1 |
| Negative regulation of T cell differentiation                 | 3             | 41          | 1.30E−02| ZC3H12A NRARP BMP4 |
| Positive regulation of nitrogen compound metabolic process     | 35            | 3351        | 1.30E−02| HGF DLX5 ZBTR16 BMP4 IL36G IL1RN HEYL JUP SOX11 FGF7 FGF18 S100A9 ELF3 ZC3H12A GREM1 ADRB2 ALOX12B CCB1 PITX1 NOTCH3 WNT11 ALOX12 KRT17 CD36 CREB3L1 ROR2 SIX2 OVO1 MAFB SH3RF3 GLIS3 TBX1 SMPD3 NGF S100A12 |
| Metanephric nephron development                                | 3             | 41          | 1.30E−02| BMP4 GREM1 SIX2 |
| Negative regulation of cell communication                     | 19            | 1440        | 1.31E−02| SK1 NRARP HGF MYOC BMP4 KLK14 IL1RN CREB3L1 HEYL GREM1 ADRB2 FAM3D WNT11 RASL11B ZC3H12A AVPR1A ROR2 MDFI NOTCH3 |
| Positive regulation of cellular metabolic process              | 36            | 3482        | 1.31E−02| HGF DLX5 ZBTR16 BMP4 IL36G IL1RN HEYL JUP SOX11 FGF7 FGF18 S100A9 ELF3 ZC3H12A GREM1 ADRB2 ALOX12B CCB1 PITX1 NOTCH3 WNT11 ALOX12 KRT17 CD36 CREB3L1 AVPR1A ROR2 SIX2 OVO1 MAFB SH3RF3 GLIS3 TBX1 SMPD3 NGF S100A12 |
| Antimicrobial humoral response                                 | 19            | 1444        | 1.35E−02| SK1 NRARP HGF MYOC BMP4 KLK14 IL1RN CREB3L1 HEYL GREM1 ADRB2 FAM3D WNT11 RASL11B ZC3H12A AVPR1A ROR2 MDFI NOTCH3 |
| Hair follicle development                                      | 5             | 145         | 1.38E−02| SLP1 S100A9 S100A12 DEF8A4 PI3 |
| Regulation of epidermis development                           | 4             | 88          | 1.38E−02| KRT17 FGF7 TEM79 KRT25 |
| Artery development                                             | 4             | 88          | 1.38E−02| BMP4 KRT17 TEM79 SULT2B1 |
| Positive regulation of cell proliferation                     | 15            | 1022        | 1.39E−02| NRARP BMP4 FGF7 GREM1 NOTCH3 SMPD3 DLX5 ALOX12 FGF18 AVPR1A SIX2 SOX11 TM1 TBX1 ADR1A |
| Functional category                              | Genes in list | Total genes | FDR     | Genes                                                                 |
|-------------------------------------------------|---------------|-------------|---------|----------------------------------------------------------------------|
| Positive regulation of response to stimulus     | 29            | 2621        | 1.39E−02| BMP4, IL36G, IL1RN, RSPC3, NRARP, JUP, HGF, MYOC, KLLK14, FGF18, S100A9, S100A12, ZC3H12A, ADRB2, ALOX12B, CCBE1, DLK5, CHN1, CD36, GREM1, ROR2, SOX11, DLOX1, WNT11, SH3RF3, NGF, TBX1, FGF7, ELF3 |
| Positive regulation of RNA metabolic process    | 22            | 1789        | 1.39E−02| DLK5, ZBTB16, HEYL, JUP, SOX11, BMP4, ELF3, ZC3H12A, PITX1, NOTCH3, FG7, CREB3L1, GREM1, ROR2, ADRB2, S102, Ovol1, MAFA, WNT11, GLI53, TBX1, HGF |
| Positive regulation of membrane depolarization  | 2             | 12          | 1.39E−02| MYOC, ALOX12 |
| Regulation of intracellular signal transduction  | 24            | 2027        | 1.42E−02| BMP4, IL36G, IL1RN, SIK1, HGF, MYOC, FGF18, S100A9, S100A12, ZC3H12A, ADRB2, ALOX12, CHN1, CD36, CREB3L1, GREM1, ROR2, SOX11, WNT11, SH3RF3, NGF, TBX1, FGF7, RHO |
| Positive regulation of cellular protein metabolic process | 21          | 1680        | 1.42E−02| HGF, BMP4, IL36G, IL1RN, FGF7, FGF18, S100A9, ZC3H12A, ADRB2, ALOX12, CCBE1, WNT11, ALOX12, KRT17, CD36, GREM1, ROR2, SH3RF3, TBX1, NGF, S100A12 |
| Lung alveolus development                        | 3             | 43          | 1.42E−02| BMP4, SMDP3, PKDCC |
| Pattern specification process                    | 9             | 451         | 1.42E−02| BMP4, ZBTB16, GREM1, ROR2, S102, NRARP, MAFA, MDFI, TBX1 |
| Growth                                           | 15            | 1028        | 1.4E−02 | NGF, BMP4, FGF7, CDA, WNT11, BDKRB1, SMDP3, KRT17, CD36, CYP19A1, PKDCC, AVPR1A, GREM1, ADRB2, S100A9 |
| Molting cycle process                            | 4             | 90          | 1.4E−02 | KRT17, FGF7, TMEM79, KRT25 |
| Hair cycle process                               | 4             | 90          | 1.4E−02 | KRT17, FGF7, TMEM79, KRT25 |
| Wnt signaling pathway                            | 10            | 543         | 1.48E−02| WNT11, RSPC3, NRARP, JUP, GREM1, DLK5, SDC1, ROR2, MYOC, MDFI |
| Negative regulation of osteoblast differentiation | 3             | 44          | 1.49E−02| TWIST2, ID3, GREM1 |
| Skin epidermis development                       | 4             | 91          | 1.49E−02| KRT17, FGF7, TMEM79, KRT25 |
| Regulation of Wnt signaling pathway              | 8             | 371         | 1.49E−02| RSPC3, NRARP, JUP, GREM1, WNT11, DLK5, ROR2, MDFI |
| Cell–cell signaling by wnt                       | 10            | 545         | 1.5E−02 | WNT11, RSPC3, NRARP, JUP, GREM1, DLK5, SDC1, ROR2, MYOC, MDFI |
| Digestive system development                     | 5             | 151         | 1.54E−02| BMP4, PKDCC, S102, S101, WNT11 |
| Insemination                                     | 2             | 13          | 1.57E−02| KLF14, AVPR1A |
| Extracellular structure organization              | 9             | 460         | 1.57E−02| COL8A1, COL28A1, SMDP3, CD36, ADAMTSL4, ELF3, SDC1, GREM1, CREB3L1 |
| Protein import into nucleus                      | 5             | 152         | 1.57E−02| BMP4, ZC3H12A, JUP, CD36, S10X |
| Positive regulation of cell death                | 12            | 741         | 1.61E−02| BMP4, ADAMTS4, S100A9, ZC3H12A, WNT11, ALOX12, ZBTB16, ID3, CD36, SIK1, PNMA5, NGF |
| Muscle structure development                     | 11            | 645         | 1.61E−02| BMP4, USP2, PITA1, SCD1, ID3, HEYL, AVPR1A, GREM1, S101, SIK1, TBX1 |
| Anterior/posterior pattern specification          | 6             | 222         | 1.63E−02| BMP4, ZBTB16, ROR2, S102, NRARP, TBX1 |
| Epithelial cell differentiation involved in kidney development | 3             | 46          | 1.63E−02| BMP4, GREM1, S10X |
| Regulation of canonical Wnt signaling pathway    | 7             | 298         | 1.65E−02| RSPC3, NRARP, JUP, GREM1, WNT11, DLK5, ROR2 |
| Regulation of blood vessel endothelial cell migration | 4           | 95          | 1.68E−02| ANGPT2, ALOX12, FGF18, JUP |
| Secretion by cell                                | 21            | 1715        | 1.70E−02| CYP19A1, FGF7, FAM3D, CHRNA4, SMDP3, CD36, IL1RN, TMEM79, ZC3H12A, AVPR1A, S10X, SDC1, GP6C2, CREB3L1, HGF, GLP1R, SLPI, CD36, S100A9, S100A12, JUP |
| Gland development                                | 9             | 468         | 1.71E−02| HGF, PITA1, BMP4, CYP19A1, FGF7, ELF3, MAFB, WNT11, TBX1 |
| Embryonic cranial skeleton morphogenesis         | 3             | 47          | 1.71E−02| BMP4, S102, TBX1 |
| Regulation of biomineral tissue development      | 4             | 97          | 1.78E−02| BMP4, PKDCC, ADRB2, GREM1 |
| Mating                                           | 3             | 48          | 1.80E−02| KLF14, PI, AVPR1A |
| Response to external stimulus                    | 28            | 2561        | 1.80E−02| IL36G, IL1RN, SIK1, DEF8B4A, HGF, ANGPT2, SLPI, CD36, FGF7, FGF18, S100A9, S100A12, ZC3H12A, JUP, A1AP1, USP2, WNT11, BDKRB1, DLK5, ALOX12, BMP4, CHN1, CYP19A1, AVPR1A, GREM1, ADRB2, PI3, LTBP2 |
| Positive regulation of canonical Wnt signaling pathway | 5             | 159         | 1.83E−02| RSPC3, NRARP, JUP, DLK5, ROR2 |
| Positive regulation of protein modification process | 17          | 1279        | 1.83E−02| BMP4, IL36G, IL1RN, FGF7, FGF18, ZC3H12A, ADRB2, ALOX12B, WNT11, CD36, GREM1, ROR2, SH3RF3, TBX1, NGF, S100A12 |
| Somite development                               | 4             | 98          | 1.83E−02| WNT11, ROR2, S10X, NRARP |
| Regulation of binding                            | 8             | 388         | 1.83E−02| ID3, SLPI, BMP4, ADRB2, S10X, CD36, MDFI, NGF |
| Genitalia development                            | 3             | 49          | 1.87E−02| CYP19A1, GREB3L1, ROR2 |
| Positive regulation of biomineral tissue develop- | 3             | 49          | 1.87E−02| BMP4, PKDCC, ADRB2 |
### Table 3 (continued)

| Functional category                                      | Genes in list | Total genes | FDR      | Genes                                      |
|----------------------------------------------------------|---------------|-------------|----------|--------------------------------------------|
| Ear development                                          | 6             | 232         | 1.93E−02 | DLX5 BMP4 ROR2 SIX2 MAFB TBX1              |
| Branching morphogenesis of an epithelial tube             | 5             | 162         | 1.94E−02 | NRARP BMP4 GREB1L GREM1 SIX2              |
| Positive regulation of exosomal secretion                | 2             | 15          | 1.95E−02 | SDC1 SMPD3                                 |
| Regulation of transcription by RNA polymerase II         | 30            | 2830        | 1.95E−02 | DLX5 ZBTB16 BMP4 ELF3 HEYL JUP SOX11 ZC3H12A GREM1 FAH38G PITX1 NOTCH3 SMPD3 GLIS3 ID3 TBX4 CD36 CREBL1 ROR2 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB ZNF469 USP2 MDFI TBX1 HGF |
| Activation of transmembrane receptor protein tyrosine kinase activity | 2             | 15          | 1.95E−02 | GREM1 ADRB2                                |
| Positive regulation of macromolecule biosynthetic process | 23            | 1978        | 1.96E−02 | IL36G IL1RN ZC3H12A BMP4 ELF3 HEYL JUP ANGPT2 5LC8A3 SDC1 ID3 CD36 ZC3H12A AVPR1A 5LC8A3 BMP4 CATSPERB |
| Secretion                                               | 22            | 1861        | 1.96E−02 | CYP19A1 FGF7 FAM3D CHRNA4 SMPD3 CD36 IL1RN TIMEM79 ZC3H12A AVPR1A SOX11 SDC1 G6PC2 ALOX12B CREBL1 HGF GLP1R SLPI CDA S100A9 S100A12 JUP |
| Positive regulation of smooth muscle cell proliferation  | 4             | 101         | 1.97E−02 | BMP4 NOTCH3 SMPD3 ALOX12                  |
| Negative regulation of hemopoiesis                       | 5             | 164         | 2.00E−02 | MAFB ZBTB16 ZC3H12A NRARP BMP4           |
| Regulation of interleukin-6 production                    | 5             | 164         | 2.00E−02 | IL36G IL1RN ZC3H12A BMP4 ELF3 HEYL JUP ANGPT2 5LC8A3 SDC1 ID3 CD36 ZC3H12A AVPR1A 5LC8A3 BMP4 CATSPERB |
| Response to organic cyclic compound                       | 14            | 975         | 2.02E−02 | IL1RN DUOX1 HEYL JUP ANGPT2 5LC8A3 SDC1 ID3 CD36 ZC3H12A AVPR1A 5LC8A3 BMP4 CATSPERB |
| Intermediate filament cytoskeleton organization           | 3             | 51          | 2.02E−02 | TCHH KRT17 KRT25                         |
| Negative regulation of lymphocyte differentiation         | 3             | 51          | 2.02E−02 | ZC3H12A NRARP BMP4                        |
| Negative regulation of cell differentiation              | 12            | 774         | 2.08E−02 | ID3 BMP4 MAFA TWIST2 NOTCH3 ZBTB16 ZC3H12A SIX2 OVO1 2NF154 NRARP MAFB GREM1 BMP4 |
| Regulation of signaling receptor activity                 | 11            | 676         | 2.08E−02 | LYNX1 HGF BMP4 NGF IL36G IL1RN FGF7 FGF7 IL1RN GREM1 FAH38G ADRB2 |
| Negative regulation of transcription by RNA polymerase II | 13            | 876         | 2.08E−02 | BMP4 SOX11 ZBTB16 NOTCH3 ID3 CD36 CREBL1 HEYL OVO1 2NF154 USP2 GLIS3 MDFI |
| Anterior/posterior axis specification                     | 3             | 52          | 2.10E−02 | MYOC CD36 JUP                             |
| Positive regulation of cell–matrix adhesion              | 3             | 52          | 2.10E−02 | TCHH KRT17 KRT25                         |
| Intermediate filament-based process                       | 3             | 52          | 2.10E−02 | BMP4 GREM1                                |
| Regulation of transcription from RNA polymerase II promoter involved in heart development | 2             | 16          | 2.11E−02 | BMP4 GREM1                                |
| Regulation of exosomal secretion                         | 2             | 16          | 2.11E−02 | BMP4 S100A9 WNT11 BMP4 BMP4 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Cellular response to oxygen-containing compound           | 16            | 1199        | 2.13E−02 | BMP4 S100A9 WNT11 BMP4 BMP4 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Positive regulation of apoptotic process                 | 11            | 680         | 2.13E−02 | SMPD3 BMP4 AMAP11 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Negative regulation of response to stimulus              | 21            | 1764        | 2.13E−02 | BMP4 S100A9 WNT11 BMP4 BMP4 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Negative regulation of binding                           | 5             | 168         | 2.13E−02 | BMP4 S100A9 WNT11 BMP4 BMP4 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Positive regulation of macromolecule metabolic process   | 35            | 3498        | 2.13E−02 | BMP4 S100A9 WNT11 BMP4 BMP4 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Muscle organ development                                  | 8             | 404         | 2.15E−02 | BMP4 S100A9 WNT11 BMP4 BMP4 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Negative regulation of DNA binding                       | 3             | 53          | 2.16E−02 | BMP4 S100A9 WNT11 BMP4 BMP4 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Mesenchyme morphogenesis                                  | 3             | 53          | 2.16E−02 | BMP4 S100A9 WNT11 BMP4 BMP4 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Regulation of phosphorylation                            | 20            | 1653        | 2.17E−02 | BMP4 S100A9 WNT11 BMP4 BMP4 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Positive regulation of programmed cell death             | 11            | 686         | 2.23E−02 | BMP4 S100A9 WNT11 BMP4 BMP4 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Unsaturated fatty acid biosynthetic process               | 3             | 54          | 2.25E−02 | BMP4 S100A9 WNT11 BMP4 BMP4 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Inner ear morphogenesis                                  | 4             | 107         | 2.25E−02 | BMP4 S100A9 WNT11 BMP4 BMP4 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Regulation of striated muscle cell differentiation        | 4             | 107         | 2.25E−02 | BMP4 S100A9 WNT11 BMP4 BMP4 ADRB2 SIX2 OVO1 2NF154 NRARP MAFB GREM1 |
| Functional category                                      | Genes in list | Total genes | FDR    | Genes                                      |
|----------------------------------------------------------|---------------|-------------|--------|--------------------------------------------|
| Negative regulation of signal transduction               | 17            | 1321        | 2.27E−02 | SIK1 NRARP HGF MYOC BMP4 KLK14 IL1RN CRPB3L1 HEYL GREM1 ADRB2 WNT11 RASL11B ZC3H12A ROR2 MDF1 NOTCH3 |
| Lipoxigenase pathway                                      | 2             | 17          | 2.27E−02 | ALOX12 ALOX12B                             |
| Interleukin-6 production                                  | 5             | 172         | 2.27E−02 | IL36G IL1RN ZC3H12A HGF CD36               |
| Linoleic acid metabolic process                           | 2             | 17          | 2.27E−02 | ALOX12 ALOX12B                             |
| Pulmonary valve morphogenesis                             | 2             | 17          | 2.27E−02 | BMP4 HEYL                                  |
| Negative regulation of molecular function                 | 16            | 1216        | 2.33E−02 | ID3 HGF SLPI BMP4 ZC3H12A HEYL ADRB2 P3 NGF S0X11 LYNX1 SPINK9 COL28A1 MDF1 SIK1 LTB4R2 |
| Positive regulation of metabolic process                  | 37            | 3789        | 2.34E−02 | HGF DLX5 ZBTB16 BMP4 IL36G IL1RN HEYL JUP S0X11 FGF7 FGF18 CREB3L1 S100A9 ELF3 ZC3H12A GREM1 ADRB2 ALOX12B CCB1 E1 PITX1 NOTCH3 WNT11 ALOX12 KRT17 CD36 AVPR1A ROR2 S6X2 OVOL1 MAFB NGF SH3RF3 GLI3 ACTG2 TBX1 SMPD3 S100A12 |
| Segmentation                                              | 4             | 109         | 2.35E−02 | BMP4 ROR2 NRARP MAFB                       |
| Regulation of stress-activated MAPK cascade               | 6             | 248         | 2.38E−02 | IL36G IL1RN ZC3H12A HGF ROR2 SH3RF3       |
| Cell differentiation involved in kidney development        | 3             | 56          | 2.42E−02 | BMP4 GREM1 SIK2                             |
| Regulation of epithelial cell proliferation               | 7             | 330         | 2.43E−02 | NRARP BMP4 FGF7 DLX5 S0X11 TGM1 TBX1       |
| Import into nucleus                                       | 5             | 176         | 2.44E−02 | BMP4 ZC3H12A JUP CD36 SIK2                 |
| Regulation of stress-activated protein kinase signaling cascade | 6             | 250         | 2.44E−02 | IL36G IL1RN ZC3H12A HGF ROR2 SH3RF3       |
| Positive regulation of protein metabolic process          | 21            | 1796        | 2.45E−02 | HGF BMP4 IL36G IL1RN FGF7 FGF18 S100A9 ZC3H12A ADRB2 ALOX12B CCB1 E1 WNT11 ALOX12 KRT17 CD36 GREM1 ROR2 SH3RF3 TBX1 NGF S100A12 |
| Carbohydrate derivative biosynthetic process              | 12            | 800         | 2.45E−02 | HS3ST3A1 B3GNT4 FUT2 B3GNT8 B3GNT3 PAPS52 CHST8 ST8SIA2 SMPD3 ST6GAL2 SDC1 CDA |
| Regulation of nucleocytoplasmic transport                | 4             | 111         | 2.46E−02 | BMP4 ZC3H12A JUP CD36                      |
| Embryonic digestive tract morphogenesis                   | 2             | 18          | 2.47E−02 | SIK2 S0X11                                 |
| Regulation of kidney development                          | 3             | 57          | 2.49E−02 | BMP4 GREM1 SIK2                             |
| Negative regulation of leukocyte differentiation          | 4             | 112         | 2.52E−02 | ZC3H12A NRARP MAFB BMP4                    |
| Lung morphogenesis                                        | 3             | 58          | 2.60E−02 | BMP4 FGF7 S0X11                             |
| Leukocyte chemotaxis                                      | 6             | 255         | 2.62E−02 | IL36G IL1RN S100A9 CYP19A1 GREM1 S100A12  |
| Cell junction assembly                                     | 6             | 255         | 2.62E−02 | MYOC JUP WNT11 GREM1 RHOD FBLM1            |
| Mucopolysaccharide metabolic process                      | 4             | 114         | 2.65E−02 | HGF SMPD3 B3GNT4 B3GNT3                    |
| Regulation of growth                                      | 11            | 710         | 2.67E−02 | NGF BMP4 CDA BDKRB1 KRT17 CD36 AVPR1A GREM1 ADRB2 WNT11 S100A9 |
| Regulation of production of miRNAs involved in gene silencing by miRNA | 2             | 19          | 2.68E−02 | BMP4 ZC3H12A                                 |
| Exosomal secretion                                        | 2             | 19          | 2.68E−02 | SDC1 SMPD3                                 |
| Keratinocyte migration                                    | 2             | 19          | 2.68E−02 | FGF7 LTB4R2                                |
| Kidney mesenchyme development                             | 2             | 19          | 2.68E−02 | BMP4 SIK2                                  |
| Negative regulation of DNA-binding transcription factor activity | 5             | 182         | 2.69E−02 | ID3 ZC3H12A HEYL SIK1 MDF1                |
| Bone morphogenesis                                        | 4             | 115         | 2.69E−02 | SMPD3 DLX5 BMP4 FGF18                      |
| Polysaccharide metabolic process                          | 4             | 116         | 2.76E−02 | B3GNT4 B3GNT8 B3GNT3 SMPD3                 |
| Cellular oxidant detoxification                           | 4             | 117         | 2.83E−02 | CD36 DUOX1 S100A9 GXP3                     |
| Molting cycle                                             | 4             | 117         | 2.83E−02 | KRT25 KRT17 FGF7 TMEM79                    |
| Hair cycle                                                | 4             | 117         | 2.83E−02 | KRT25 KRT17 FGF7 TMEM79                    |
| Extracellular exosome biogenesis                          | 2             | 20          | 2.84E−02 | SDC1 SMPD3                                 |
| Positive regulation of cardiac muscle cell differentiation | 2             | 20          | 2.84E−02 | GREM1 BMP4                                 |
| Regulation of cell growth                                 | 8             | 432         | 2.84E−02 | NGF CDA BDKRB1 KRT17 AVPR1A GREM1 WNT11 S100A9 |
| Glycoprotein metabolic process                            | 8             | 432         | 2.84E−02 | HS3ST3A1 B3GNT4 FUT2 B3GNT8 B3GNT3 CHST8 ST8SIA2 ST6GAL2 |
| Notochond development                                     | 2             | 20          | 2.84E−02 | WNT11 ID3                                 |
| Blood vessel endothelial cell proliferation involved in sprouting angiogenesis | 2             | 20          | 2.84E−02 | NRARP BMP4                                 |
Table 3 (continued)

| Functional category                              | Genes in list | Total genes | FDR          | Genes                                      |
|--------------------------------------------------|---------------|-------------|--------------|--------------------------------------------|
| Down-regulated genes in alive × dead             |               |             |              |                                            |
| Positive regulation of stress-activated MAPK      | 5             | 186         | 2.84E−02     | IL36G IL1RN ZC3H12A ROR2 SH3RF3            |
| cascade                                          |               |             |              |                                            |
| Response to hydroperoxide                         | 2             | 20          | 2.84E−02     | CD36 GPX3                                  |
| Middle ear morphogenesis                          | 2             | 20          | 2.84E−02     | SOX2 TBX1                                  |
| Entrainment of circadian clock by photoperiod     | 2             | 20          | 2.84E−02     | USP2 SKI                                   |
| Negative regulation of leukocyte chemotaxis       | 2             | 20          | 2.84E−02     | CYP19A1 GREM1                              |
| Regulation of production of small RNA involved in | 2             | 20          | 2.84E−02     | BMP4 ZC3H12A                                |
| gene silencing by RNA                             |               |             |              |                                            |
| Hormone secretion                                 | 7             | 346         | 2.86E−02     | CYP19A1 FAM3D SMPD3 IL1RN SOX11 G6PC2 GLP1R |
| Positive regulation of stress-activated protein   | 5             | 187         | 2.88E−02     | IL36G IL1RN ZC3H12A ROR2 SH3RF3            |
| kinase signaling cascade                          |               |             |              |                                            |
| Negative regulation of endopeptidase activity     | 6             | 264         | 2.89E−02     | HGF P13 SLPI SPINK9 COL28A1 NGF             |
| Neutrophil chemotaxis                             | 4             | 119         | 2.91E−02     | BMP4 BMP4 ROR2                             |
| Embryonic digit morphogenesis                     | 2             | 62          | 2.91E−02     | ZBTB16 BMP4 ROR2                           |
| Modification of morphology or physiology of other | 5             | 188         | 2.91E−02     | SLPI S100A9 ZC3H12A DEF84A                  |
| organism                                         |               |             |              |                                            |
| Positive regulation of cell-substrate adhesion    | 4             | 120         | 2.95E−02     | MYOC CD36 COL8A1 JUP                       |
| Positive regulation of protein serine/threonine   | 7             | 349         | 2.95E−02     | BMP4 FGF18 HGF ROR2 ADRB2 S100A12          |
| kinase activity                                   |               |             |              |                                            |
| Regulation of cell-substrate junction assembly    | 3             | 63          | 3.00E−02     | MYOC GREM1 RHOD                           |
| Sialylation                                       | 2             | 21          | 3.00E−02     | ST8SIA2 S6GAL2                             |
| Regulation of protein localization to nucleus     | 4             | 121         | 3.00E−02     | BMP4 ZC3H12A JUP CD36                      |
| Protein glycosylation                             | 6             | 268         | 3.00E−02     | B3GNT4 FUT2 B3GNT8 B3GNT3 ST8SIA2 S6GAL2   |
| Positive regulation of Wnt signaling pathway      | 5             | 190         | 3.00E−02     | RSP33 NRARP JUP DLX5 ROR2                  |
| Negative regulation of chondrocyte differentiation | 2             | 21          | 3.00E−02     | BMP4 GREM1                                |
| Macromolecule glycosylation                       | 6             | 268         | 3.00E−02     | B3GNT4 FUT2 B3GNT8 B3GNT3 ST8SIA2 S6GAL2   |
| Regulation of focal adhesion assembly             | 3             | 63          | 3.00E−02     | MYOC GREM1 RHOD                           |
| Regulation of mitochondrial depolarization        | 2             | 21          | 3.00E−02     | MYOC ALOX12                                |
| Pulmonary valve development                       | 2             | 21          | 3.00E−02     | MYOC ALOX12                                |
| Chordate embryonic development                    | 10            | 632         | 3.02E−02     | MDF1 BMP4 RPSO3 ELF3 ROR2 S100A11 NRARP WNT11 TBX1 |
| O-glycan processing                               | 3             | 64          | 3.07E−02     | B3GNT4 B3GNT8 B3GNT3                      |
| Respiratory gaseous exchange                      | 3             | 65          | 3.18E−02     | CHRNA4 CCB1 MAFB                          |
| Gland morphogenesis                               | 4             | 124         | 3.18E−02     | HGF BMP4 FGF7 ELF3                        |
| Negative regulation of chemotaxis                | 3             | 65          | 3.18E−02     | ANGPT2 CYP19A1 GREM1                      |
| Positive regulation of striated muscle cell       | 3             | 65          | 3.18E−02     | GREM1 BMP4 TBX1                            |
| differentiation                                   |               |             |              |                                            |
| Hormone transport                                 | 7             | 357         | 3.19E−02     | CYP19A1 FAM3D SMPD3 IL1RN SOX11 G6PC2 GLP1R |
| Extracellular vesicle biogenesis                  | 2             | 22          | 3.21E−02     | SDC1 SMPD3                                |
| Positive regulation of transcription of Notch     | 2             | 22          | 3.21E−02     | NOTCH3 HEYL                               |
| receptor target                                   |               |             |              |                                            |
| Gastrulation                                      | 5             | 195         | 3.21E−02     | BMP4 IL1RN WNT11 S100A1 COL8A1            |
| Negative regulation of peptidase activity         | 6             | 273         | 3.21E−02     | HGF P13 SLPI NGF SPINK9 COL28A1            |
| Negative regulation of response to external       | 7             | 358         | 3.21E−02     | ANGPT2 AJAP1 HGF ALOX12 CYP19A1 ZC3H12A GREM1 |
| stimulus                                         |               |             |              |                                            |
| Proteolysis                                       | 22            | 1988        | 3.24E−02     | CPXM2 HGF USP2 KLK14 S100A9 ZC3H12A CCB1 KLK12 PRSS50 ALOX12 P13 SLPI KLK10 NGF ADAMTS4 PRSS5 PRSS5 ADAMTS15 KLK13 PRSS2 SPINK9 COL28A1 ADRB2 |
| Negative regulation of cell-substrate adhesion    | 3             | 66          | 3.25E−02     | MYOC AJAP1 ANGPT2                         |
| Transcription by RNA polymerase II                | 30            | 2992        | 3.25E−02     | DLX5 ZBTB16 BMP4 ELF3 HEYP JUP SOX11 ZC3H12A GREM1 FAM83G PITX1 NOTCH3 SMPD3 GLS3 ID3 TBX4 CD36 GREB3L1 ROR2 ADRB2 S100A9 OXOL1 ZNF154 NRARP MAFB ZNF469 USP2 MDF1 TBX1 HGF |
| Regulation of phosphate metabolic process         | 21            | 1870        | 3.25E−02     | HGF BMP4 IL36G IL1RN MYOC FGF7 FGF18 CDA ZC3H12A GREM1 ADRB2 ALOX12B WNT11 BDKR81 SMPD3 CD36 ROR2 SH3RF3 TBX1 NGF S100A12 |
Table 3

Down-regulated genes in alive × dead

| Functional category                                           | Genes in list | Total genes | FDR     | Genes                                                                 |
|---------------------------------------------------------------|---------------|-------------|---------|----------------------------------------------------------------------|
| Regulation of membrane potential                              | 8             | 450         | 3.25E−02| CHRNA4 KCNK12 MYOC SLClB3 ALOX12 CD36 ADRB2 JUP                      |
| Positive regulation of MAP kinase activity                    | 6             | 275         | 3.25E−02| BMP4 FGFB HGF ROR2 NGF S100A12                                       |
| Cellular detoxification                                        | 4             | 126         | 3.26E−02| CD36 DUOX1 S100A9 GPX3                                               |
| Carbohydrate metabolic process                                | 10            | 644         | 3.27E−02| G6PC2 B3GNT4 B3GNT8 B3GNT3 ST6SIA2 ST6GAL2 SMPD3 CHST8 FUT2 SK1      |
| Positive regulation of nucleobase-containing compound metabolic process | 22            | 1993        | 3.27E−02| DLX5 ZBTB16 HEYL JUP SOX11 HGF BMP4 ELF3 ZC3H12A GREM1 PITX1 NOTCH3 FGFB GREB1L ROR2 ADRB2 SIK2 OVOL1 MAP2B WNT11 GLI5 TBX1 |
| Regulation of phosphorus metabolic process                    | 21            | 1872        | 3.27E−02| HGF BMP4 IL36G IL1 RN MYOC FGFB FGFB IL18 CDA ZC3H12A GREM1 ADRB2 ALOX12B WNT11 BDKRB1 SMPD3 CD36 ROR2 SH3RF3 TBX1 NGF S100A12 |
| Protein localization to nucleus                                | 6             | 276         | 3.27E−02| BMP4 ZC3H12A JUP ZBTB16 CD36 SIK2                                   |
| Cofactor catabolic process                                     | 3             | 67          | 3.31E−02| GPX3 DUOX1 ALDH1L1                                                   |
| Male genitalia development                                     | 2             | 23          | 3.35E−02| GREB1L ROR2                                                          |
| Lens morphogenesis in camera-type eye                         | 2             | 23          | 3.35E−02| BMP4 SOX11                                                           |
| Positive regulation of epidermal cell differentiation          | 2             | 23          | 3.35E−02| BMP4 SULT2B1                                                         |
| Negative regulation of muscle contraction                     | 2             | 23          | 3.35E−02| ADRB2 ZC3H12A                                                       |
| Branching involved in salivary gland morphogenesis            | 2             | 23          | 3.35E−02| HGF FGFB                                                            |
| Endothelial tube morphogenesis                                 | 2             | 23          | 3.35E−02| BMP4 ALOX12                                                          |
| Morphogenesis of an endothelium                               | 2             | 23          | 3.35E−02| BMP4 ALOX12                                                          |
| Cranial skeletal system development                            | 3             | 68          | 3.39E−02| BMP4 SIK2 TBX1                                                       |
| Inflammatory response                                         | 12            | 856         | 3.39E−02| BDKRB1 LTBR42 S100A9 HGF SDC1 IL36G IL1 RN CYP19A1 S100A12 ELF3 ZC3H12A CD36 |
| Negative regulation of cell–cell adhesion                     | 5             | 200         | 3.39E−02| IL1 RN ALOX12 ZC3H12A NARAP BMP4                                    |
| Glycosylation                                                 | 6             | 280         | 3.41E−02| B3GNT4 FUT2 B3GNT8 B3GNT3 ST6SIA2 ST6GAL2                            |
| Embryo development ending in birth or egg hatching            | 10            | 652         | 3.44E−02| MDFI BMP4 RSP03 ELF3 ROR2 SIK2 SOX11 NARAP WNT11 TBX1               |
| Regulation of adherens junction organization                  | 3             | 69          | 3.48E−02| MYOC GREM1 RHCO                                                     |
| Skeletal muscle cell differentiation                           | 3             | 69          | 3.48E−02| HEYL SOX11 TBX1                                                      |
| Ceramide biosynthetic process                                 | 3             | 69          | 3.48E−02| ST6SIA2 ALOX12 SMPD3                                                 |
| Response to toxic substance                                   | 9             | 555         | 3.49E−02| CHRNA4 HGF SMPD3 SDC1 CD36 DUOX1 S100A9 ZC3H12A GPX3                 |
| Photoperiodism                                                | 2             | 24          | 3.51E−02| USP2 SIK1                                                           |
| Positive regulation of lipid storage                          | 2             | 24          | 3.51E−02| ZC3H12A CD36                                                        |
| Purine ribonucleoside bisphosphate metabolic process          | 2             | 24          | 3.51E−02| PAPS52 SULT2B1                                                      |
| Myelination                                                   | 4             | 131         | 3.51E−02| MALL HGF MYOC SLClB3                                                |
| Positive regulation of protein kinase activity                | 9             | 557         | 3.51E−02| BMP4 FGFB HGF WNT11 GREM1 ROR2 ADRB2 NGF S100A12                   |
| 3-phosphoadenosine 5′-phosphosulfate metabolic process        | 2             | 24          | 3.51E−02| PAPS52 SULT2B1                                                      |
| Mitochondrial depolarization                                  | 2             | 24          | 3.51E−02| MYOC ALOX12                                                          |
| Uterus development                                            | 2             | 24          | 3.51E−02| CYP19A1 GREB1L                                                     |
| Regulation of cellular protein metabolic process              | 28            | 2768        | 3.54E−02| HGF BMP4 IL36G IL1 RN MYOC FGFB FGFB IL18 S100A9 ZC3H12A GREM1 ADRB2 ALOX12B CCB1 WNT11 BDKRB1 SMPD3 ALOX12 P3 SL8P KRT17 NGF CD36 ROR2 SPINK9 COL2B8 SMH3RF3 TBX1 S100A12 |
| Formation of primary germ layer                               | 4             | 132         | 3.56E−02| BMP4 WNT11 SIK2 COLBA1                                               |
| Endocrine system development                                  | 4             | 132         | 3.56E−02| BMP4 PITX1 WNT11 TBX1                                               |
| Regulation of DNA binding                                     | 4             | 132         | 3.56E−02| ID3 SOX11 MDFI NGF                                                  |
| Striated muscle cell differentiation                          | 6             | 285         | 3.56E−02| BMP4 AVPR1A GREM1 SDC1 SIK1 TBX1                                    |
| Inner ear development                                         | 5             | 205         | 3.59E−02| DLX5 BMP4 ROR2 MAF2 TBX1                                            |
| Neutrophil migration                                          | 4             | 133         | 3.62E−02| IL36G IL1 RN S100A9 S100A12                                         |
directly with several components within the tumor microenvironment, including cancer cells, immune cells, endothelial cells and others [4, 6, 17, 25, 26, 30, 35, 61, 80, 81, 93, 95, 117, 120, 142, 143, 148]. We detected an increase in intra-tumoral CGRP after over-activation of sensory neurons (Fig. 10). However, it remains unknown whether the decrease in the tumor size after sensory neurons overactivation is due to this increase in the intra-tumoral concentration of CGRP. Future studies will need to genetically eliminate specific receptors for this and other neuropeptides (such as receptor activity-modifying protein 1 (RAMP1) for CGRP) from different cellular components of the

Table 4 Sensory neuron markers

| Gene   |
|--------|
| CALCA  |
| CHRNA3 |
| DIO3   |
| FAM19A |
| GRM3   |
| GRP    |
| KCNQ2  |
| MDGA1  |
| MMD2   |
| NECAB2 |
| NTRK1  |
| NTRK2  |
| NTRK3  |
| ONECUT3|
| P2RX3  |
| PCP4   |
| POU4F3 |
| POU6F2 |
| PRDM12 |
| PRDM8  |
| RET    |
| RUNX1  |
| RUNX3  |
| SCN10A |
| SCN11A |
| SCN9A  |
| STRA6  |
| SYT13  |
| TAC1   |
| TRPA1  |
| TRPM3  |
| TRPM8  |
| TRPV1  |
| VIP    |

Table 5 Angiogenesis-related genes down-regulated in alive x dead

| Gene   | symbol | log2FC  | FDR     |
|--------|--------|---------|---------|
| ANGPT2 | −1.42767| 3.23E−02|
| BMP4   | −1.23615| 3.99E−02|
| CCB1E1 | −1.04094| 4.44E−02|
| COL8A1 | −1.22669| 4.09E−02|
| CREB3L1| −1.04632| 4.44E−02|
| FGF18  | −1.35348| 3.80E−03|
| GREM1  | −1.36182| 1.10E−02|
| HGF    | −1.19158| 4.28E−03|
| JUP    | −1.81687| 1.10E−02|
| NOTCH3 | −1.22979| 3.57E−03|
| NRARP  | −1.42862| 5.97E−03|
| RSPO3  | −1.48997| 6.13E−04|
| TBBX1  | −1.26122| 3.69E−02|
| TBX4   | −1.84817| 5.39E−03|
| ZC3H12A| −1.35742| 4.67E−03|

Fig. 9 Schematic illustration summarizing the results of sensory neurons’ activity inhibition and overactivation in the melanoma microenvironment
tumor microenvironment to reveal whether those communications are relevant for melanoma outcomes.

**Conclusion**

In conclusion, this work identifies sensory neurons over-activation as a potential strategy for blocking melanoma progression and improving patient outcomes. We also anticipate that drugs that reduce sensory neurons hyperexcitability used for analgesic treatment may cause undesired effects in cancer patients and need to be carefully evaluated in pre-clinical models of cancer before using them in these patients [34, 125]. Moreover, the relationship between sensory hyperexcitability and cancer outcomes is likely to inform studies of other cancers that are also infiltrated by sensory neurons.

**Supplementary Information**

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**Additional file 1.** Gate strategy for regulatory markers. Representative contour plots showing proportion of CTLA-4 and PD-1 (top to bottom) in CD4+ and CD8+ (right to left) T viable lymphocytes within CD45+ alive cells from tumor infiltrate.

**Additional file 2.** Interactions among genes related to angiogenesis which are overexpressed in SKCM patients presenting worse prognosis (dead vs. alive).

**Additional file 3.** Inferred proportion of immune infiltrated cells in SKCM patients from the TCGA cohort.

**Additional file 4.** Differences observed on inferred proportions of tumor-infiltrating CD4+ T cells, CD8+ T cells, dendritic cells and NK cells between samples of alive and dead SKCM patients from the TCGA cohort.

**Additional file 5.** Impact of Th17 immune response in melanoma. (A) Microarray analysis of skin samples from Melanoma (n=46) and healthy (n=16) individuals from GEO database: GSE15605 was analyzed by Phantasmus [156]. Expression of Th17 immune response markers in melanoma samples, normalized to health samples, as Log2 Fold Change. (B) Survival curve from melanoma patients. The prognostic impact of IL17A expression in melanoma patients was evaluated using the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). We evaluated the survival probability of melanoma patients with melanoma based on their tumor transcriptome (n = 214) [19]. High expression of IL17A in melanoma is correlated with increased patient survival. Differences were considered significant at P value < 0.05.

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**Availability of data and materials**

Data will be made available on reasonable request.

**Declarations**

**Competing interests**

The authors have no competing interests to declare.

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