Supplementary Information

"Targeted disruption of glutathione peroxidase 4 (GPx4) in mouse skin epithelial cells impairs postnatal hair follicle morphogenesis that is partially rescued through inhibition of COX-2"

Supplementary Materials and Methods:

Reagents

Primary antibodies against keratin-1 (PRB-165P, Covance, Princeton, NJ, USA), keratin-6 (PRB-169P, Covance), keratin-14 (PRB-155P, Covance), loricrin (PRB-145P, Covance), Ki67 (VP-K451, Vector Laboratories, Burlingame, CA, USA), F4/80 (MF48000, CALTAG, Burlingame, CA, USA), myeloperoxidase (A0398, DAKO, Carpinteria, CA, USA), COX-2 (160106, Cayman Chemical, Ann Arbor, MI, USA), GPx4 (3649-1, Epitomics, Burlingame, CA, USA) and TR1 (3928-1, Epitomics) were used in this study. Vectastain Elite ABC kit and DAB were procured from Vector Laboratories.

Maintenance of knockout mice

The breeding pattern used to generate the knockout mice was followed thereafter for maintenance of offspring and all the breeders were maintained on a selenium adequate diet (Kumaraswamy *et al.*, 2003). All mice were handled and humanely sacrificed in accordance with the National Institutes of Health Institutional Guidelines under the expert direction of Dr. John Dennis (NCI, NIH, Bethesda, MD, USA).

Genotyping of mice and analysis for tissue-specific recombination

Genomic DNA from mouse tails was isolated using the REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO, USA), according to manufacturer's instructions and genotyped using previously described primer sets for *GPx4* (Seiler *et al.*, 2008) and *TR1* (Jakupoglu *et al.*, 2005). PCR amplification for *GPx4* using the designated primers yielded 180-bp and 240-bp fragments for the wild type ($GPx4^{+/+}$) and floxed alleles ($GPx4^{fl/fl}$), respectively, while the wild type ($TR1^{+/+}$) and floxed alleles ($TR1^{fl/fl}$) for *TR1* yielded 66-bp and 130-bp, respectively. Fragments amplified through PCR, corresponding to the Δ*GPx4* or Δ*TR1* alleles were 500-bp and 320-bp, respectively. The *cre* transgene was amplified using the *Cre*-forward and *Cre*-reverse primers (Sengupta, *et al.*, 2010), amplifying a 700-bp product.

At the time of autopsy, DNA samples were extracted from various tissues of control and knockout animals and examined for tissue-specific recombination of the conditional allele by genotyping PCR as described above.

Protein isolation and western blotting

Proteins were extracted from cultured keratinocytes, quantified by BCA protein assay, resolved by SDS-PAGE and electro-transferred to PVDF membranes (Sengupta *et al.*, 2010). The membranes were blocked with Tris-buffered saline (TBS) containing 5% non-fat dried milk and 0.02% Tween 20 for 1 h at room temperature and incubated overnight at 4°C with the indicated antibodies. Specific binding was detected using corresponding horseradish-peroxidase-linked secondary antibodies followed by an enhanced chemiluminescence method (Thermo Fisher Scientific Inc., Rockford, IL, USA). Antibodies for detecting selenoproteins through western blots were generated in this laboratory, while antibody against tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used to assess protein loading.

Microarray and quantitative real-time PCR (q-PCR)

Total RNA was isolated from cultured keratinocytes using Trizol (Invitrogen, Carlsbad, CA, USA) and microarray analysis on GeneChip Mouse 430 2.0 Array (Affymetrix, Santa Clara, CA, USA) were performed at the Molecular Technology Microarray Laboratory (Frederick, MD, USA). The data were normalized and statistically analyzed using software tools (Expression Console; Affymetrix) provided by the National Cancer Institute, Center for Cancer Research in collaboration with the National Institutes of Health, Center for Information Technology, Bioinformatics and Molecular Analysis Section. Three independent arrays were analyzed for mRNA samples from cultured keratinocytes of control and knockout mice, respectively and the data submitted to the GEO repository (GEO accession number GSE34215). Genes differentially regulated in knockout cultures were subjected to functional analysis using DAVID (Huang *et al.*, 2009) and Panther (Thomas *et al.*, 2003) analysis tool. Expression of some differentially regulated genes was validated by a two-step q-PCR (Sengupta *et al.*, 2008), with reactions being carried out in triplicate and RNA levels normalized to *Gapdh*. The expression levels were compared to keratinocyte cultures from control mice.

To examine gene expression in skin, 8 mm full-thickness skin punch biopsies from control and knockout mice were ground in Trizol (Invitrogen) and total RNA was extracted according to the manufacturer's protocol. The RNA was further purified on RNeasy columns (Qiagen,

Valencia, CA, USA) with DNase I treatment prior to cDNA synthesis. Some of the primers used in the study and the protocol for q-PCR have been previously described (Cataisson *et al.*, 2012), while rest of the primers were purchased from Qiagen (Quantitect).

Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems Inc., Redwood City, CA, USA), was used for grouping genes associated with similar biological processes and functions, which were most relevant to the experimental datasets, assigning the differentially expressed genes to pathways and networks. Significant genes ($p \leq 0.05$ and fold change ≥ 2.0) from the microarray data were grouped into upregulated and downregulated genes and subjected to IPA core analysis. Based on the molecular mechanisms stored in Ingenuity database, individual networks were generated for upregulated and downregulated genes. A subsequent network was generated for all differentially regulated genes and subjecting them to IPA core analysis.

Measurement of lipid peroxidation

Lipid peroxidation in lysates of cultured keratinocytes from knockout and control mice was performed using the Oxiselect™ HNE-His Adduct ELISA kit according to manufacturer's recommendations (Cell Biolabs Inc., San Diego, CA, USA). The analysis was carried out with three different sample sets in duplicate and data plotted relative to values obtained for control samples.

Statistical analysis

For statistical analysis, a Student's t-test was performed to determine whether there were differences between the experimental and control groups. A p-value of ≤ 0.05 was considered significant.

References

Cataisson C, Salcedo R, Hakim S *et al.* (2012) IL-1R-MyD88 signaling in keratinocyte transformation and carcinogenesis. *J Exp Med.* 209: 1689-1702.

Huang DW, Sherman BT, Lempicki RA (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 37: 1-13.

Jakupoglu C, Przemeck GK, Schneider M *et al.* (2005) Cytoplasmic thioredoxin reductase is essential for embryogenesis but dispensable for cardiac development. *Mol Cell Biol* 25: 1980-1988.

Kumaraswamy E, Carlson BA, Morgan F *et al.* (2003) Selective removal of the selenocysteine tRNA [Ser]Sec gene (Trsp) in mouse mammary epithelium. *Mol Cell Biol* 23: 1477-1488.

Seiler A, Schneider M, Förster H *et al.* (2008) Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. *Cell Metab* 8: 237-248.

Sengupta A, Carlson BA, Weaver JA *et al.* (2008) A functional link between housekeeping selenoproteins and phase II enzymes. *Biochem J* 413: 151-161.

Sengupta A, Lichti UF, Carlson BA *et al.* (2010) Selenoproteins are essential for proper keratinocyte function and skin development. *PLoS One* 5: e12249.

Thomas PD, Kejariwal A, Campbell MJ *et al.* (2003) PANTHER: a browsable database of gene products organized by biological function, using curated protein family and subfamily classification. *Nucleic Acids Res* 31: 334-341.

Supplementary Table S1. The top 25 up- and down-regulated genes in cultured keratinocytes lacking *GPx4*.

Up-regulated genes			**Down-regulated genes**		
Gene symbol	**Fold change**	**p-value**	**Gene symbol**	**Fold change**	**p-value**
Ereg	9.939	0.0503	*Ddx3y*	-38.952	0.0011
Prl2c2	8.408	0.0218	*Eif2s3y*	-14.23	0.0001
Ptgs2	5.962	0.0403	*Jarid1d*	-9.106	0.0173
Gsta4	5.556	0.0019	*Gm106*	-8.246	0.0112
Fosl1	5.525	0.0473	*Krt79*	-6.138	0.0365
Slc2a3	5.227	0.0336	*Chdh*	-6.058	0.0083
Gm566	5.189	0.0139	*Uty*	-4.787	0
Ifi205	4.548	0.0437	*Syt4*	-4.657	0.0021
Actg2	4.475	0.0272	*Ucp2*	-4.645	0.0007
Ch25h	4.347	0.019	*Tgfbi*	-4.55	0.009
Efemp1	4.252	0.0388	*Itga1*	-4.239	0.003
Mal	4.115	0.0082	*Gas1*	-4.108	0.0029
Procr	4.102	0.028	*Sostdc1*	-4.093	0.0058
Prl8a9	3.229	0.0004	*Clca2*	-4.033	0.005
Myl7	3.132	0.0009	*Ssbp2*	-4.016	0.0095
Xist	2.963	0.0023	*Maf*	-3.982	0.0019
Cd28	2.948	0.0069	*Slc16a7*	-3.925	0.0009
Nr2f2	2.874	0.006	*Arid5b*	-3.835	0.0196
Ntf3	2.816	0.011	*Rbp1*	-3.83	0.0191
Fst	2.6	0.0018	*S100g*	-3.795	0.0057
Gpm6a	2.576	0.0051	*Tbx1*	-3.738	0.0023
Nrp1	2.485	0.0001	*Scara3*	-3.68	0.0001
Wnt7a	2.48	0.0059	*Cspg4*	-3.566	0.0001
Ngfb	2.458	0.0007	*Irx4*	-3.353	0.0014
Csf1	2.335	0.0007	*Itgb8*	-3.299	0

Supplementary Table S2. List of primer sets used for q-PCR.

AlcamF	5'-ACTGTGGTGTGGATGAAGGA-3'
AlcamR	5'-TCTACAATGAGGGTCAGCGA-3'
Csf1F	5'-CAGCAGTTGATCGACAGTCA-3'
Csf1R	5'-CTTTAAAGCGCATGGTCTCA-3'
EregF	5'-GCTGCTTTGTCTAGGTTCCC-3'
EregR	5'-AATCTGCACTTGAGCCACAC-3'
Foxq1F	5'-AGCATTCTCAGCAAGCCTTT-3'
Foxq1R	5'-GTAAGCACAGAGCGGTAGCA-3'
Gpx1F	5’-CAGGAGAATGGCAAGAATGA-3’
Gpx1R	5’-GAAGGTAAAGAGCGGGTGAG-3’
GapdhF	5'-ATGTGTCCGTCGTGGATCT-3'
GapdhR	5'-GTTGAAGTCGCAGGAGACAA-3'
Hoxc5F	5’-CGGAGGTTGTCCTATGCTCT-3’
Hoxc5R	5’-GGGTCCAAAGACAAGAGACC-3’
Hoxc8F	5'-CCGGTATCAGACCTTGGAAC-3'
Hoxc8R	5'-TTCCACTTCATCCTTCGATTC-3'
Il18F	5'-GAACAAGATCATTTCCTTTGAGG-3'
Il18R	5'-TTGGCAAGCAAGAAAGTGTC-3'
Nr2f2F	5'-GGCCATAGTCCTGTTCACCT-3'
Nr2f2R	5'-GAAGGGAGACGAAGCAAGAG-3'
Nrp1F	5'-GGTGAGCCCTGTGGTCTATT-3'
Nrp1R	5'-AACCACCATCCAGACCAGTT-3'
Ntf3F	5'-CAATAGAACCTCACCACGGA-3'
Ntf3R	5'-CAGCACTGTGACCTGGTGT-3'
Ptgs2F	5'-AGCCTTCTCCAACCTCTCCT-3'
Ptgs2R	5'-CAGGGATGAACTCTCTCCGT-3'
TR1F	5’-CTACAGACCATTGCCTTGCT-3’
TR1R	5’-ACCTCCTACCCACAAGATCC-3’
Vav3F	5'-CAGGCGACACTGTTGAACTT-3'
Vav3R	5'-CCAGGGTTGGCAAGAATAAT-3'
Wnt7aF	5'-CATAGGAGAAGGCTCCCAAA-3'
Wnt7aR	5'-TAATCGCATAGGTGAAGGCA-3'

Supplementary Figure legends:

Supplementary Figure S1. Genotyping and tissue-specific deletion of *GPx4* in knockout mice. (**a**) Tabular representation of offspring generated from mating K14-*cre*; GPx4$^{fl/+}$ males and GPx4$^{fl/fl}$ females. (**b**) Genotype of progeny resulting from breeding heterozygous males bearing K14-*cre* (K14-*cre*; *GPx4*$^{fl/+}$) with floxed females (*GPx4*$^{fl/fl}$). The *GPx4*$^{fl/fl}$, *GPx4*$^{+/+}$ and the Δ*GPx4* products correspond to 240, 180 and 500 bp, respectively, while the amplified product from *cre* was 700 bp. Phenotype of knockout (**A,** K14-*cre*; *GPx4*$^{fl/fl}$) or control (**B,** K14-*cre*; *GPx4*$^{fl/+}$, **D,** *GPx4*$^{fl/fl}$) mice used in the study. (**c**) Genotyping PCR of various tissues from knockout mice and heterozygous control littermates. The upper panel shows *GPx4*$^{fl/fl}$ (upper band) and *GPx4*$^{+/+}$ (lower band) products, while the middle and lower panels show amplification for Δ*GPx4* and *cre* respectively. (**d**) Control and knockout mice (10 d old) with different hair color due to absence and presence of melanin, from the same litter exhibit similar skin and hair phenotype. (**e**) Average number of hair follicles observed per field in skin sections from control and knockout mice, under identical magnification. CT and KO designate control and knockout mice respectively.

Supplementary Figure S2. Morphology of newborn-skin and adult tongue and whiskers in *GPx4* knockout mice. (**a**) Histological examinations of back skin sections from newborn littermates at 1, 2 and 4 days of age, stained with H&E. Scale bar: 100 μm. (**b**) Sections of tongue from littermate control and knockout mice (19 d) stained with H&E. Scale bar: 100 μm. (**c**) Image and histology of whiskers stained with H&E in control and knockout pups (19 d). Scale bar: 200 μm.

Supplementary Figure S3. Time course of changes in histochemical detection of keratinocyte differentiation markers. Histochemical analysis of keratinocyte differentiation markers in back skin sections of age matched control and knockout mice. Scale bar: 100 μm.

Supplementary Figure S4. Time course of cellular infiltration in skin of control and knockout mice. Back skin sections of age matched control and knockout mice were stained for infiltrating (**a**) macrophages with F4/80 (macrophage/monocyte specific antigen) and (**b**) granulocytes with myeloperoxidase. Scale bar: 100 μm.

Supplementary Figure S5. Functional class for genes differentially regulated by ablation of *GPx4* in keratinocytes and validation of some regulated genes through q-PCR. (**a**) Pie-chart representation of major functional classes influenced by the loss of GPx4 in keratinocytes. The number of genes in each functional class is marked in the chart. (**b**) q-PCR analysis of some up-

and down-regulated genes upon ablation of *GPx4*, from cultured keratinocytes of control (CT) and knockout mice (KO).

Supplementary Figure S6. Network of genes regulated by the loss of *GPx4* expression in mouse keratinocytes. IPA Knowledge Base was used to deduce network of genes differentially regulated by the ablation of *GPx4*. Regulated genes form nodes of the pathway and lines and arrows represent biological relationship between two nodes. Nodes are displayed using various shapes that represent the functional classes of the gene product and are color coded for up-regulation (red) and down-regulation (green), with the color intensity signifying the degree of regulation. Solid lines depict genes directly related in a pathway, while dotted lines show genes with indirect relations.

Supplementary Figure S7. COX-2 expression in skin sections of mice lacking selenoproteins in keratinocytes. Immunodetection of COX-2 in back skin sections from 10 d old control and knockout mice with complete lack of selenoproteins in keratinocytes reveals prominent COX-2 staining in ORS of hair follicles (arrows) and at the epidermal-dermal junction (arrowhead) in knockout skin sections. Scale bar: 100 μm.

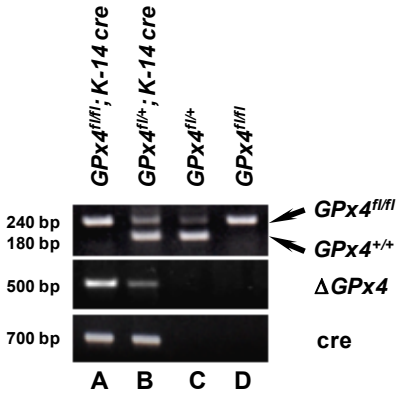
Supplementary Figure S8. GPx4 deficiency alters COX-2 expression in cultured keratinocytes through lipid peroxidation. Schematic diagram showing putative pathway for elevation of COX-2 in keratinocytes upon ablation of GPx4. GPx4 prevents lipid peroxidation and reduces hydroperoxides (ROOH) in cells, with membrane-bound ROOH being reduced solely by GPx4. Hydroperoxides activate COX-2 and GPx4 inhibits this process. In keratinocytes lacking GPx4, lipid peroxidation and ROOH levels are elevated, leading to an increase in COX-2 expression, which could contribute to modified hair follicle morphogenesis.

Supplementary Figure S1.

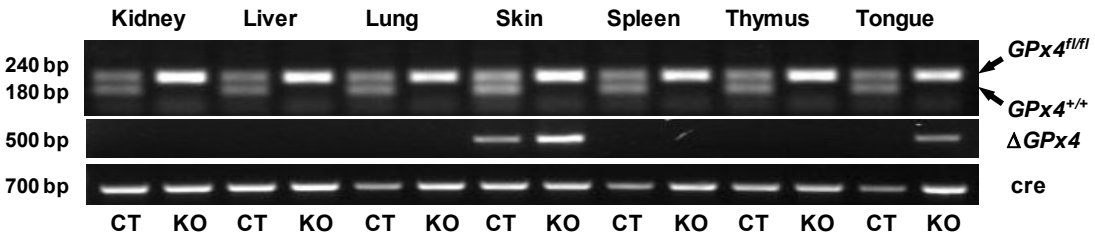
a

Genotype	Number of pups (%)	*ΔGPx4*	Phenotype
GPx4$^{fl/+}$ --	91 (20.63)	--	Normal
GPx4$^{fl/+}$ *K14-Cre*	170 (38.55)	+	Normal
GPx4$^{fl/fl}$ --	72 (16.33)	--	Normal
GPx4$^{fl/fl}$ *K14-Cre*	108 (24.49)	+	Skin abnormality, delayed hair coat development
Total	441 (100)		

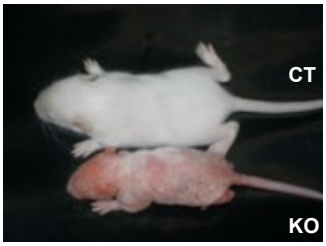
b

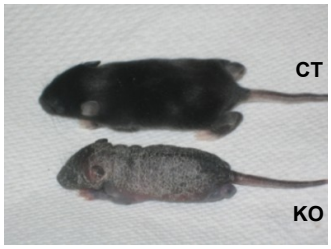


c

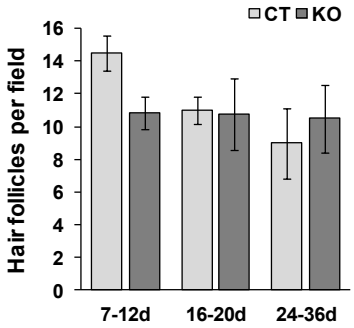


d

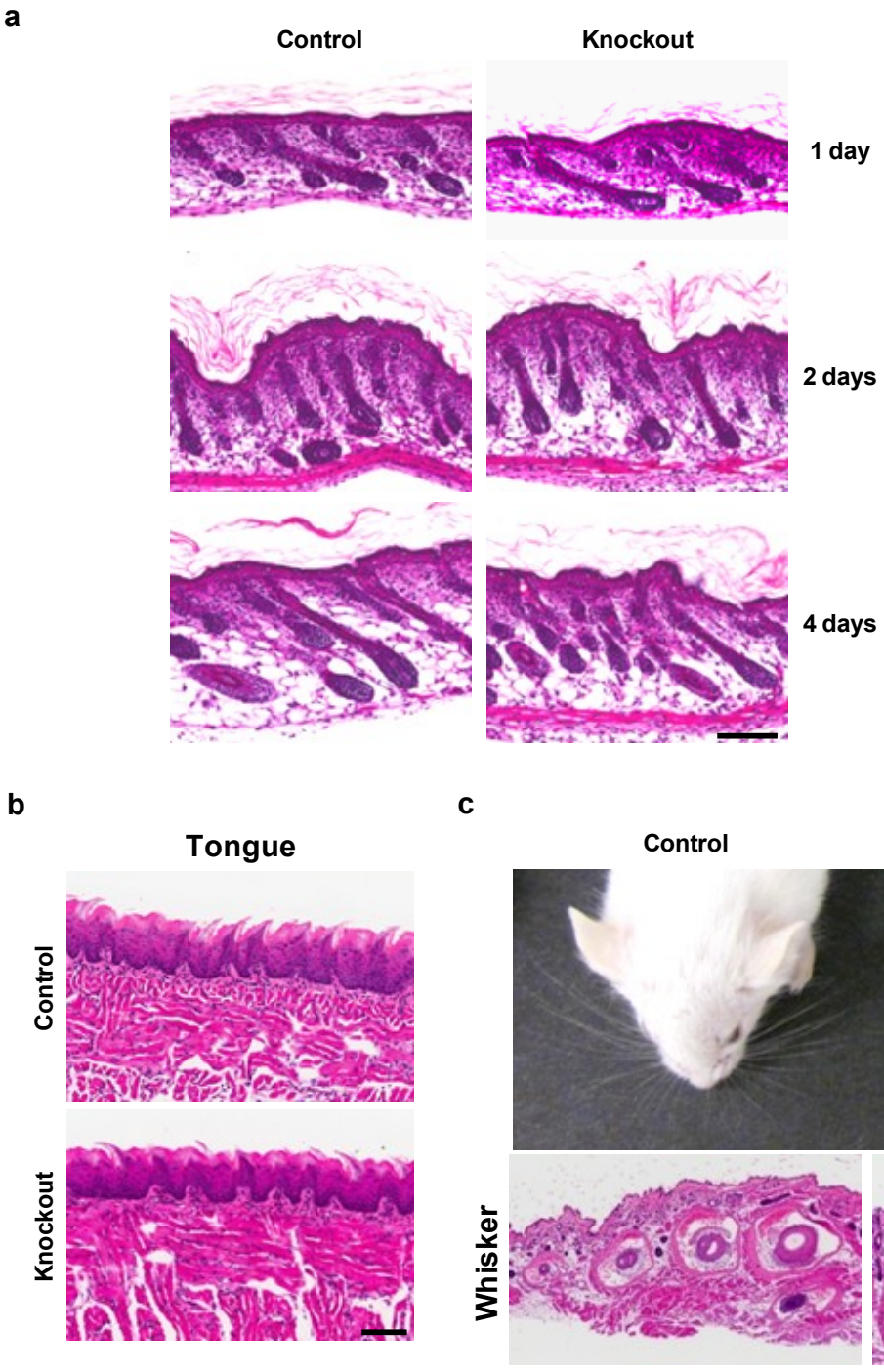




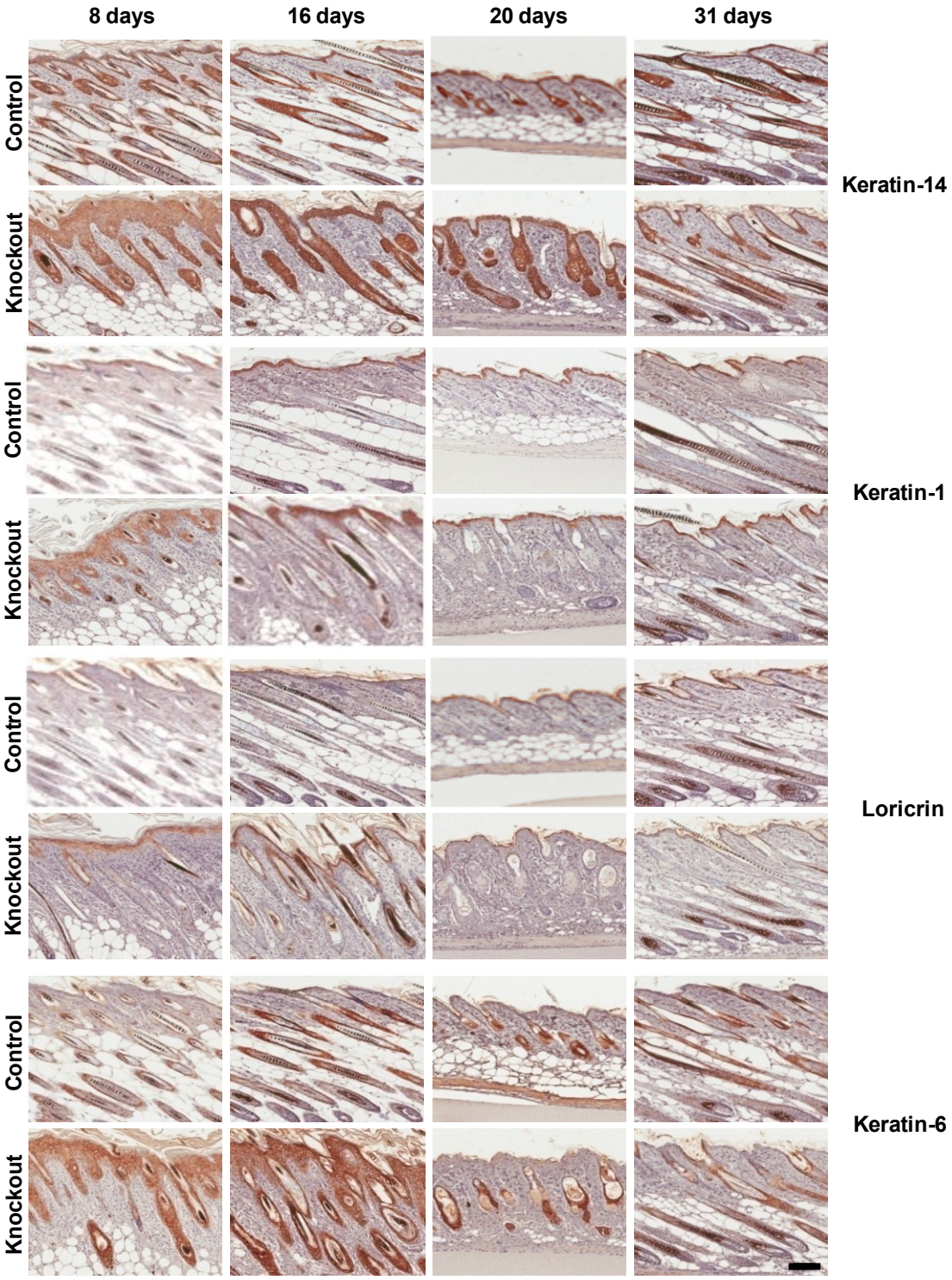
e



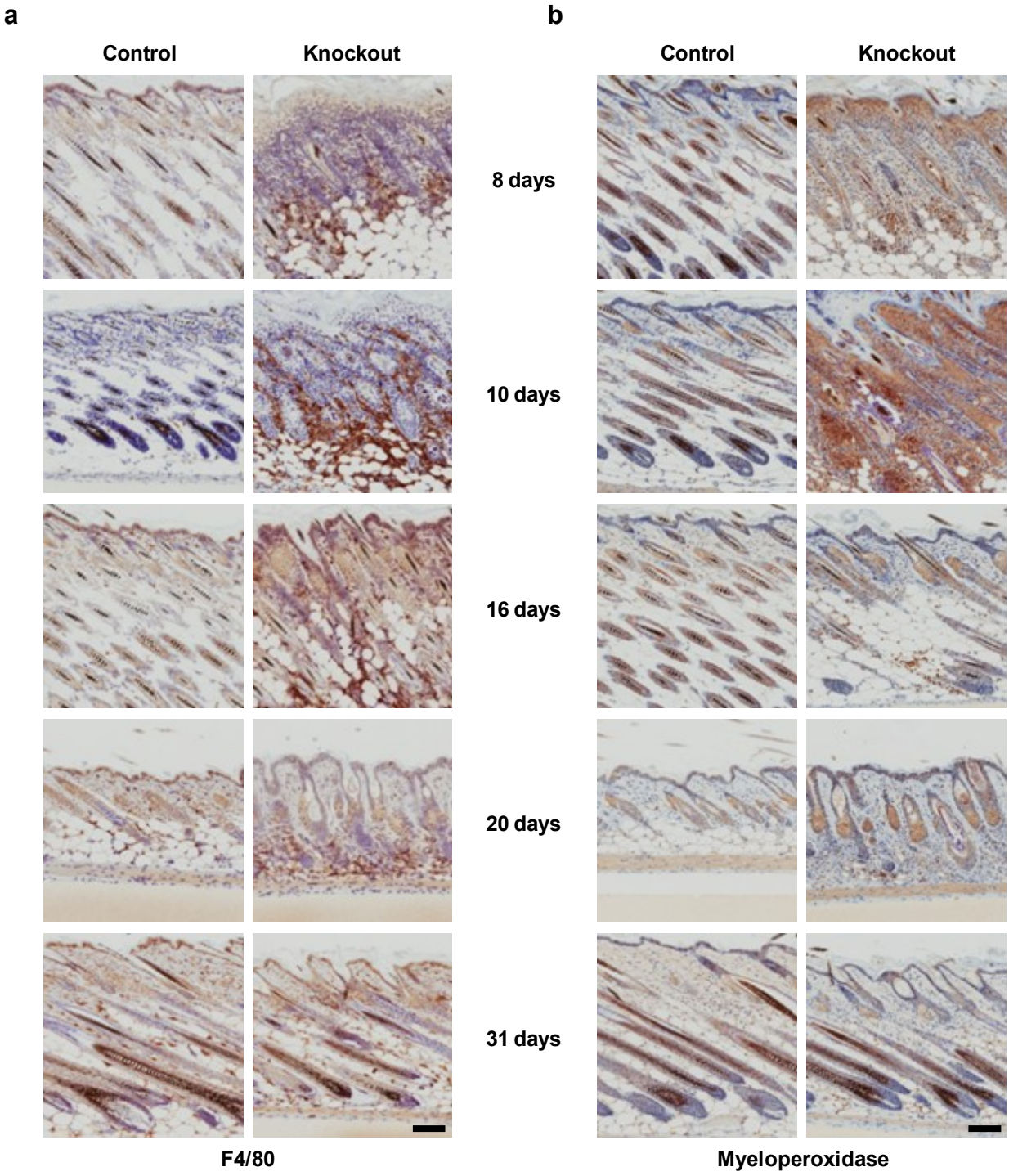
Supplementary Figure S2.



Supplementary Figure S3.

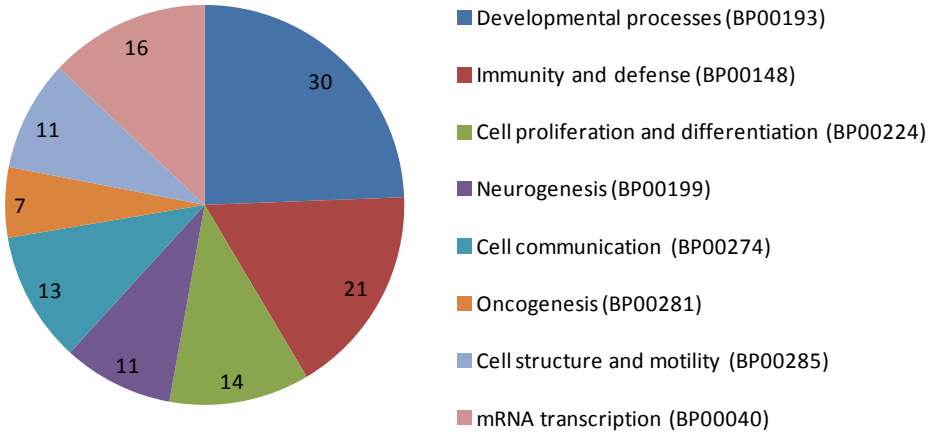


Supplementary Figure S4.

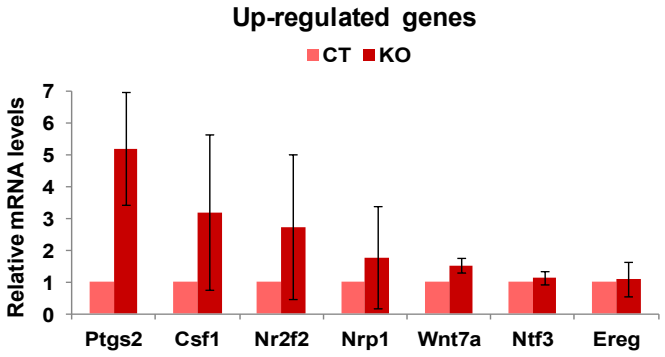


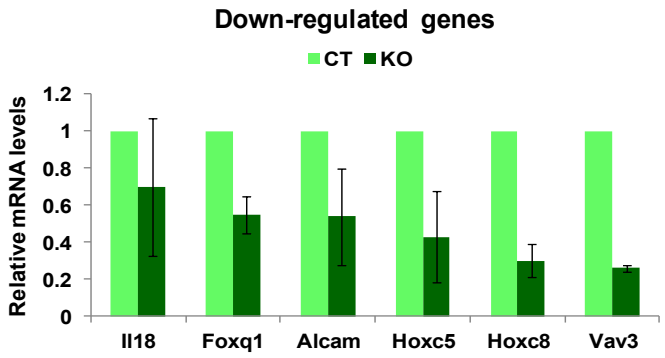
Supplementary Figure S5.

a

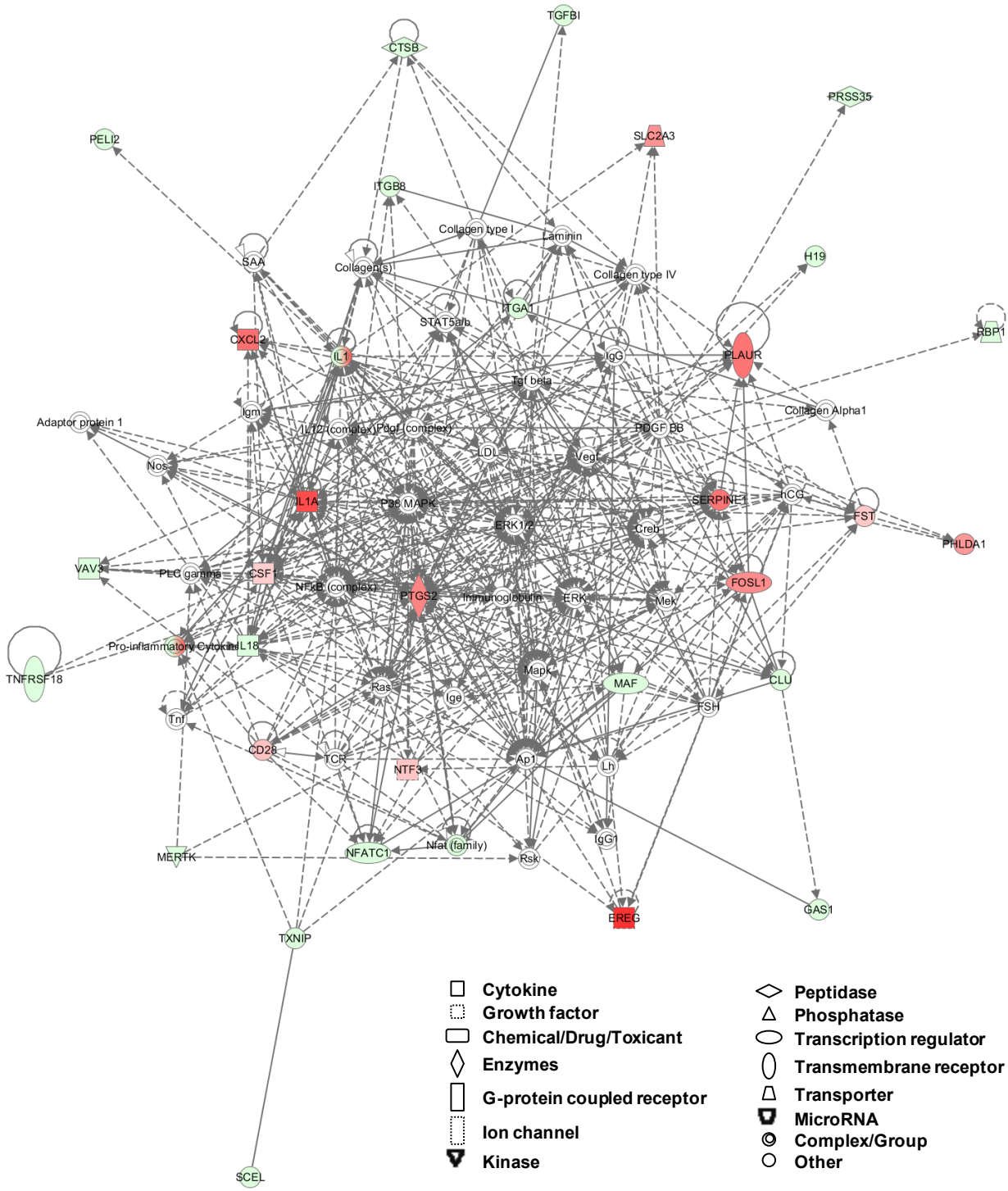


b

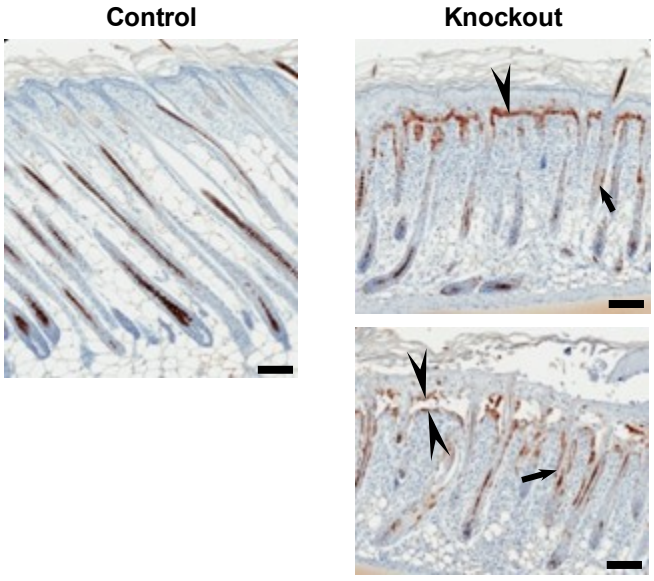




Supplementary Figure S6.



Supplementary Figure S7.



Supplementary Figure S8.