Additional File

Continuous ZnO nanoparticle exposure induces melanoma-like skin lesions in epidermal barrier dysfunction model mice through anti-apoptotic effects mediated by the oxidative stress–activated NF-κB pathway

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1. Materials & Methods

1.1 Functional analyses of the epidermal barrier and skin permeability

In order to determine the rate of fluid loss, animals were placed on a heating pad set at 37°C. Transepidermal water loss (TEWL) was measured using an Aquaflux AF-200 Tewameter (Courage and Khazaka, UK). Dye exclusion assays were performed as described previously.[1] Unfixed embryos were immersed in a low-pH X-gal substrate solution at 30-37°C for several hours to overnight until the color developed. The tails were removed with scissors to serve as a positive control for staining.

1.2 RNA sequencing

RNA from the skin of *Cdc42* knockout (KO) and wild-type (WT) mice after ZnO NP or control treatment for 14 days was prepared for RNA-seq (three biological replicates for each group). RNA-seq experiments were performed by Novogene (Beijing, China). Briefly, total RNA was isolated from fresh skin tissue using TRIzol. mRNA was then purified from total RNA using poly-T oligo-attached magnetic beads. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations, and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBotHS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform and 125/150 bp paired-end reads were generated. For the data analysis, raw data (raw reads) in fastq format were first processed through in-house Perl scripts. Clean data (clean reads) were obtained by removing reads containing adapters, reads containing poly-N, and low-quality reads from raw data. Reference genome and gene model annotation files were downloaded from genome website directly. An index of the reference genome was built using STAR, and paired-end clean reads were aligned to the reference genome using STAR (v2.5.1b). STAR uses the method of Maximal Mappable Prefix (MMP). HTSeq v0.6.0 was used to count the read numbers mapped to each gene. And then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. Differential expression analysis of three biological replicates per condition

was performed using the DESeq2 R package (1.10.1). DESeq2 provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg approach for controlling the false discovery rate. Genes with an adjusted P-value <0.05 found by DESeq2 were assigned as differentially expressed. Analysis of differential expression was performed using the edgeR R package (3.12.1). The P values were adjusted using the Benjamini and Hochberg method. The hierarchical clustering heat map was generated with the ggplot library.

2. Additional Figures

2.1. *Cdc42* knockout mice show abnormal inside-out and outside-in barrier function.

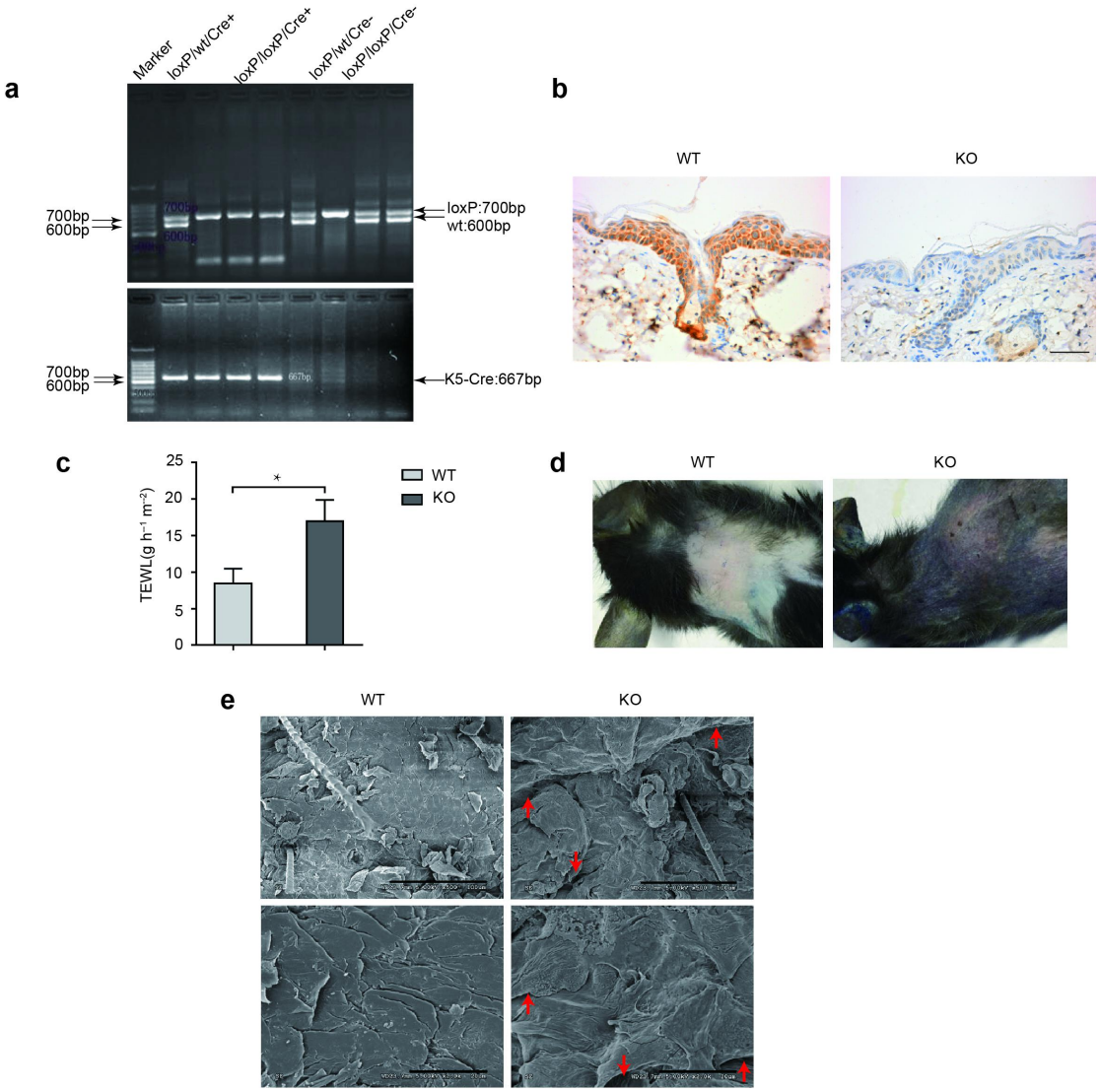

Figure S1. Epidermal barrier dysfunction caused by *Cdc42* knockout (KO) **a** PCR verification of *Cdc42* deletion in the epidermis. The upper panel shows PCR products for the *Cdc42*$^{loxp/loxp}$ allele and the bottom panel shows those of the *K5*-Cre (+) allele. The 700-base-pair (bp) band represents the flox allele; the 600 bp band represents the wild-type (WT) allele; the 667 bp band represents the *K5*-Cre (+) allele; and the blank band represents the internal positive control. The *Cdc42*$^{loxp/loxp/Cre+}$ mice were *Cdc42* KO, and the *Cdc42*$^{loxp/wt/Cre-}$/*Cdc42*$^{loxp/loxp/Cre-}$ were *Cdc42* WT.

b In immunohistochemistry experiments, there was negligible *Cdc42* expression in the epidermis of the KO mice. Scale bar = 50 μm. **c** TEWL measurement (n = 3), *p<0.05. **d** Skin permeability assay using X-gal staining in control and *Cdc42* KO mice. **e** Scanning electron micrograph (SEM) of dorsal skin of KO and WT mice. n = 3. Scale bars = 100 or 20 μm.

2.2. In vivo experiments

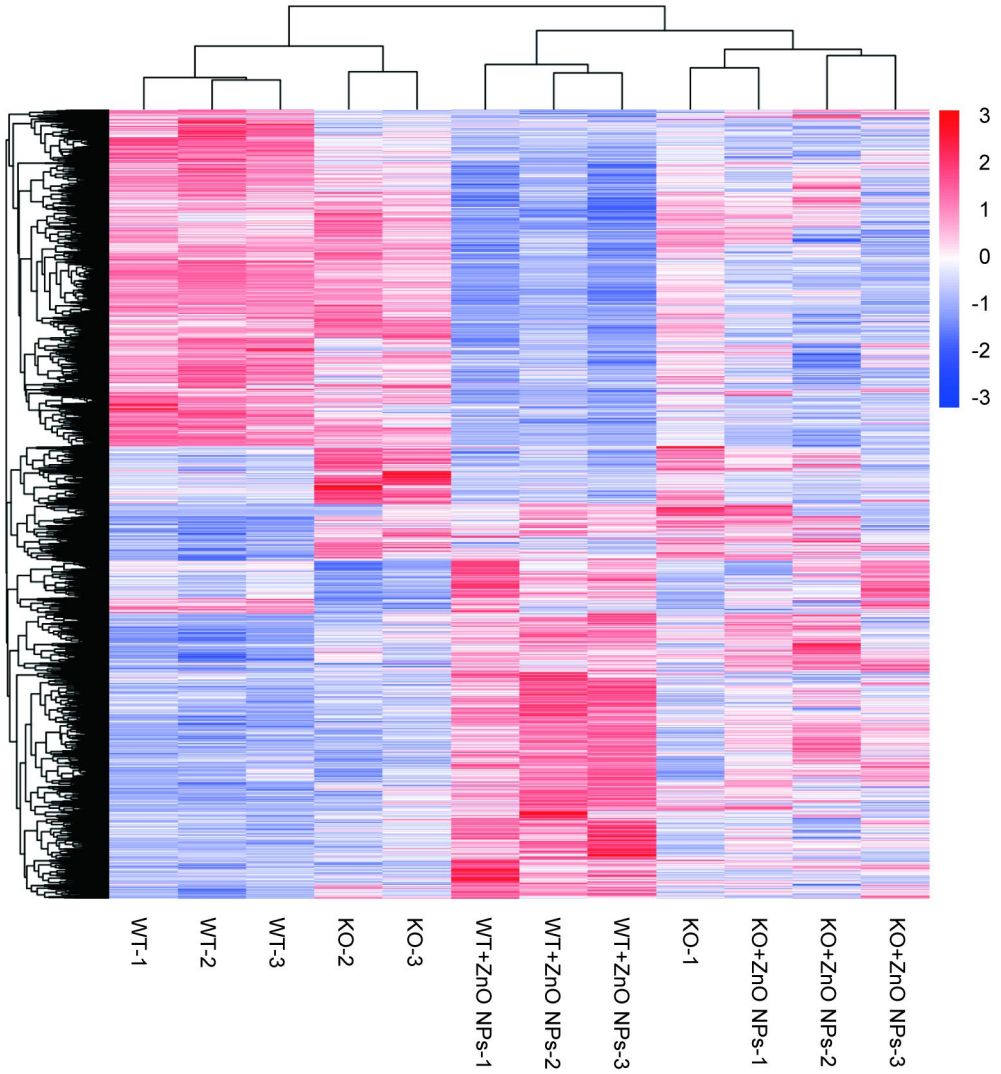

Figure S2. Cluster Analysis Using a Heatmap. Cluster analysis of differentially expressed mRNA in the skin on day 14 of *Cdc42* KO and WT mice continuously treated in the negative control group and the *Cdc42* KO and WT mice continuously treated with zinc oxide nanoparticles. Red indicates increased expression, and blue indicates decreased expression. n=3.

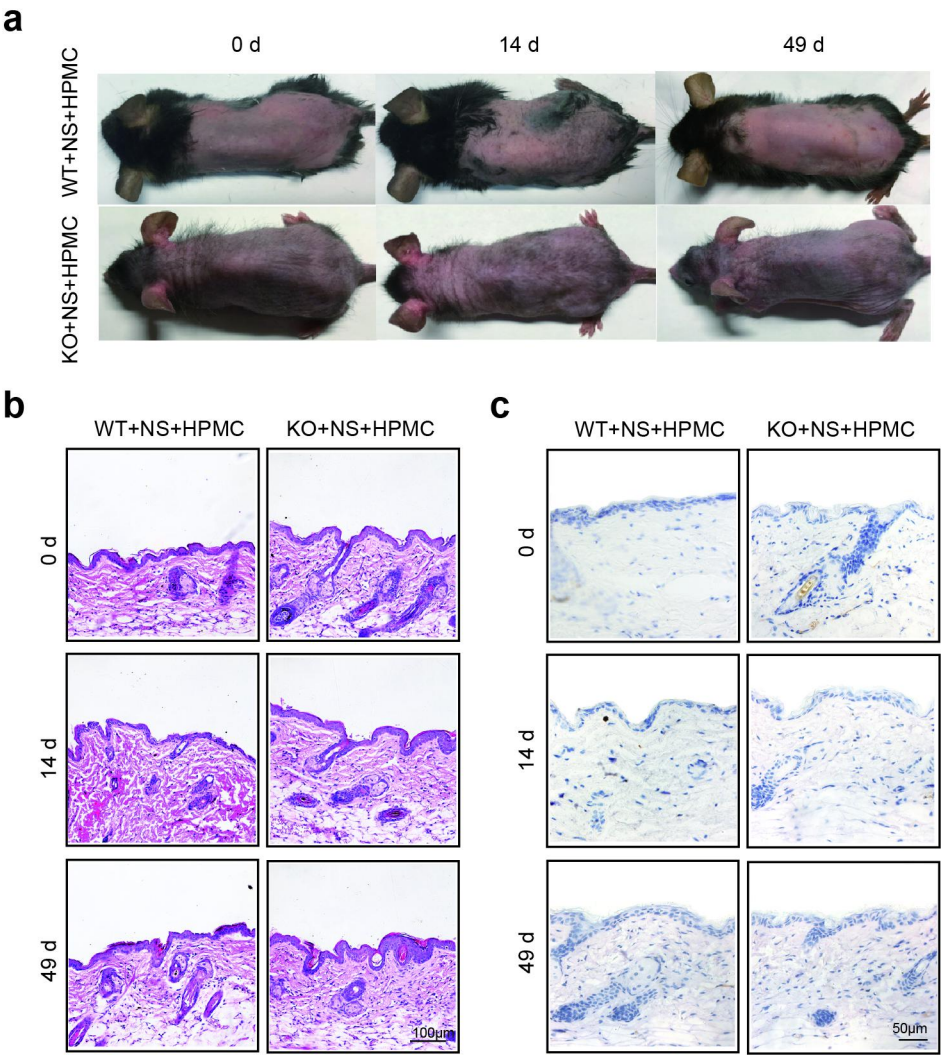

Figure S3. Changes in skin of *Cdc42* KO and WT mice continuously treated in the negative control group. **a** The gross morphology of *Cdc42* KO and WT mice continuously treated in the negative control group, observed on days 0, 14, and 49. **b** Histological changes in the skin of Cdc42 KO and WT mice continuously treated in the negative control group, observed on days 0, 14, and 49. Scale bar = 100 μm. **c** Immunohistochemical detection of tyrosinase in the skin of *Cdc42* KO and WT mice continuously treated in the negative control group, observed on days 0, 14, and 49. Scale bar = 50 μm.

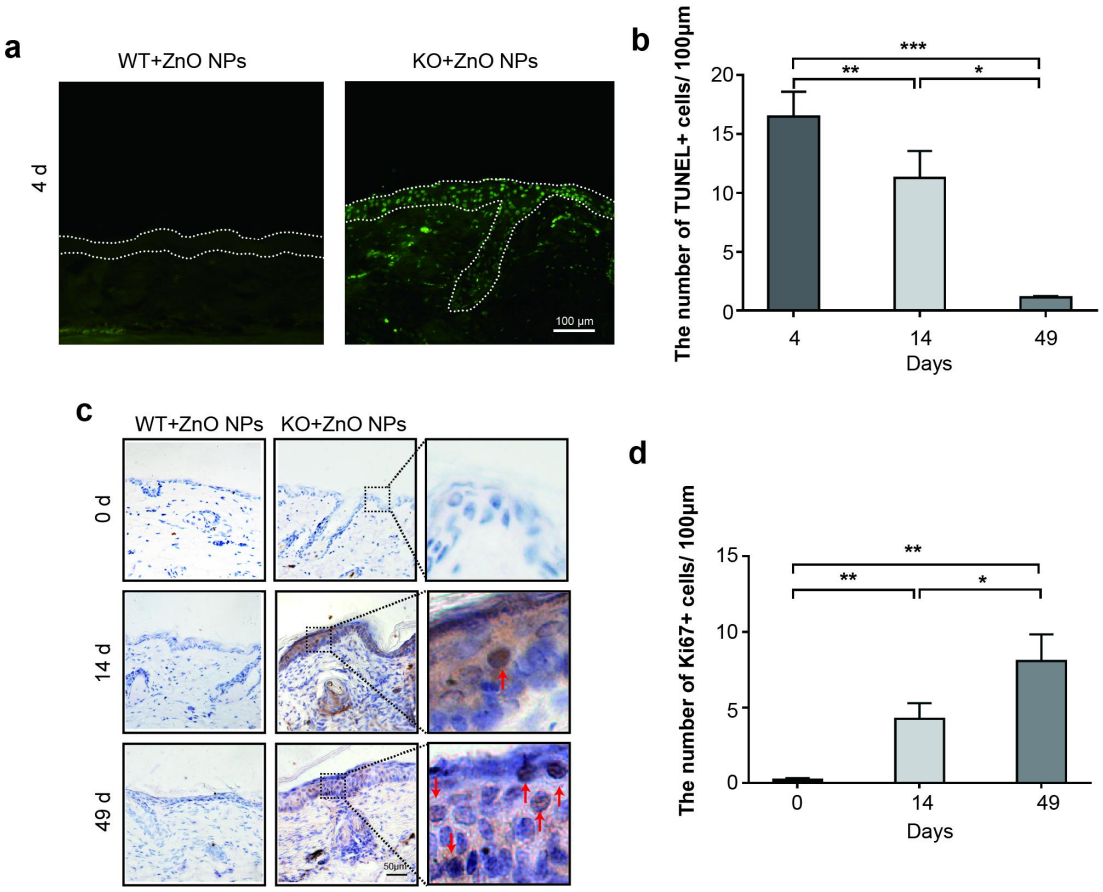

Figure S4. Abnormal apoptosis and proliferation in the skin of *Cdc42* KO and WT mice treated with zinc oxide nanoparticles (ZnO NPs). **a** TUNEL staining in the skin of *Cdc42* KO and WT mice continuously treated with ZnO NPs, observed on days 4. Scale bar = 100 μm. **b** Quantitation of TUNEL-positive cells per 100 μm of skin of *Cdc42* KO continuously treated with ZnO NPs on days 4, 14, and 49. n=3. *p < 0.05, **$p < 0.01$, and ***$p < 0.001$. **c** Immunohistochemical detection of Ki67-positive cells in the epidermis of *Cdc42* KO and WT mice continuously treated with ZnO NPs, observed on days 0, 14, and 49. Scale bar = 50 μm. **d** Quantitative analysis of the number of Ki67-positive cells per 100 μm in the epidermis of *Cdc42* KO and WT mice continuously treated with ZnO NPs on days 0, 14, and 49. n = 3. *$p<0.05$ and **$p<0.001$.

2.3. In vitro experiments

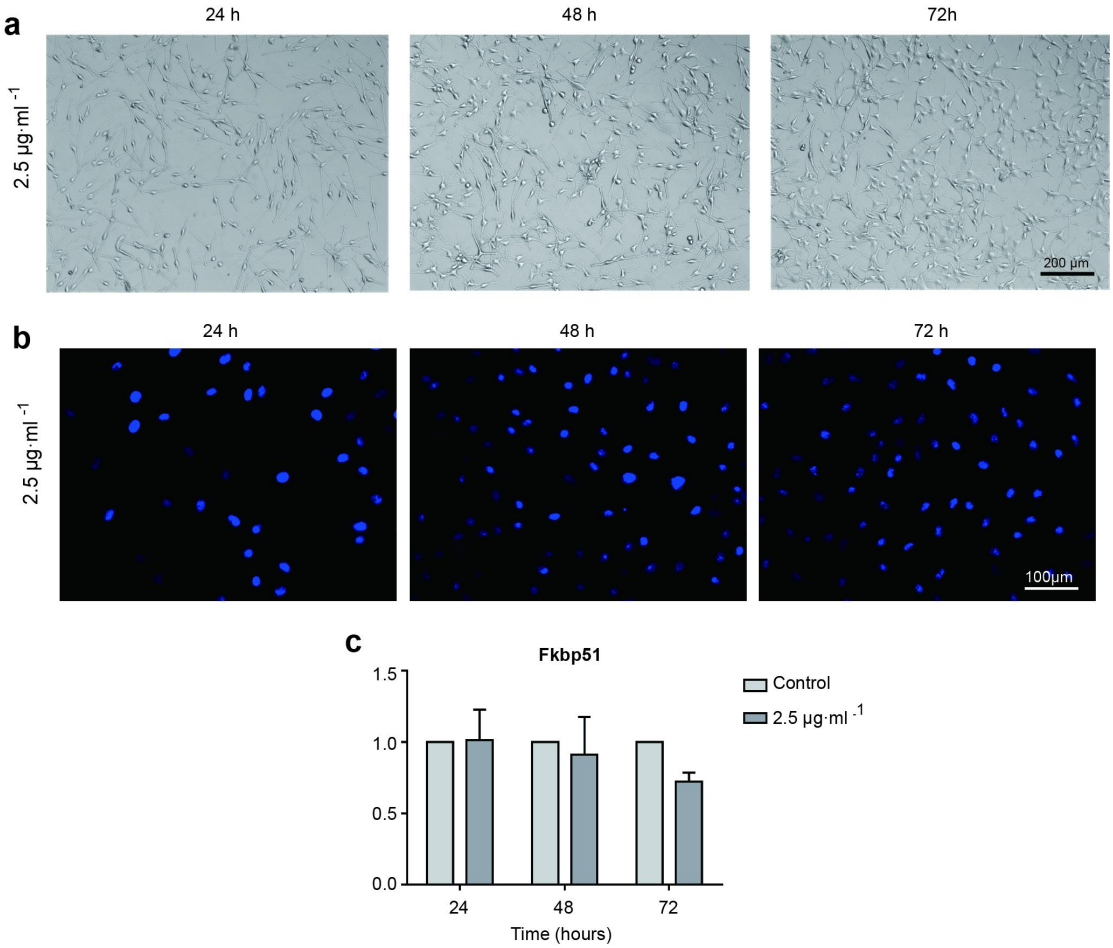

Figure S5. Changes in human epidermal melanocytes (HEMs) in culture after treatment wi th 2.5 µg·mL^{-1} ZnO NPs. **a** Images of HEMs in culture after treatment with 2.5 µg·mL^{-1} ZnO NPs, monitored using inverted phase-contrast microscopy.Scale bar = 200 µm. **b** Ter minal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of HEMs aft er treatment with 2.5 µg·mL^{-1} ZnO NPs. Scale bar = 100 µm. **c** *Fkbp51* mRNA levels w ere measured by quantitative real-time reverse transcriptase PCR (qRT-PCR) and normalize d to *Gapdh* expression. Data are presented as the mean ± SEM (n = 3).

References

1. M. J. Hardman, P. Sisi, D. N. Banbury, C. Byrne, *Development* **1998**, *125*, 1541.