Developmental Cell, Volume *26*

Supplemental Information

Insulin/IGF-1 Controls Epidermal Morphogenesis via Regulation of FoxO-Mediated p63 Inhibition

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Inventory of supplementary data:

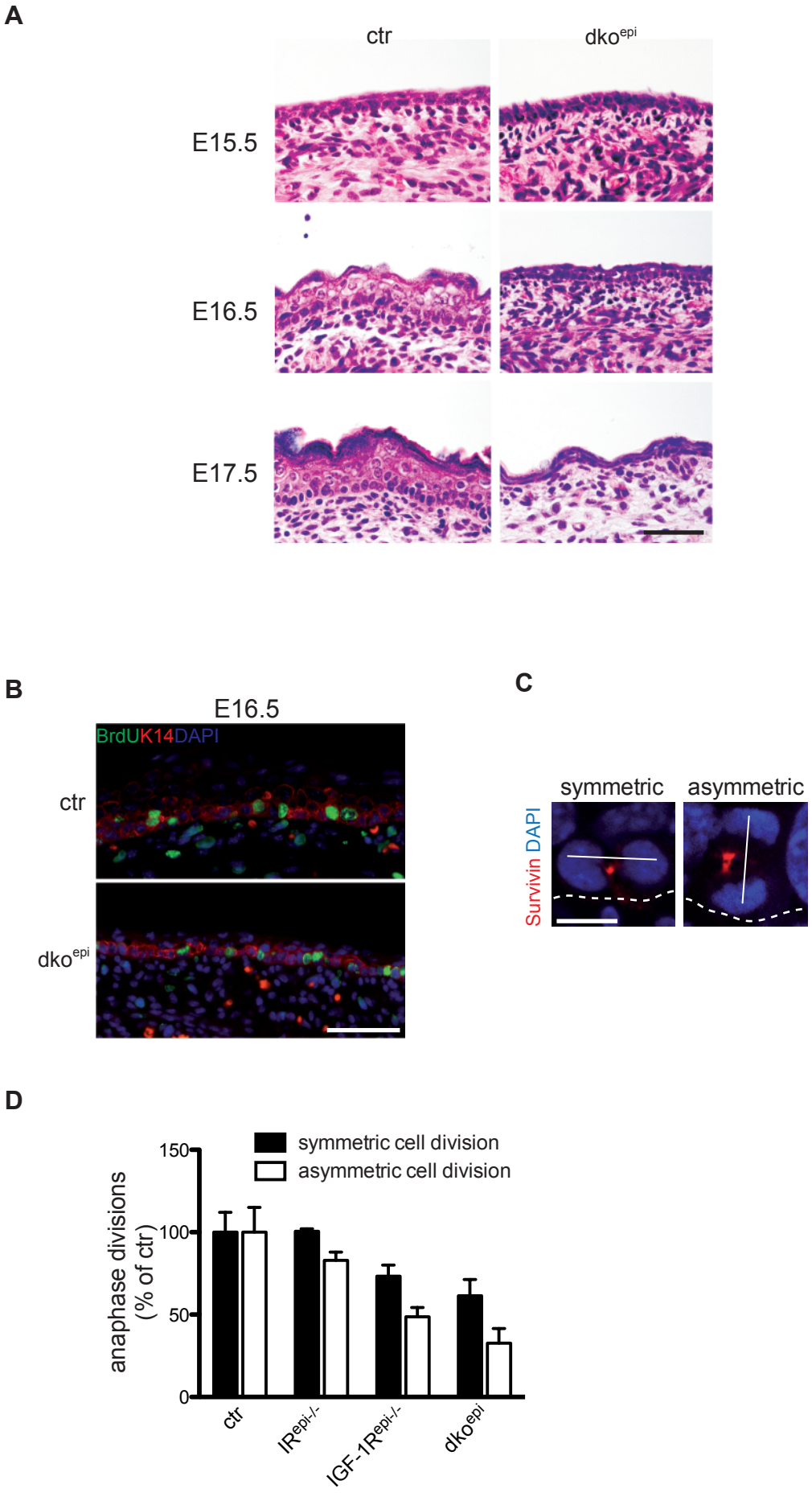


Figure S1 C.Guenschmann et al.

Figure S1 related to Figure 1.

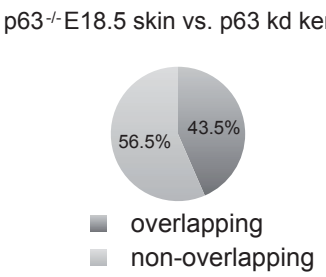
(A) Hematoxilin/Eosin staining of E15.5, E16.5 and E17.5 control and dko^{epi} reveals a reduced number of suprabasal layers in the epidermis in dko^{epi} mice. Scale bar is 100 µm.

(B) Immunfluorescence analysis of keratin 14 (K14, red), BrdU (green) and DAPI-stained nuclei (blue) in E16.5 control and dko^{epi} mice injected with BrdU 1h before sacrificing.

(C) Representative axes of division using the anaphase and telophase marker survivin (red). Dashed line represents the basement membrane. Scale bar is 10 µm.

(D) Quantification of symmetric and asymmetric divisions in control (N=349 divisions), IR$^{epi-/-}$ (N=186), IGF-1R$^{epi-/-}$ (N=119) and DKO^{epi} (N=118) E16.5 epidermis reveals an increasing loss of asymmetric cell divisions (ACD). Symmetric and asymmetric divisions were set to 100% Quantification was based using DAPI staining to mark anaphase dividing cells and collagen IV as a basement membrane markers with spindles parallel to the basement membrane scored as symmetric and spindles perpendicular scored as asymmetric. (N=5 control embryos, N=3 IR$^{epi-/-}$ and N=4 IGF-1R$^{epi-/-}$ embryos and dko^{epi} E16.5 embryos, mean ± SD).

A



B

Common GO terms in arrays of p63-/- E18.5 skin and p63 kd keratinocytes

GO Term	PValue	Fold Enrichment
epidermis development	3,23E-12	3,076759789
ectoderm development	4,81E-12	2,963378356
response to organic substance	1,21E-10	1,848474039
response to endogenous stimulus	9,47E-10	2,125876414
regulation of cell proliferation	2,84E-09	1,738414892

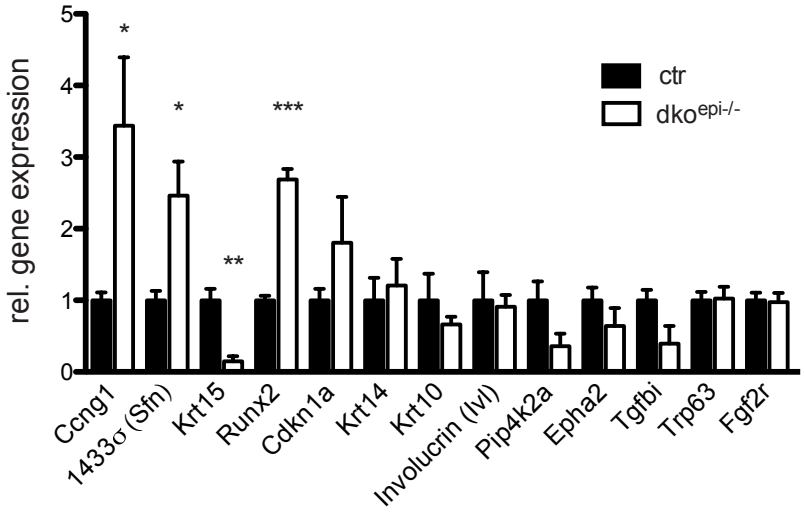
Unique GO terms in p63-/- E18.5 skin array

GO Term	PValue	Fold Enrichment
chemical homeostasis	4,15E-14	2,060255643
response to wounding	4,85E-13	1,990284697
cellular chemical homeostasis	3,21E-11	2,094117647
homeostatic process	6,52E-11	1,7249413
muscle organ development	9,56E-11	2,499646657

Unique GO terms in p63 kd keratinocytes array

GO Term	PValue	Fold Enrichment
regulation of programmed cell death	1,24E-11	1,512121392
regulation of apoptosis	1,37E-11	1,51365262
regulation of cell death	1,80E-11	1,506555301
protein localization	7,50E-10	1,44139005
establishment of protein localization	2,93E-08	1,427116006

C



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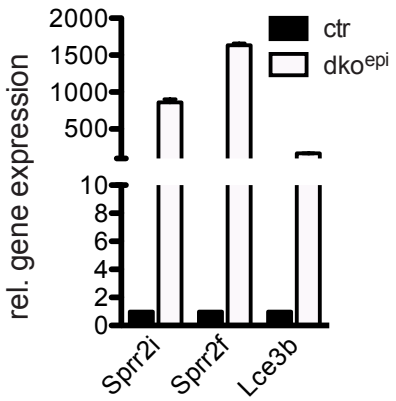

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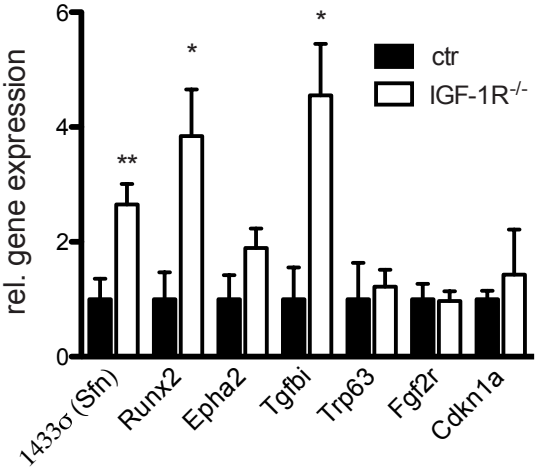

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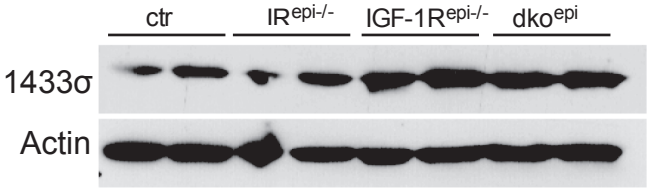

G

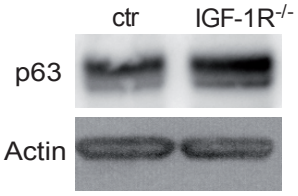

Figure S2 C.Guenschmann et al.

Figure S2 related to Figure 3.

(A) Pie chart showing the percentage of overlapping and non-overlapping genes of the $p63^{-/-}$ E18.5 skin global gene set with the p63 kd keratinocytes global gene expression set.

(B) GO terms for the overlapping genes from (A) and the unique genes for either the $p63^{-/-}$ E18.5 skin or the p63 kd keratinocyte gene sets.

(C and D) Quantitative real time PCR analysis showing relative gene expression of p63 regulated genes (C) and selected EDC genes (D) in control (ctr) and dko^{epi} newborn epidermis (N=3 newborn epidermis/genotype, mean ± SEM).

(E) Quantitative Real time PCR showing relative gene expression of p63 regulated genes in control (set to 1) and IGF-$1R^{-/-}$ primary cultured keratinocytes (mean of three independently isolated primary keratinocytes/genotype ± SEM).

(F) Increased 14-3-3σ protein expression in newborn epidermis of $IR^{epi-/-}$, IGF-$1R^{epi-/-}$ and dko^{epi} mice compared to control (ctr). Actin was a loading control.

(G) Western blot analysis for p63 in keratinocytes lysate reveals no change of protein expression upon loss of IGF-1R.

Values in (C), (D) and (E) are means ± SEM and statistical significance was tested by student's T-test and indicated as *P<0.05, **P<0.01, ***p<0.001.

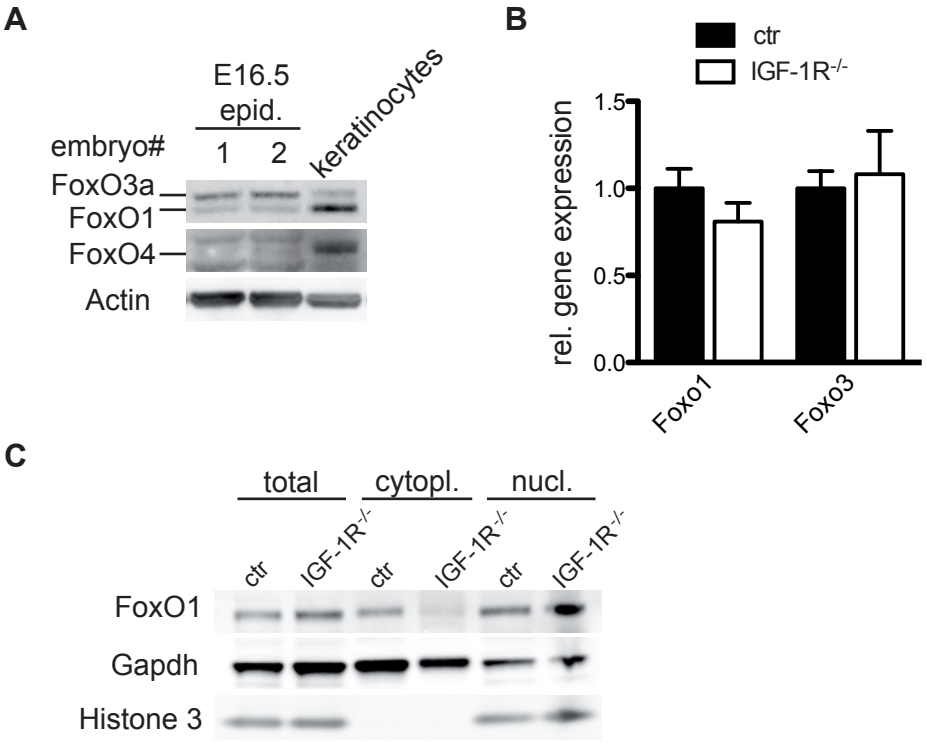

Figure S3 C.Guenschmann et al.

Figure S3 related to Figure 4.

(A) Western blot analysis for FoxO1, 3 and 4 protein in E16.5 epidermis and in primary mouse keratinocytes.

(B) Quantitative real time PCR analysis of E16.5 epidermis reveals no obvious changes in either FoxO1 or Foxo3 expression in IGF-1R$^{epi-/-}$. Control is set to 1 (mean of N=3 embryos/ genotype ± SEM). Statistical significance was tested by student's T-test.

(C) Fractionation of keratinocytes shows more FoxO1 in the nucleus upon loss of IGF-1R.

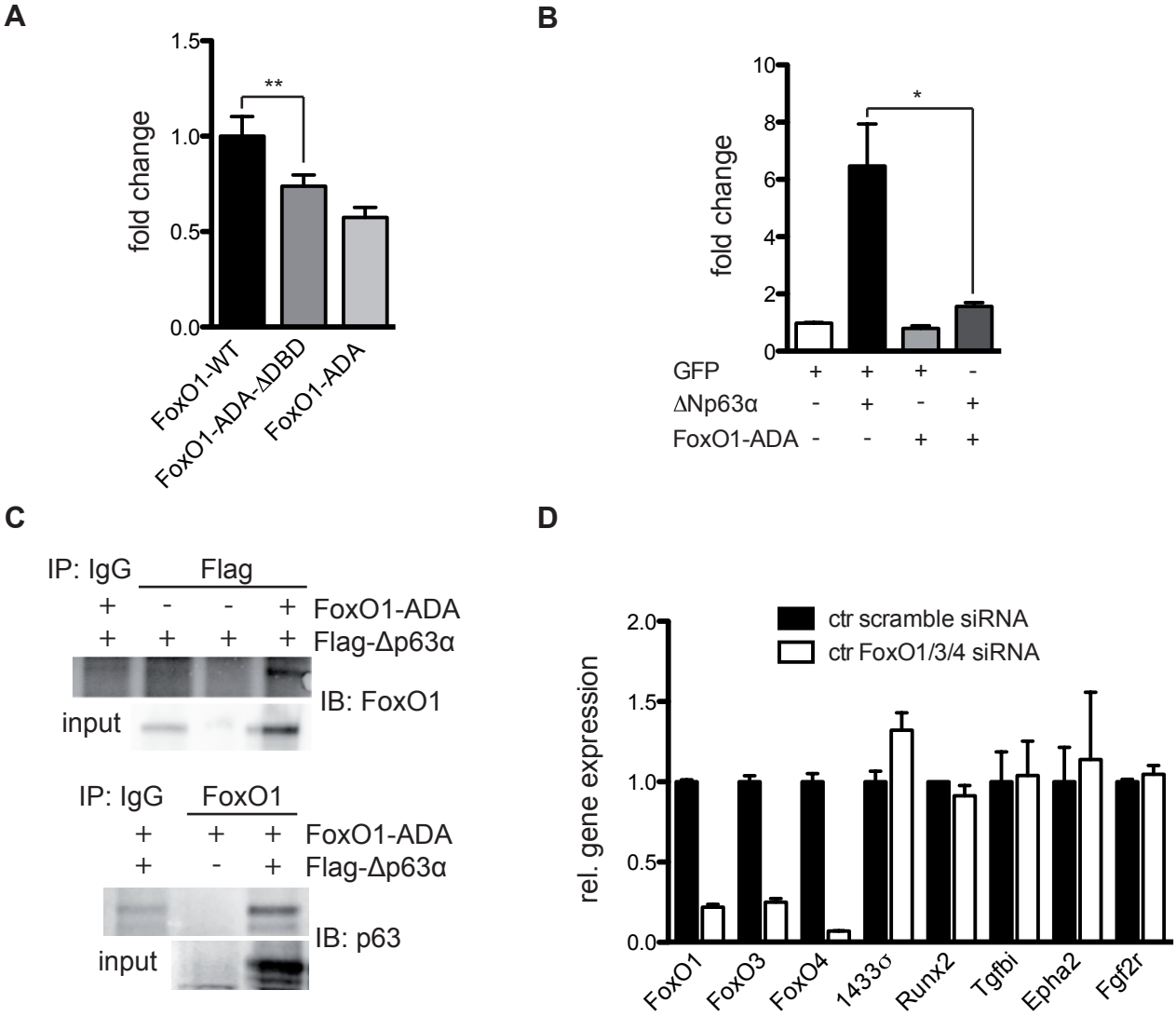

Figure S4 C.Guenschmann et al.

Figure S4 related to Figure 5.

(A) Luciferase reporter assay showing that both FoxO1-ADA and the DNA binding deficient Foxo1 mutant (FoxO1-ADA-ΔDBD) repress the activity of the p63-transactivated reporter (BDS-2,3x) in keratinocytes (mean of N=4 independent experiments ± SEM).

(B) Luciferase reporter assay in CHO cells using the p63-transactivated (BDS-2 (3x)) reporter and co-transfected with GFP and FoxO1-ADA or Δ Np63α showing that p63 expression is required for FoxO-mediated regulation of the p63 reporter (mean of N=4 independent experiments ± SEM).

(C) Immunoprecipitation analysis of CHO cells transfected with FoxO1-ADA and Flag-ΔNp63α shows that FoxO-ADA can immunoprecipitate p63 and, vice versa, Flag-P63 co-precipitates FoxO1-ADA. Shown is one representative experiment of three independent experiments.

(D) Real time PCR analysis on control primary mouse keratinocytes transiently transfected with either scramble or combined knockdown of FoxO1/3/4 using smart pool siRNAs to each of these FoxOs. FoxO1/3/4 knockdown did not alter p63 target expression in control keratinocytes compared to scrambled siRNAs (mean of N=4 independent experiments ± SEM).

Values in (A), (B) and (D) are means ± SEM. Statistical significance in (D) and (E) was tested by student's T-test and is indicated as *P<0.05, **P<0.01.

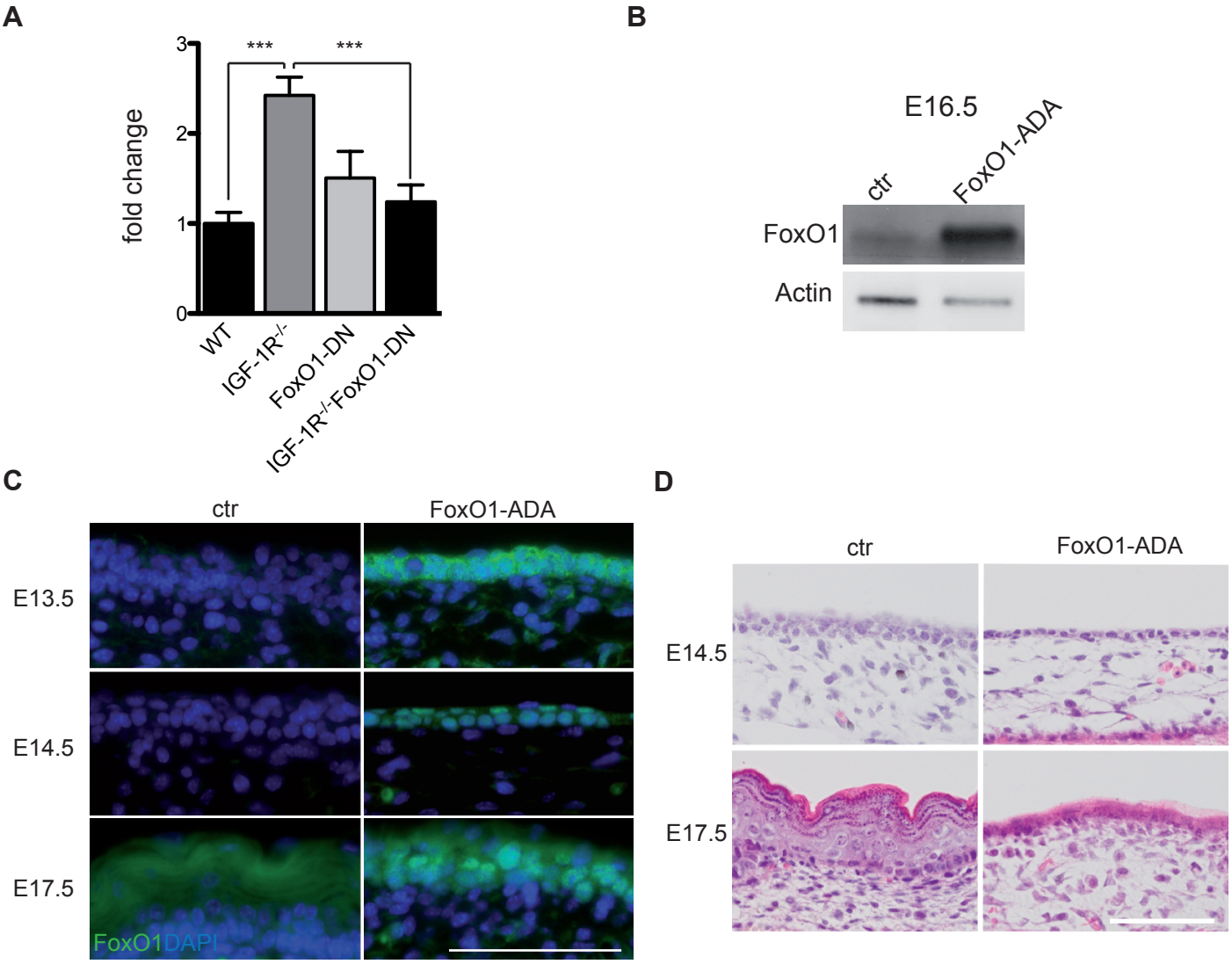

Figure S5 C.Guenschmann et al.

Figure S5 related to Figure 6.

(A) Luciferase reporter assays using the FoxO luciferase reporter (6xDBE) in primary keratinocytes isolated from control, IGF-1R$^{epi-/-}$, FoxO-DN^{epi} and IGF-1R$^{epi-/-}$ mice show that expression of FoxO-DN represses FoxO transcriptional activity in IGF-1R$^{-/-}$ keratinocytes (N=4 independent experiments, mean ± SEM). Statistical significance was tested by student's T-test and is indicated as ***P<0.001.

(B) Western blot analysis of FoxO1-ADA on protein lysates of E16.5 control and FoxO1-ADA epidermis.

(C) Immunofluorescence analysis for FoxO1 (green) on skin sections from indicated embryonic stages in control and FoxO1-ADA mice. Nuclei are counterstained with DAPI (blue). Scale bar 50 μm.

(D) H&E staining on paraffin sections of FoxO1-ADA embryos reveals an hypomorphic epidermis at E14.5. Scale bar is 50 μm.

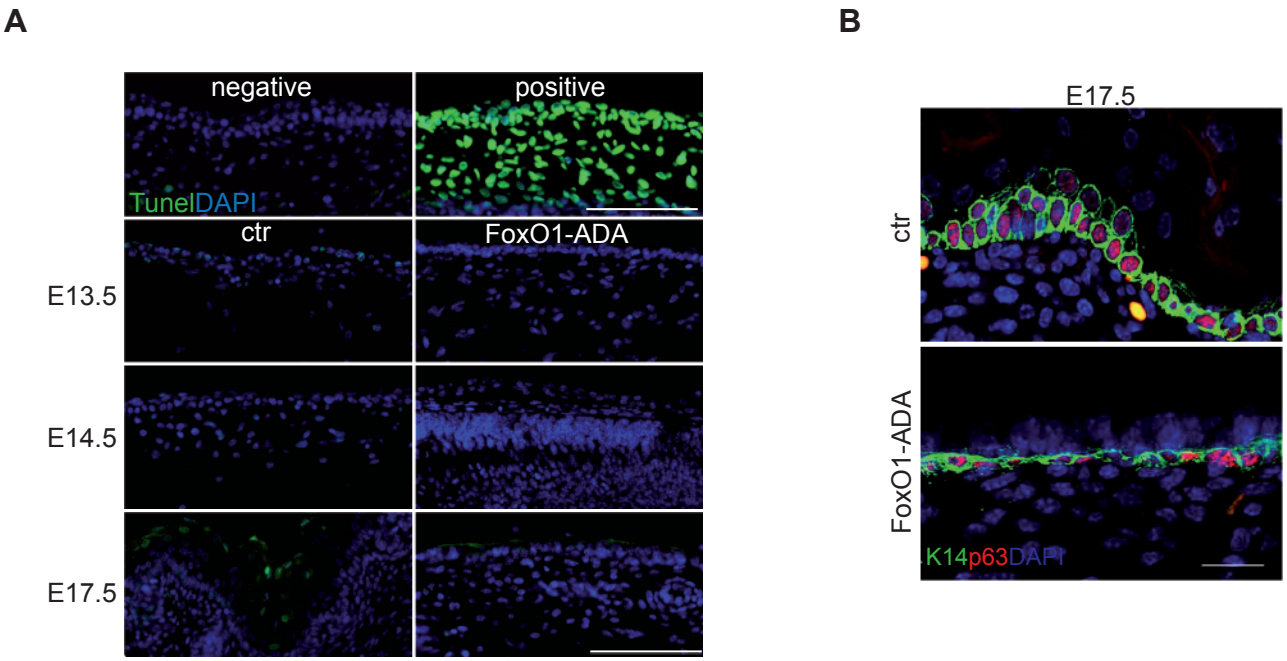

Figure S6 C.Guenschmann et al.

Figure S6 related to Figure 7.

(A) Apoptosis is not increased in FoxO1-ADA epidermis compared to control at indicated embryonic stages as shown by TUNEL staining (green). Scale bar is 50 μm. Nuclei were counterstained by DAPI (Blue).

(B) Immunfluorescence analysis for p63 (red) and K14 (green) in epidermis of E17.5 FoxO1-ADA mice. Nuclei were counterstained with DAPI (Blue) Scale bar is 10 μm.

Supplementary experimental procedures

Cellular fractionation of primary keratinocytes

Detergent-free lysis of primary keratinocytes was performed as previously described (Maher et al., J. Cell Biol. 186:219). Nuclei were extracted using high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF.

Taqman probes used for realtime qPCR

Gene Symbol	Probe number
Foxo1	Mm00490672_m1
Foxo3	Mm01185733_m1
Foxo4	Mm00840140_g1
Foxo6	Mm00809934_s1
Hprt	Mm00446986_m1
18s	Mm03928990_g1
Runx2	Mm00501584_s1
Sfn	Mm01180869_s1
Trp63	Mm01144752_m1
Krt15	Mm00492972_m1
Krt14	Mm00516876_m1
Krt10	Mm03009921_m1
Ccng1	Mm00438084_m1
Fgfr2	Mm01269930_m1
Sprr2f	Mm00448855_s1
Sprr2i	Mm007268832_s1
Lce3b	Mm01333146_g1
Cdkn1a	Mm01303209_m1
Tgfbi	Mm00493634_m1
Pip4k2a	Mm00435721_m1
Epha2	Mm00438726_m1
Jag1	Mm00496902_m1

Primary antibodies used for IF, WB or IP

Antigen	**Host**	**Manufacturer**
Actin (C4)	mouse	MP Biomedicals, Santa Ana, CA
Akt1 (C73H10)	rabbit	Cell Signaling Technology, Danvers, MA
phospho-Akt (S473)(D9E)	rabbit	Cell Signaling Technology, Danvers, MA
BrdU (B44)	mouse	BD Bioscience, Franklin Lakes, NJ
FoxO1 (C29H4)	rabbit	Cell Signaling Technology, Danvers, MA
FoxO3a (75D8)	rabbit	Cell Signaling Technology, Danvers, MA
FoxO4	rabbit	Santa Cruz Biotechnology, Santa Cruz, CA
phospho-FoxO1 (S256)	rabbit	Cell Signaling Technology, Danvers, MA
Flag M2	mouse	Sigma-Aldrich, St Louis, MO
GFP	chicken	Abcam, Cambridge, MA
IGF-1Rβ (C20)	rabbit	Santa Cruz Biotechnology, Santa Cruz, CA
Keratin 10	rabbit	Covance, Princeton, NJ
Keratin 14	rabbit	Covance, Princeton, NJ
Loricrin	rabbit	Covance, Princeton, NJ
p63α (H129)	rabbit	Santa Cruz Biotechnology, Santa Cruz, CA
p63 (4A4)	mouse	Santa Cruz Biotechnology, Santa Cruz, CA
Survivin (71G4B7)	rabbit	Cell Signaling Technology, Danvers, MA
14-3-3σ (N14)	goat	Santa Cruz Biotechnology, Santa Cruz, CA

List of primers used for Chromatin immunoprecipitation assays

Gene Symbol	**Sense/ Antisense Primer Sequence**
1433sigma (Sfn)	CGATGTGGAGAACCAGAGAG CCAATATGTTTGTTGGACACCT
Cdkn1a	CATGTTCAGCCCTGGAATTG GTAGTTGGGTATCATCAGGTCTC
Runx2	GACTGTCAGGAGCTGGGAAG GGCCATATAGCCTTGCATCA
Fgfr2 (Ferone et al., Embo Mol Med, 2011)	AATGAGCGCGCAAGTTAGAAC GCCGCGCCGAGATGT
negative control (Ferone et al., Embo Mol Med, 2011)	ACTCTGACGGATGGCTCTTCA AGGCAGACTTGTGTGGAGATGA