Additional file 1

Figure S1 CISD2 is mainly expressed in the proliferating keratinocytes of the epidermis in normal human skin from an older person. Related to Figure 1.

Figure S2 Toxicity testing of different dosages of hesperetin against HEK001 human keratinocytes from an older person. Related to Figure 2.

Figure S3 Oral administration of hesperetin is able to ameliorate UVB-induced skin photoaging in WT mice. Related to Figure 3.

Figure S4 Hesperetin enhances Cisd2 expression in the skin of WT mice. Related to Figure 4.

Figure S5 Hesperetin modulated gene expression profiles using HEK001 human keratinocytes from an older person, These are related to proteostasis, cellular senescence, stress response and redox homeostasis. Related to Figure 5.

Figure S6 Hesperetin modulates the activity of FOXM1 and IL-1α, as well as the expression of FOXO3a downstream target genes in HEK001 keratinocytes. Related to Figure 6.

Table S1 Hesperetin-modulated changes in the upstream regulators and their downstream target genes in HEK001 keratinocytes

Figure S1

A

Face 57	Face 57	Face 47	Face 47	Face 48	Face 48	Face 52	Face 52	Neck 53	Neck 53	Chest 63	Chest 63
Chest 62	Chest 62	Chest 47	Chest 47	Chest 37	Chest 37	Chest 49	Chest 49	Chest 53	Chest 53	Chest 73	Chest 73
Chest 73	Chest 73	Chest 71	Chest 71	Chest 50	Chest 50	Chest 71	Chest 71	Chest 44	Chest 44	Chest 51	Chest 51
Chest 50	Chest 50	Chest 45	Chest 45	Chest 54	Chest 54	Chest 40	Chest 40	Chest 35	Chest 35	Chest 50	Chest 50
Chest 56	Chest 56	Chest 61	Chest 61	Chest 43	Chest 43	Chest 53	Chest 53	Chest 59	Chest 59	Chest 40	Chest 40
Chest 64	Chest 64	Chest 42	Chest 42	Chest 56	Chest 56	Chest 67	Chest 67	Back 50	Back 50	Back 29	Back 29
Back 78	Back 78	Groin 19	Groin 19	Groin 66	Groin 66	Anus 47	Anus 47	Anus 42	Anus 42	Anus 58	Anus 58
Anus 49	Anus 49	Anus 33	Anus 33	Anus 51	Anus 51	Peri 71	Peri 71	Hip 43	Hip 43	Thign 52	Thign 52
Thign 49	Thign 49	Thign 50	Thign 50		♂	♀	Back: Humeral back Peri: Perineum			SKN1001	

B

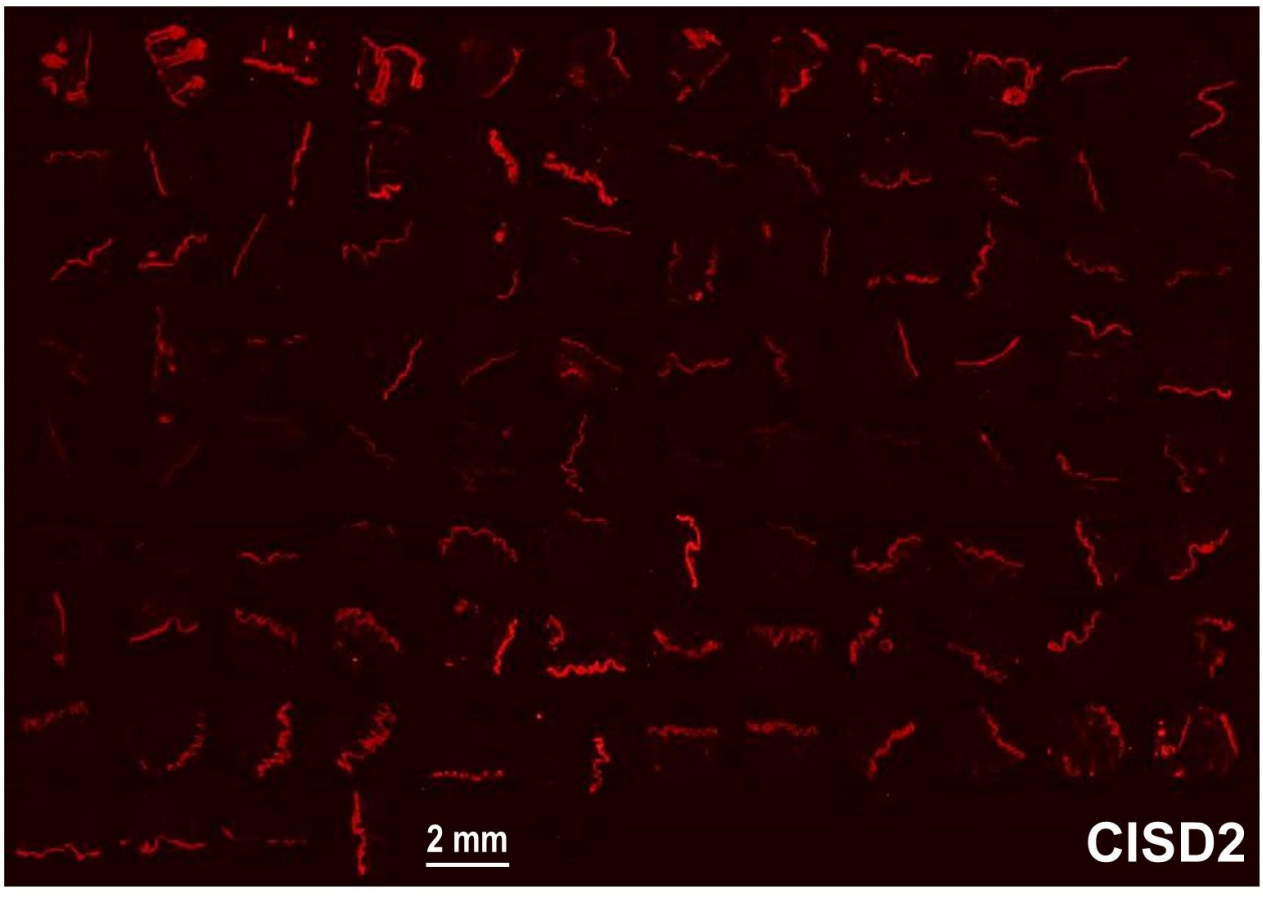


Figure S1 continued

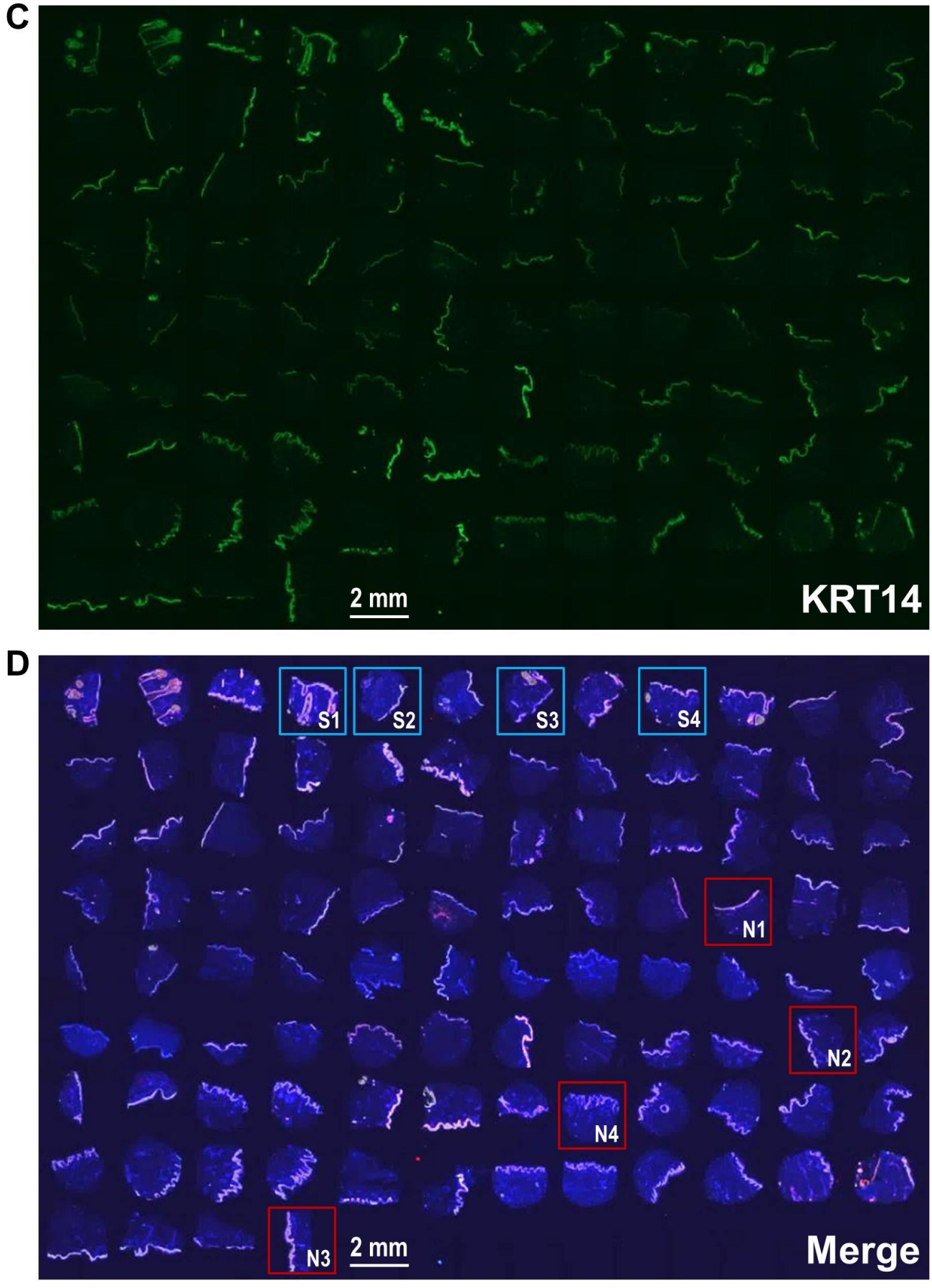


Figure S1 continued

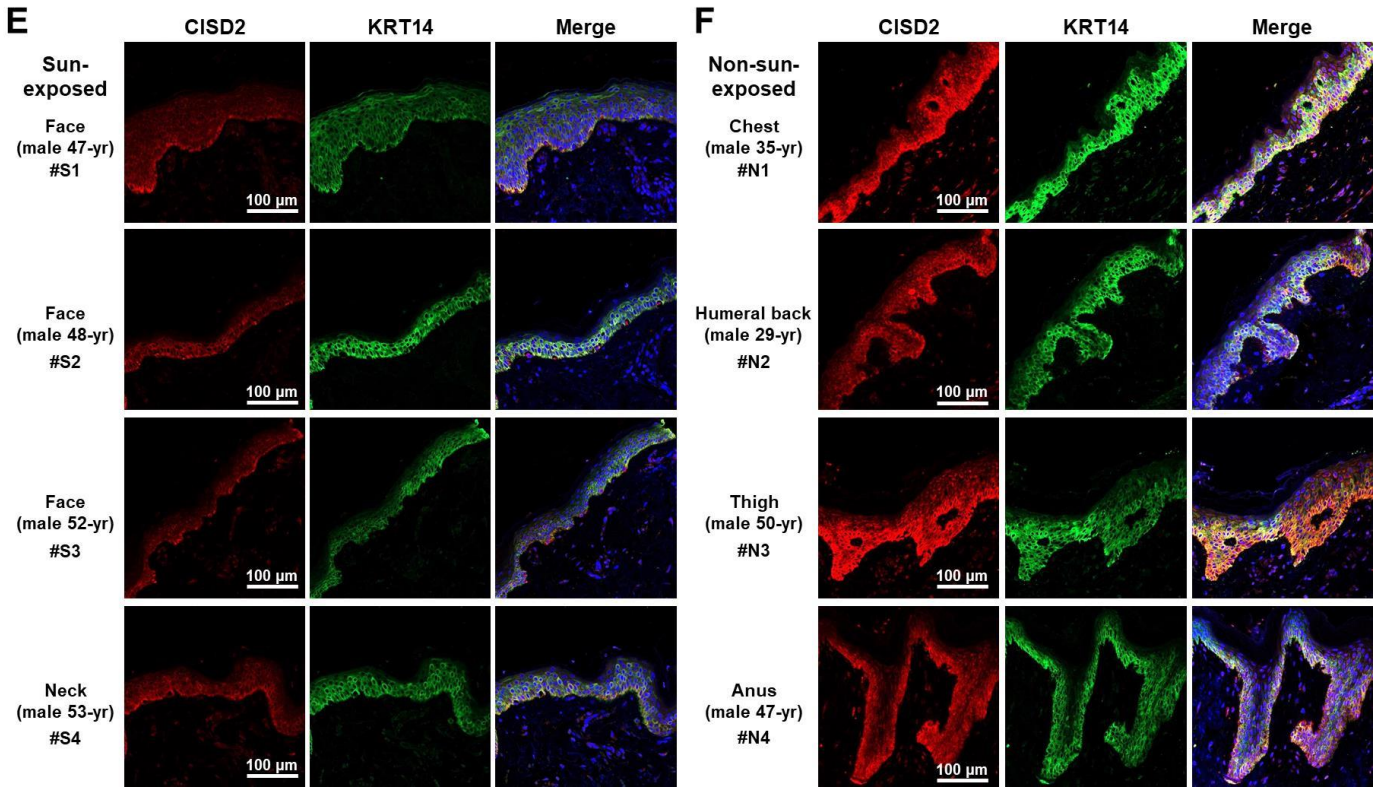


Figure S1. CISD2 is mainly expressed in the proliferating keratinocytes of the epidermis in normal human skin from an older person. Related to Figure 1.

(A) The age, sex and collection sites of the skin samples of the human tissues array SKN1001. **(B and C)** Fluorescent immunohistochemistry (IHC) staining of CISD2 (B) and KRT14 (a marker of proliferating keratinocytes in the epidermis) (C) in the normal human skin of the SKN1001 tissue array. **(D)** The merged IHC image of CISD2, KRT14 and DAPI in the SKN1001 tissue array. Blue boxes indicate the representative samples in panel E (#S1 to #S4). Red boxes indicate the representative samples in panel F (#N1 to #N4). Scale bars in (B-D), 2 mm. **(E)** Representative images of IHC staining of CISD2 and KRT14 from the sun-exposed sites (Face and Neck) of human skin. **(F)** Representative images of IHC staining of CISD2 and KRT14 from the non-sun-exposed sites, including chest, humeral back, thigh and anus of human skin. Scale bars in (E) and (F), 100 μm.

Figure S2

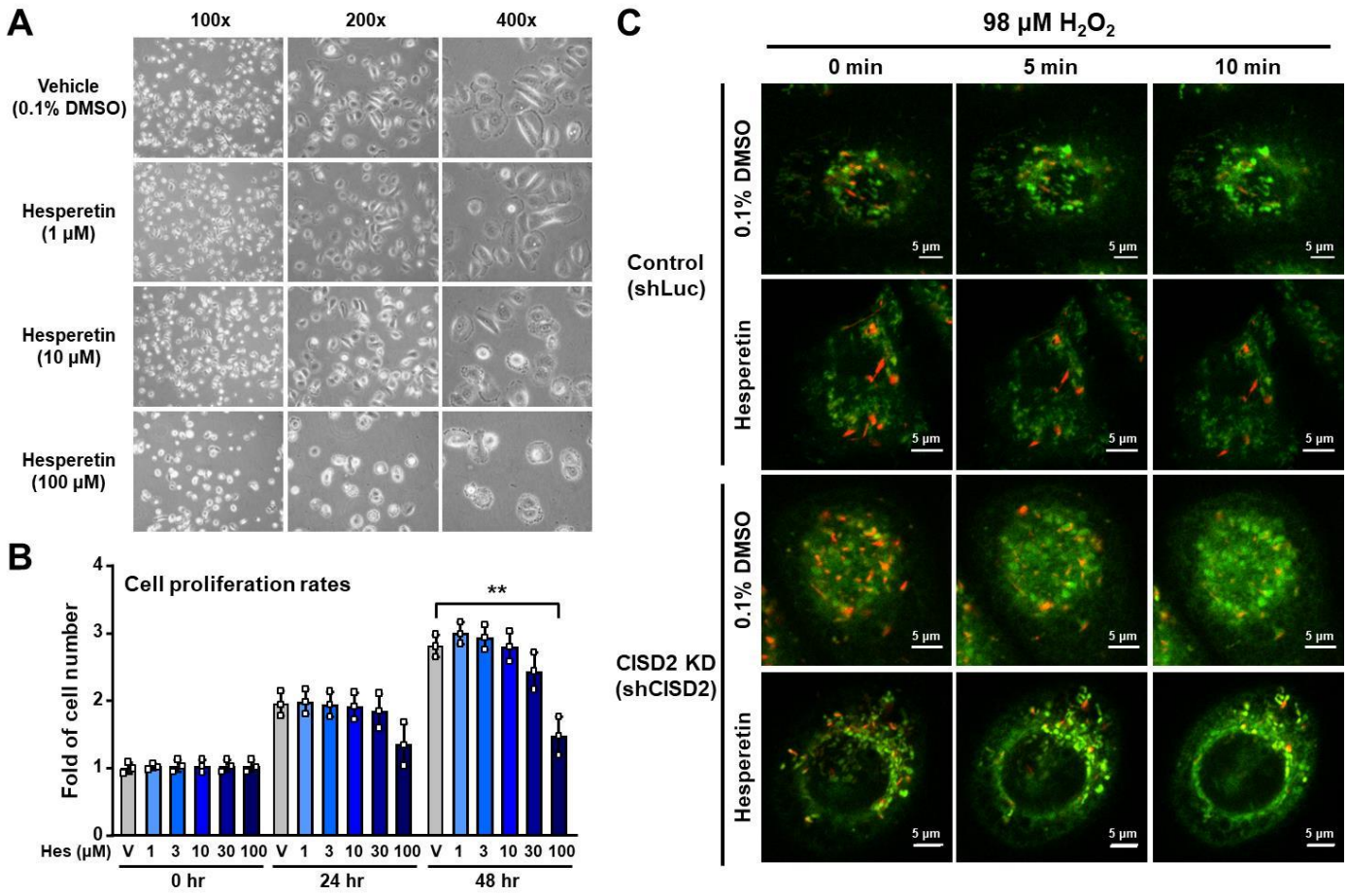


Figure S2. Toxicity testing of different dosages of hesperetin against HEK001 human keratinocytes from an older person. Related to Figure 2.

(A) Toxicity testing of different dosages of hesperetin against HEK001 keratinocytes. Cell morphology of the HEK001 keratinocytes after treatment with hesperetin at different dosages (1 µM, 10 µM and 100 µM) for 4 days. The cytotoxic effects, including a reduced cell density and a smaller cell size are present in the HEK001 keratinocytes at a high concentration (100 µM) of hesperetin treatment. **(B)** Analysis of cell proliferation rates after treatment of HEK001 keratinocytes with different concentrations of hesperetin (1-100 µM). The HEK001 keratinocytes were treated with different doses of hesperetin as indicated. Vehicle (V), 0.1% DMSO. **(C)** Representative images of JC-1 staining of the different groups of HEK001 keratinocytes. Hesperetin (10 µM) protects against oxidative stress-induced mitochondrial dysfunction. All experiments were performed and repeated three independent times as biological replicates using HEK001 keratinocytes. Data are presented as mean ± SD. The statistical analysis was performed by one-way ANOVA with Bonferroni multiple comparison test.

Figure S3

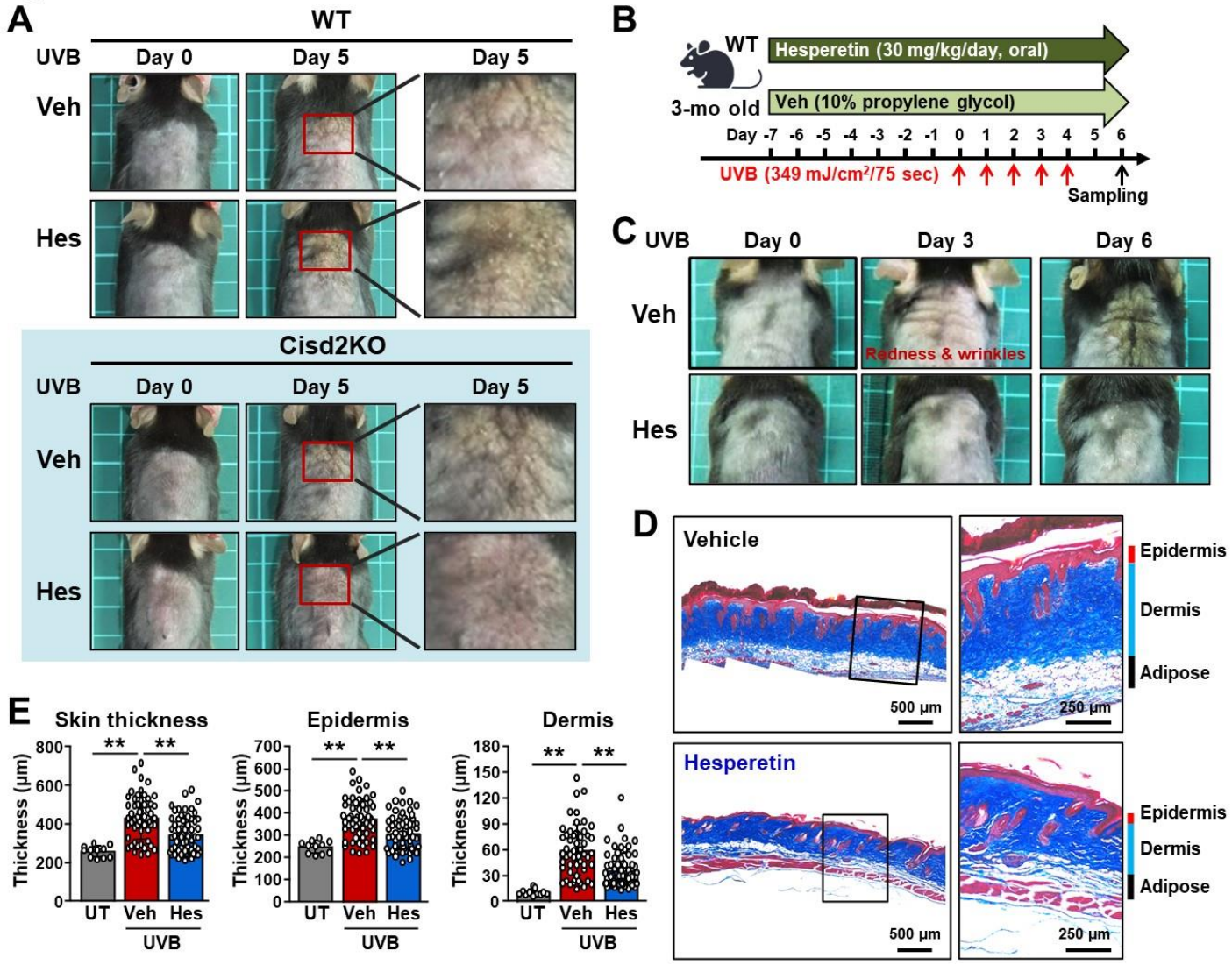


Figure S3. Oral administration of hesperetin is able to ameliorate UVB-induced skin photoaging in WT mice. Related to Figure 3.

(A) A gross view of the dorsal skin of Vehicle or Hesperetin treated WT and Cisd2KO mice before and after UVB exposure. **(B)** The protocol for oral treatment with hesperetin and its effect on UVB-induced skin damage in WT mice at 3-month old. The mice were pre-treated with hesperetin (30 mg/kg/day, oral administration) for 7 days, and then exposed to UVB (312 nm, 349 mJ/cm^2/75 seconds) light once a day for 5 days in a UVB box. The mice were sacrificed 6 days after the first UVB exposure. **(C)** A gross view of the dorsal skin of Vehicle or Hesperetin treated WT mice before and after UVB exposure. **(D)** Masson's trichrome staining of skin sections from the different groups of mice. UVB exposure significantly induces skin damage and increases skin thickness, which are the major characteristics of photoaging, while hesperetin treatment ameliorates UVB-induced skin damage. **(E)** Quantitation of the thickness of total skin layer, epidermal layer and dermal layer, in the skin of WT mice. Data are presented as mean ± SD. *$p < 0.05$; **$p < 0.005$ by one-way ANOVA with Bonferroni multiple comparison test; not significant (n.s.). UT, untreated.

Figure S4

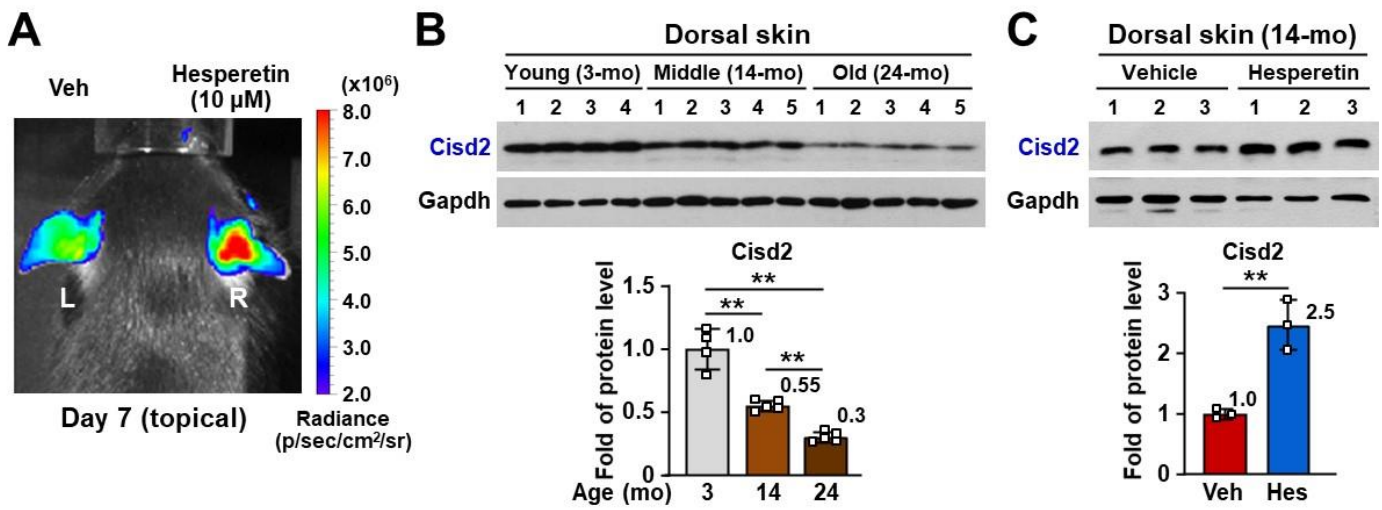
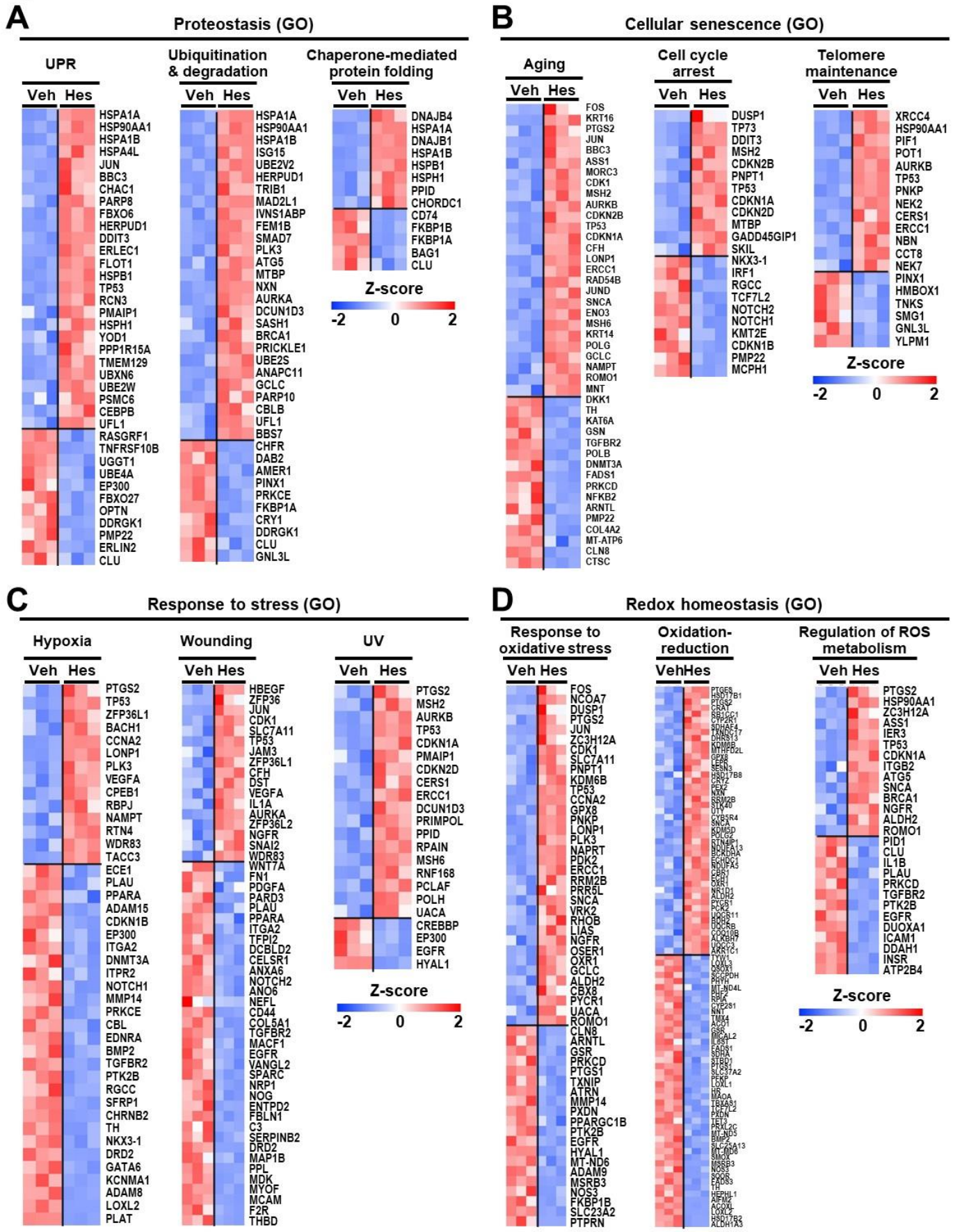


Figure S4. Hesperetin enhances Cisd2 expression in the skin of WT mice. Related to Figure 4.

(A) In vivo imaging system (IVIS) analysis of luciferase activity was examined in transgenic mice carrying the CISD2 BAC Luc reporter. Hesperetin stimulated luciferase reporter activity in the right ear of the CISD2 BAC reporter mice. Hesperetin (10 μM) and vehicle (10% glycerol with 5% menthol) were topically applied twice a day for 7 days to the right (R) and left (L) ears of mice at 3-month old, respectively. Luciferase activity was monitored for 7 days after hesperetin or Vehicle treatment. **(B)** Western blot analysis of Cisd2 protein levels revealed an age-dependent decline in Cisd2 protein expression in the skin from the young (3-month), mid-age (14-month) and old (24-month) wild-type C57BL/6 male mice (n = 4-5). **(C)** Hesperetin (10 mg/kg/day i.p. for 30 days) enhances by 2.5-fold the Cisd2 protein levels in the skin of middle-aged mice at 14-month old (n=3). The Cisd2 protein levels were determined by Western blot analysis and normalized using Gapdh. Data are presented as mean ± SD. *$p < 0.05$; **$p < 0.005$. In (B) the statistical analysis was performed by one-way ANOVA with Bonferroni multiple comparison test. In (C) the statistical analysis was performed by Student's t test.

Figure S5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FBXO6
HERPUD1
DDIT3
ERLEC1
FLOT1
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Chaperone-mediated protein folding
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CLU
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CTSC
Cell cycle arrest
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CDKN2D
CERS1
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RNF168
PCLAF
POLH
UACA
CREBBP
EP300
EGFR
HYAL1
Z-score
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D
Redox homeostasis (GO)
Response to oxidative stress
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FOS
NCOA7
DUSP1
PTGS2
JUN
ZC3H12A
CDK1
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PRR5L
SNCA
VRK2
RHOB
LIAS
NGFR
OSER1
OXR1
GCLC
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SLC23A2
PTPRN
Oxidation-reduction
VehHes
Regulation of ROS metabolism
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ZC3H12A
ASS1
IER3
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ITGB2
ATG5
SNCA
BRCA1
NGFR
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PID1
CLU
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Figure S5 continued

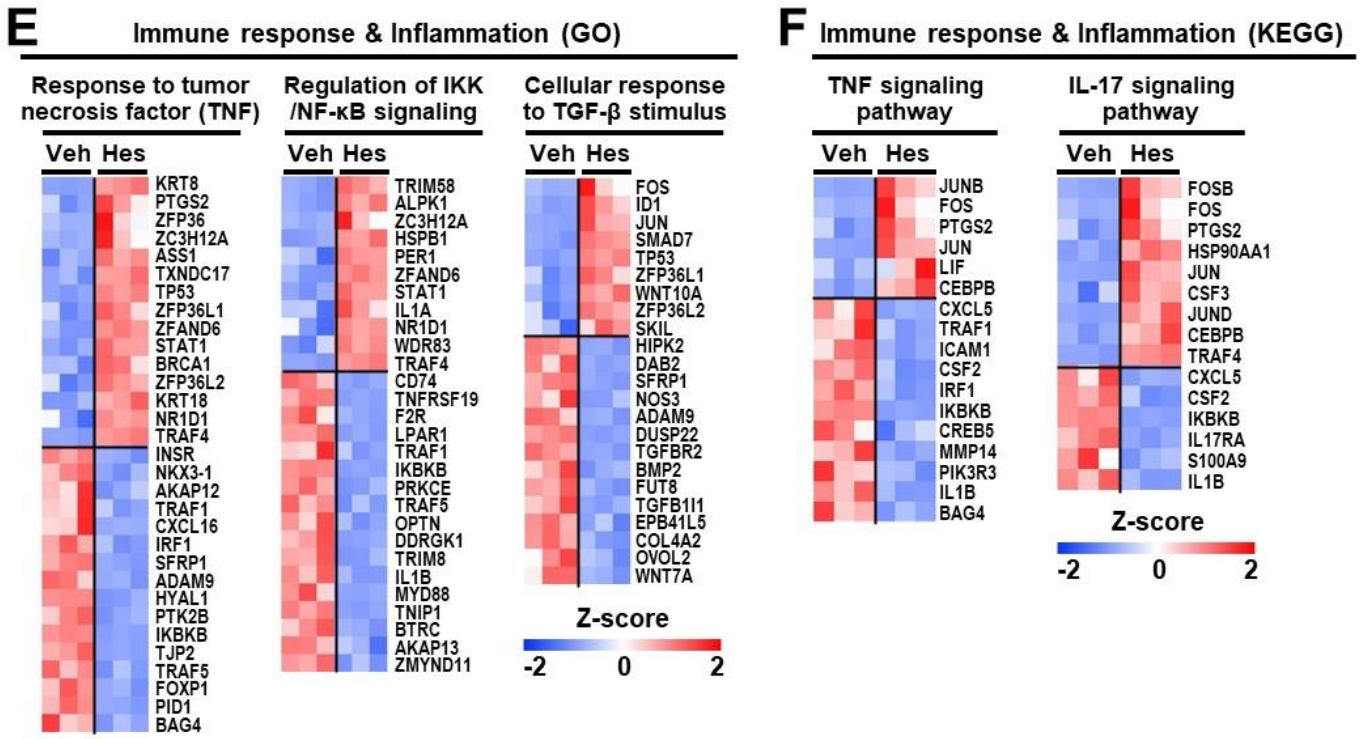

Figure S5. Hesperetin modulated gene expression profiles using HEK001 human keratinocytes from an older person, these are related to proteostasis, cellular senescence, stress response and redox homeostasis. Related to Figure 5.

(A) The heatmap pinpoints the proteostasis-related DEGs, including unfolded protein response (UPR), ubiquitination and proteasomal protein degradation, and chaperone-mediated protein folding, in HEK001 keratinocytes. **(B)** The heatmap pinpoints the cellular senescence-related DEGs, including aging, cell cycle arrest, and telomere maintenance in HEK001 keratinocytes. **(C)** The heatmap pinpoints the stress response-related DEGs, including response to UV, hypoxia, and wounding, in HEK001 keratinocytes. **(D)** The heatmap pinpoints the redox homeostasis-related DEGs, including oxidative stress response, oxidation-reduction, and regulation of reactive oxygen species (ROS) metabolism, in HEK001 keratinocytes. **(E and F)** The heatmap pinpoints the immune response and inflammation-related DEGs in HEK001 keratinocytes, including TNF signaling, IKK/NF-κB signaling, cellular response to TGF-β stimulus and IL-17 signaling.

Figure S6

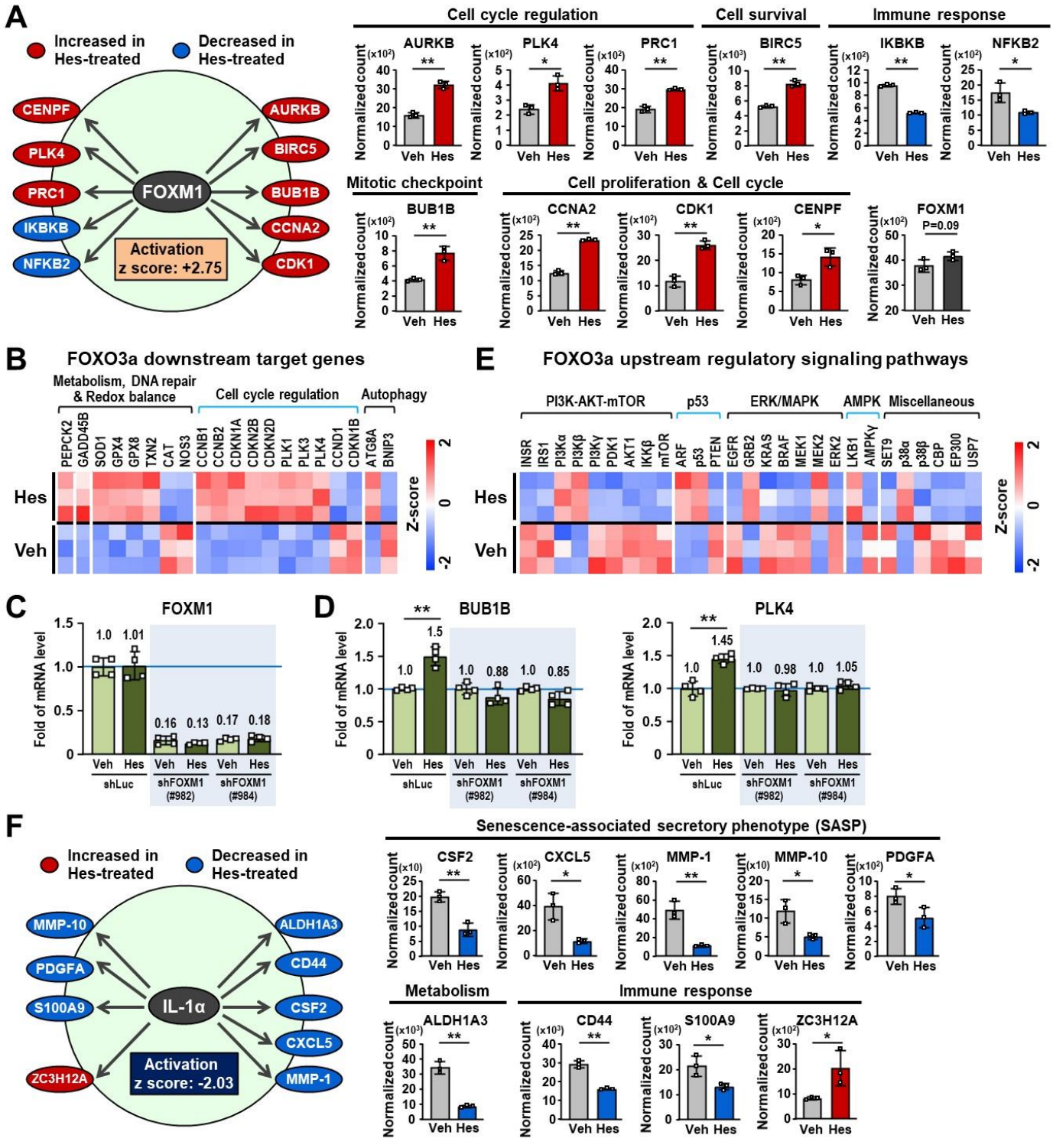

Figure S6. Hesperetin modulates the activity of FOXM1 and IL-1α, as well as the expression of FOXO3a downstream target genes in HEK001 keratinocytes. Related to Figure 6.

(A) Significant activation of FOXM1 transcriptional signaling is based on the activation z-score (z-score > +2.0 and p-value of overlap < 0.01) from IPA upstream regulator analysis of the Hesperetin modulated DEGs of HEK001 keratinocytes. The mRNA expression levels of the Hesperetin modulated DEGs are associated with

FOXM1-related transcriptional changes in HEK001 keratinocytes. The DEGs can be classified according to the different functions of the downstream target genes of FOXM1. These include cell cycle regulation, cell death prevention, mitotic checkpoint control, cell proliferation and immune response. **(B)** The heatmap shows that hesperetin modulates the expression of FOXO3a downstream target genes in HEK001 keratinocytes. **(C)** Real-time RT-qPCR analysis of FOXM1 mRNA levels revealed that >70% of the FOXM1 mRNA was knockdown (KD) by two independent FOXM1 shRNA clones (clone ID: TRCN0000273982 [#982] and TRCN0000273984 [#984]) treatments of HEK001 keratinocytes. The sequence of FOXM1 shRNA (Clone ID: TRCN0000273982; 5'-GCCAATCGTTCTCTGACAGAA-3'; Clone ID: TRCN0000273984; 5'-TTGCAGGGTGGTCCGTGTAAA-3'). The sequence of FOXM1 qPCR primers (FOXM1-F: 5'-TGCAGCTAGGGATGTGAATCTTC-3' and FOXM1-R: 5'-GGAGCCCAGTCCATCAGAACT-3'). **(D)** Real-time RT-qPCR analysis of FOXM1 downstream target genes (BUB1B and PLK4) mRNA levels in the Veh- or hesperetin-treated shLuc control and FOXM1 KD HEK001 keratinocytes. The sequence of qPCR primers (BUB1B-F: 5'-GAAGCTGAGCCCAATTATTG-3' and BUB1B-R: 5'-GAGTAGGGTTTTCTGAAGTC-3'; PLK4-F: 5'-GACACCTCAGACTGAAACCGTAC-3' and PLK4-R: 5'-GTCCTTCTGCAAATCTGGATGGC-3'). The mRNA levels by qPCR analysis were normalized against HPRT1. **(E)** The heatmap shows that hesperetin modulates the expression pattern of genes in various signaling pathways, associated with regulation of FOXO3a transcriptional activity in HEK001 keratinocytes. (D) Significant inhibition of IL1α cytokine signaling (z-score < -2.0 and p-value of overlap < 0.01) occurs in the Hesperetin modulated DEGs of HEK001 keratinocytes. The DEGs can be classified according to the different functions of the downstream target genes of IL1α. This includes senescence-associated secretory phenotype (SASP), metabolism and immune response. The DEGs were analyzed by IPA upstream analysis. The criteria for the gene list in the heatmaps are absolute fold change > 1.1 and $p < 0.05$ (Hes vs Veh). The data are presented as mean ± SD. *$p < 0.05$; **$p < 0.005$; not significant (n.s.). In (A) and (F) the statistical analysis was performed by Student's t test. In (C) and (D) the statistical analysis was performed by one-way ANOVA with Bonferroni multiple comparison test.

Table S1. Hesperetin-modulated changes in the upstream regulators and their downstream target genes in HEK001 keratinocytes.

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value	Target molecules in dataset
CKAP2L	Other	Activated	4.000	3.51E-07	AURKB,BIRC5,CCNB2,CDK1,CENPF,ERCC6L,KIF23,KIF2C,MAD2L1,NCAPG,NDC80,NEK2,NUF2,PLK4,SPC24,SPC25
AREG	Growth factor	Activated	2.892	1.21E-04	AURKB,BIRC5,C3,CCNA2,CCNB2,CENPF,IFI6,KIF14,MMP15,PLAU,PRC1,PTAFR,SLC36A1,TOP2A
FOXM1	Transcription regulator	Activated	2.754	3.79E-05	AURKB,BIRC5,BUB1B,CCNA2,CDK1,CENPF,IKBKB,NFKB2,PLK4,PRC1
Interferon-α	Group	Activated	2.433	1.95E-03	IFIH1,IFIT1,ISG15,MX1,OAS1,STAT1
FOXO4	Transcription regulator	Activated	2.429	4.66E-04	CCN2,CDKN1A,CDKN2B,GADD45B,IER3,OVOL1
SMAD4	Transcription regulator	Activated	2.343	5.14E-03	CCN2,CDKN1A,CDKN2B,DLX3,EPB41L5,GADD45B,IER3,OVOL1,SNAI2,VEGFA
EPHA2	Kinase	Activated	2.335	4.64E-07	CD274,DUSP1,FOS,FOSB,IER3,IL1B,JUN,JUNB,NR4A1,ZFP36
FOXO3	Transcription regulator	Activated	2.239	5.66E-04	CCN2,CDH1,CDKN1A,CDKN2B,GADD45B,IER3,NOS3,OVOL1
NEUROG1	Transcription regulator	Activated	2.138	5.48E-05	AMIGO2,ASS1,C3,CD82,CEMIP,CFH,FN1,GFPT2,LRIG1,NOG,PXDN,SLC43A3,SPOCK1,SQOR
Notch	Group	Activated	2.013	4.24E-03	BIRC5,CDKN1A,CDKN2B,DUSP1,GADD45B,ID1,NFATC1,TAGLN
STAG2	Other	Inhibited	-2.688	2.63E-07	CD274,DDX60,DHX58,IFI44L,IFIH1,IRF7,IRF9,ISG15,ISG20,LGALS3BP,OAS1,SAMD9,SP110,UBE2L6
KDM5B	Transcription regulator	Inhibited	-2.360	1.38E-10	AURKA,BRCA1,BUB1B,CDCA3,CDK1,DDIT3,DLGAP5,EPB41L1,FABP5,HMMR,HSD17B8,ISG15,IVNS1ABP,KIF2C,LGALS3BP,MCAM,MT1E,NDC80,NEDD9,NMB,OSER1,POLB,PSD3,SAT1,SCNN1A,SMOX,TOP2A,TTK
IgG	Complex	Inhibited	-2.255	1.71E-09	CDKN1A,CEBPB,CRABP2,CTSC,DUSP1,FABP5,HSPB1,IER2,IFITM3,IL1B,IL1RN,ISG15,JUND,KRT10,KRT15,KRT16,KRT18,LDLR,LGALS7/LGALS7B,PIM1,PPL,PPP1R15A,RND3,SLC2A3,TRIM16,ZFP36
ATF3	Transcription regulator	Inhibited	-2.236	4.14E-04	AURKA,AURKB,CDK1,GSN,NEK2
LONP1	Peptidase	Inhibited	-2.236	6.56E-03	ASNS,CHAC1,MT-ATP6,MT-ND5,MT-ND6
IL1α	Cytokine	Inhibited	-2.028	5.90E-03	ALDH1A3,CD44,CSF2,CXCL5,IL1A,MMP-1,MMP-10,PDGFA,S100A9,ZC3H12A