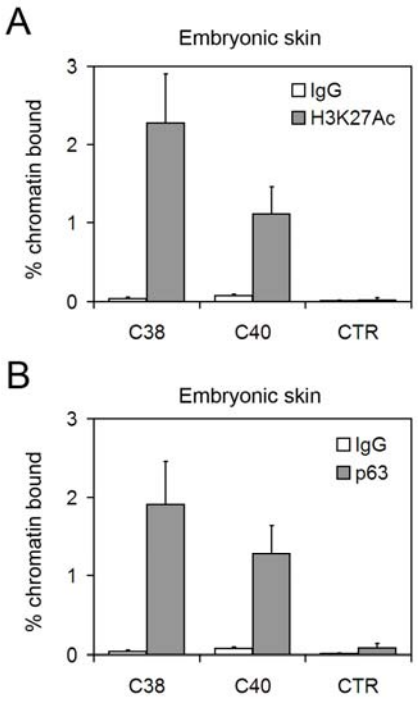
Supplementary data

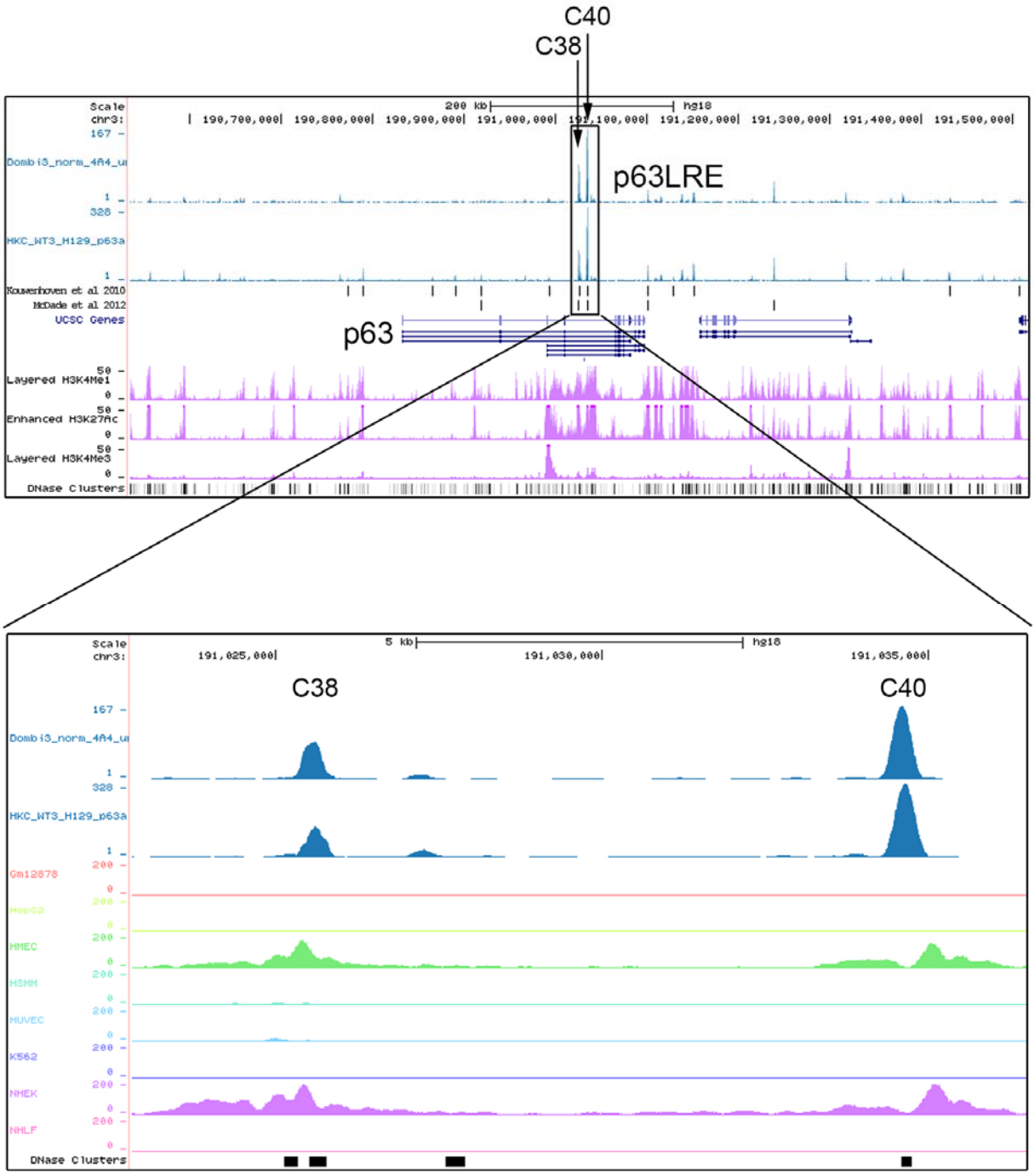
A composite enhancer regulates *p63* gene expression in epidermal morphogenesis and in keratinocyte differentiation by multiple mechanisms

Antonini et al.

Supplementary Figures

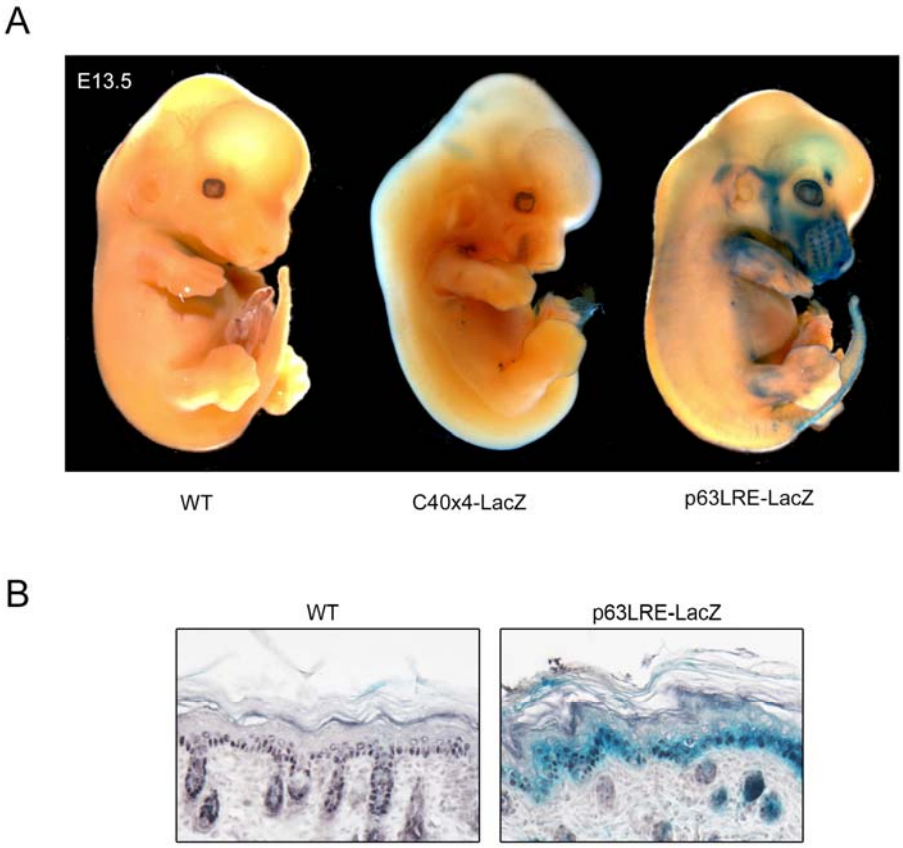


Supplementary Figure S1. (A,B) ChIP-qPCR was performed on mouse embryonic skin at E14.5 using anti-H3K27Ac (A), anti-p63 (B) (grey bars), or rabbit IgG (white bars) as negative control. Error bars denote SD.

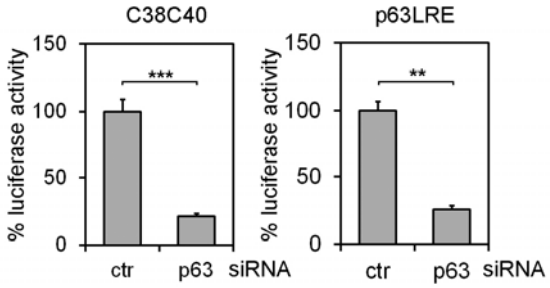


Supplementary Figure S2. The position of the p63-binding regions in the *p63* genomic loci was obtained from previously published ChIP-seq analyses in human primary keratinocytes using 4A4 and H129 antibodies, as indicated with the blue peaks and black bars (Kouwenhoven et al. 2010), and with black bars (McDade et al. 2012). C38, C40 elements and p63LRE enhancer regions corresponding to the mouse genomic regions are indicated. Genomic regions enriched for DNAse clusters and H3K27ac in

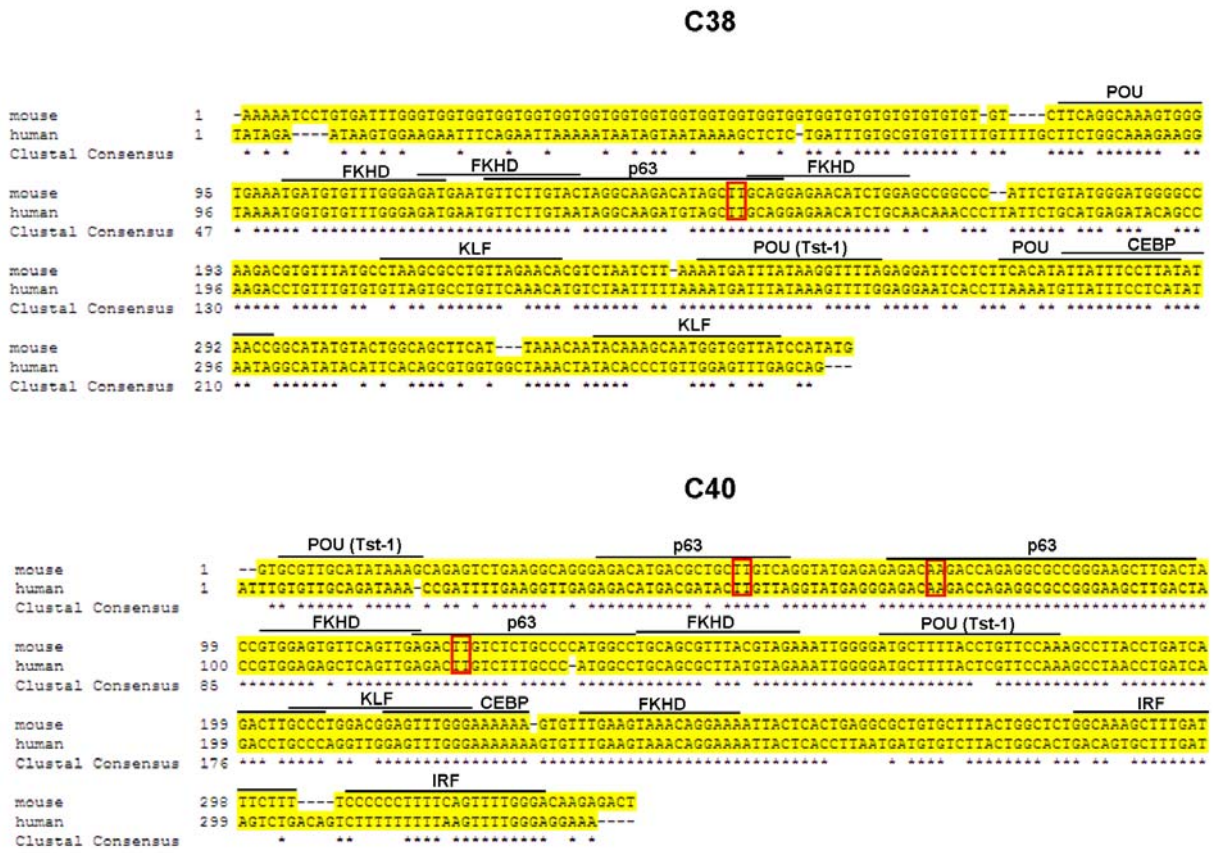
the indicated cell lines were obtained from the ENCODE project (Sabo et al. 2006; Ernst et al. 2011). The following human cells were used: B-lymphoblastoid cells (GM12878), hepatocellular carcinoma cells (HepG2), mammary epithelial cells (HMEC), skeletal muscle myoblasts (HSMM), umbilical vein endothelial cells (HUVEC), erythrocytic leukaemia cells (K562), normal epidermal keratinocytes (NHEK) and normal lung fibroblasts (NHLF).



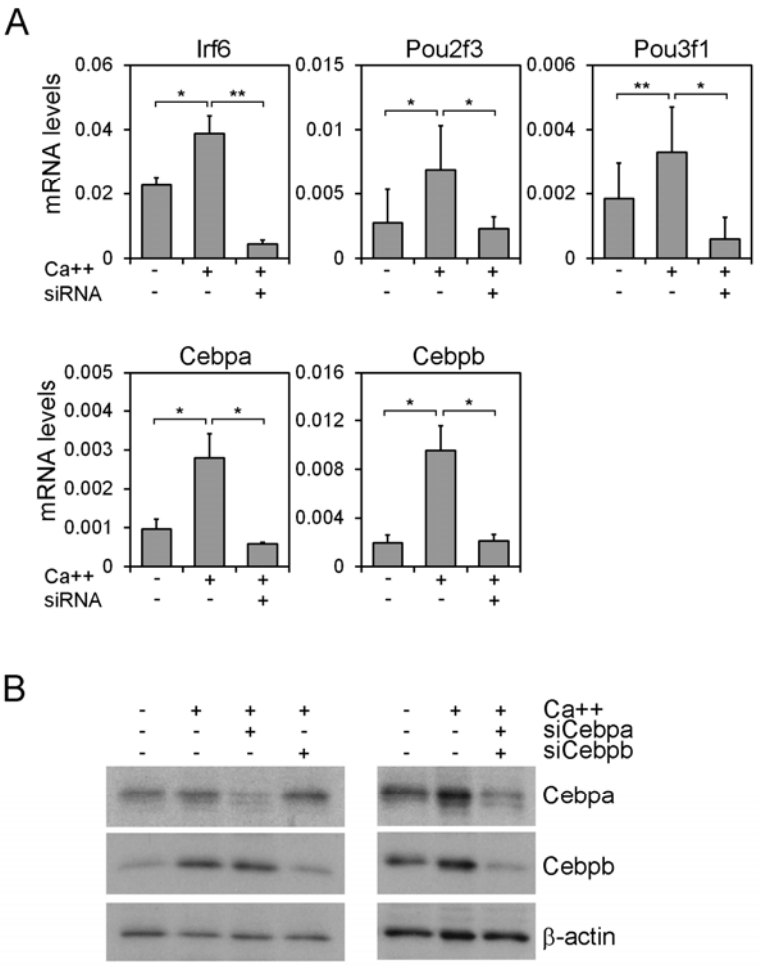
Supplementary Figure S3. (A) E13.5 wild type (WT) and transgenic embryos containing either four copies of C40 (C40x4-LacZ) or p63LRE-LacZ were tested for β-galactosidase activity. (B) Histological sections of skin at P2 of wild type (WT) and p63LRE-LacZ transgenic mice were stained for β-galactosidase activity, and subsequently immunostained with anti-p63 antibodies (H137; Santa Cruz Biotechnology).



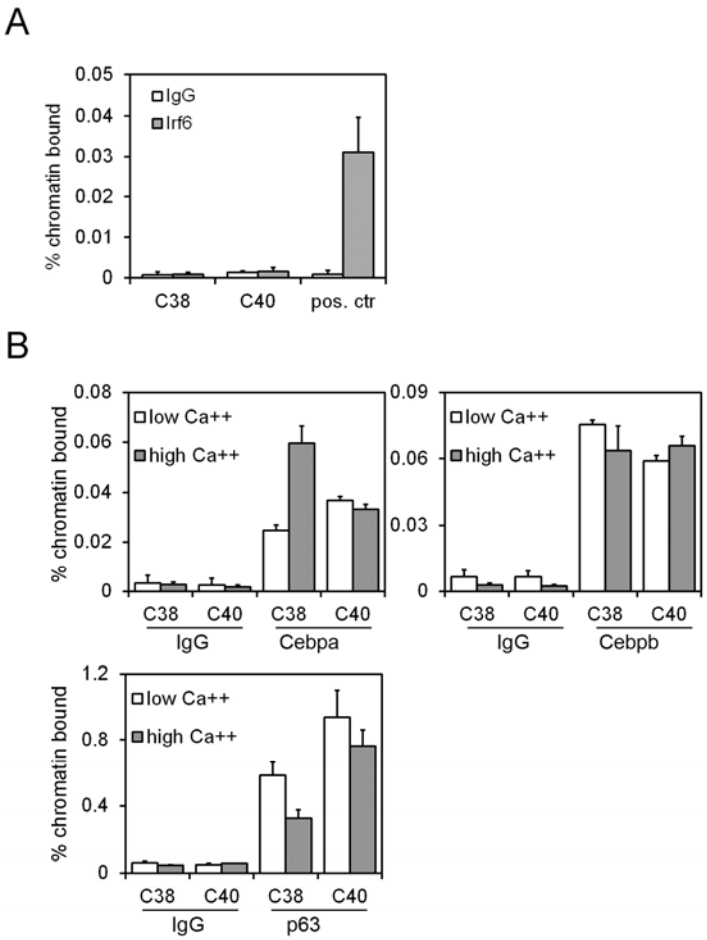
Supplementary Figure S4. C38C40-Luc and p63LRE-Luc luciferase activity were tested in mouse keratinocytes transfected with p63 siRNA. Data are expressed relative to luciferase activity in keratinocytes transfected with control siRNA (ctr). (** $P \leq 0.005$; *** $P \leq 0.0005$; n=3). Error bars denote SD.



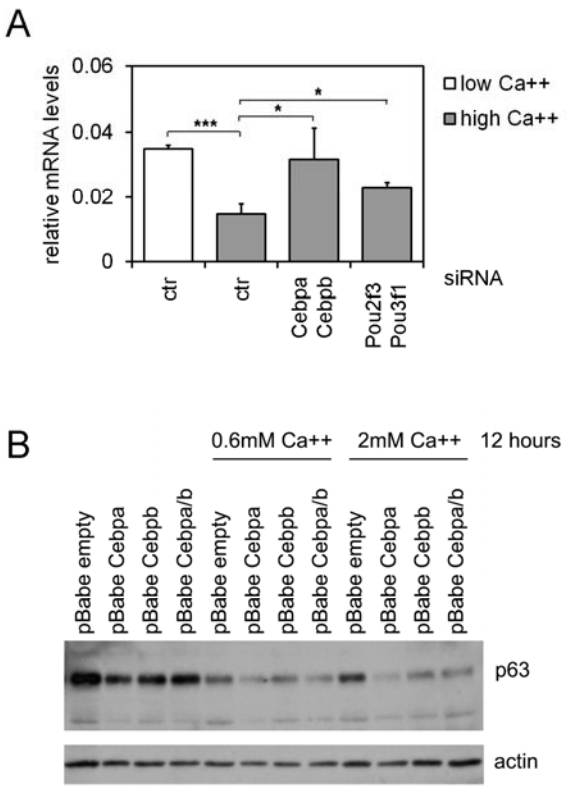
Supplementary Figure S5. Evolutionary conservation of C38 and C40 elements in mouse and human. Conserved nucleotides are shown in yellow, and nucleotides that are identical in the two species are indicated by stars (Clustal Consensus). Cloned mouse sequences of both elements were analyzed using MatInspector. TF-binding sites are indicated. Red squares indicate the two-bp of p63 binding site cores mutated in C38C40p63BSmut construct (see Suppl. Materials and Methods).



Supplementary Figure S6. (A) mRNA expression levels of the indicated genes was measured by real time RT-PCR on RNA isolated from proliferating (-) or differentiated (+) keratinocytes treated with calcium (Ca++), and transfected with the siRNA corresponding to the indicated genes or negative control (-). (* $P \leq 0.05$; ** $P \leq 0.005$; n=4). Error bars denote SD. (B) Immunoblotting of total cell extracts from primary mouse keratinocytes transfected with the indicated siRNAs using antibodies against the indicated proteins. Keratinocytes were cultured in undifferentiated or in undifferentiated conditions (0.6mM Ca^{++} for 24 hours).



Supplementary Figure S7. (A) ChIP-qPCR was performed on human keratinocytes using rabbit anti-Irf6 antibodies (kindly provided by Dr. Mike Dixon, University of Manchester) (grey bars), or rabbit IgG (white bars) as negative control. Error bars denote SD. (B) ChIP-qPCR was performed on undifferentiating (white bars) and differentiating (grey bars) mouse primary keratinocytes using antibodies specific for Cebpa, Cebpb, p63 or rabbit IgG as negative control. Error bars denote SD.



Supplementary Figure S8. (A) p63 mRNA expression levels in differentiated (grey bars) and proliferating (white bars) keratinocytes upon transfection of the indicated siRNAs. (* $P \leq 0.05$; *** $P \leq 0.0005$; n=3). Error bars denote SD. **(B)** Immunoblotting of total cell extracts from primary mouse keratinocytes infected with the indicated retroviruses using antibodies against p63 and β-actin proteins. Keratinocytes were cultured in proliferating or in differentiated conditions with 0.6 or 2mM calcium for 12 hours as indicated.

Supplementary Table S1. Predicted transcription factor binding sites using MatInspector

Element	Matrix Family	Matrix	Position	Strand	Core sim.	Matrix sim.	Human cons.	Chicken cons.
C38	V$OCT1	V$OCT1.02	80	+	0.75	0.816	Y	
C38	V$FKHD	V$HNF3.01	100	-	1	0.971	Y	Y
C38	V$FKHD	V$FOXP1_ES.01	114	-	1	0.959		
C38	V$P53F	V$P53.07	130	+	0.858	0.779	Y	Y
C38	V$FKHD	V$FOXP1_ES.01	148	+	1	0.959	Y	Y
C38	V$P53F	V$P53.05	188	+	1	0.745		
C38	V$FKHD	V$FOXP1_ES.01	193	-	1	0.981	Y	
C38	V$OCT1	V$POU3F3.01	198	-	1	0.849	Y	Y
C38	V$KLFS	V$GKLF.02	208	-	0.897	0.916		
C38	V$P53F	V$P53.03	210	+	0.859	0.896	Y	
C38	V$FKHD	V$FOXP1_ES.01	216	+	1	0.959	Y	Y
C38	V$BRNF	V$TST1.01	240	+	0.9	0.888	Y	
C38	V$KLFS	V$GKLF.02	251	+	0.862	0.936		
C38	V$FKHD	V$XFD3.01	263	-	0.783	0.774	Y	
C38	V$OCT1	V$OCT1.04	265	-	1	0.771		
C38	V$BRNF	V$TST1.01	269	-	0.9	0.924		
C38	V$FKHD	V$FHXB.01	271	-	1	0.872	Y	
C38	V$OCT1	V$OCT1.05	272	+	0.85	0.921	Y	
C38	V$FKHD	V$HNF3.01	274	-	0.971	0.959	Y	Y
C38	V$CEBP	V$CEBPB.01	278	-	1	0.916	Y	Y
C38	V$OCT1	V$OCT1.03	309	+	1	0.853		
C38	V$FKHD	V$FOXP1_ES.01	314	+	1	0.981		
C38	V$OCT1	V$OCT1.02	322	+	0.75	0.828		
C38	V$KLFS	V$GKLF.01	326	+	0.78	0.833	Y	
C38	V$OCT1	V$POU3F3.01	338	-	0.761	0.859		
C40	V$OCT1	V$POU3F3.01	3	+	1	0.821		
C40	V$P53F	V$P53.03	24	+	1	0.9	Y	Y
C40	V$P53F	V$P53.07	34	+	1	0.853	Y	Y
C40	V$P53F	V$P53.03	54	+	0.922	0.872	Y	Y
C40	V$P53F	V$P53.02	63	-	0.885	0.894	Y	Y
C40	V$FKHD	V$HFH1.01	102	-	0.75	0.798		
C40	V$P53F	V$P53.03	105	+	0.828	0.912	Y	Y
C40	V$P53F	V$P53.03	114	-	0.922	0.975	Y	Y
C40	V$FKHD	V$FREAC2.01	140	-	1	0.825		
C40	V$P53F	V$P53.02	186	+	0.885	0.92	Y	Y
C40	V$P53F	V$P53.01	196	+	0.844	0.701	Y	
C40	V$KLFS	V$BTEB3.01	204	+	1	0.906		
C40	V$CEBP	V$CEBPA.01	214	+	0.972	0.944	Y	
C40	V$RBPF	V$RBPJK.02	215	+	1	0.945	Y	
C40	V$FKHD	V$HNF3.01	217	+	0.857	0.931	Y	Y
C40	V$FKHD	V$HNF3.01	225	-	1	0.962	Y	
C40	V$CEBP	V$CEBP.02	230	+	0.971	0.898	Y	
C40	V$BRNF	V$TST1.01	232	+	0.8	0.877	Y	Y
C40	V$FKHD	V$FOXP2.01	233	+	1	0.995	Y	Y
C40	V$OCT1	V$OCT1.06	241	+	1	0.763	Y	Y
C40	V$IRFF	V$IRF7.01	284	-	1	0.846		
C40	V$IRFF	V$IRF4.01	290	-	1	0.912		
C40	V$FKHD	V$HNF3.01	294	-	0.886	0.94		
C40	V$KLFS	V$GKLF.02	302	-	1	0.931		
C40	V$IRFF	V$IRF1.01	305	-	1	0.887		

Note. Sim: similarity; cons.: conservation

SUPPLEMENTARY MATERIAL AND METHODS

Retroviral preparation and infection.

High titer retrovirus production was obtained in HEK-293T cells by transient transfection of the pBabe-puro, pBabe-Cebpa and pBabe-Cebpb constructs with pEco vector using Lipofectamine 2000 as previously described (Antonini et al. 2006). Primary keratinocytes were infected twice with the retrovirus 24 and 48 h after plating in the presence of 8 μg/ml polybrene. Cells were cultured for 4 additional days in the presence of 2μg/ml puromycin.

Immunostaining for p63

For immunohistochemistry, 7-μm thick sections were deparaffinized and stained with the Vectastain kit according to the manufacturer's instructions (Vector Laboratories) using the p63 (H137; Santa Cruz Biotechnology) primary antibodies.

Recombineering

Miniarms were amplified by PCR from the BAC RP23-239F8 containing mouse *p63* genomic region using the following oligonucleotides containing NotI and XbaI (region 1 at the 5' of C38; 540bp), XbaI and SpeI (region 2 at the 3' of C40; 440bp), KpnI and SacI (region1ΔC38 at the 3' of C38; 530bp) and SacI and NheI (region2ΔC40 at the 5' of C40; 510bp):

region1 Forward CATAAGCGGCCGCGCACACTGCTTGAATCTCTCTCTC;

region1 Reverse GTCATCTCTAGACCTGTGGTCTAACTTAGGTGTTGG;

region2 Forward GTCATCTCTAGACCACGTCTCTGATTCTAGCTGATG;

region2 Reverse TCTGAACTAGTGCTGTACAGTGTGGAGGGACTATG;

region1ΔC38 Forward CATAAGGTACCGTCTGGAATGTCCTTCTTGCCACT;

region1ΔC38 Reverse GTCAAGAGCTCCAGGCTTGCAAGATGGAGCTTAGT;

region2ΔC40 Forward GTCAAGAGCTCAGCTCAGTAGGTTCTTCCTCATGC;

region2ΔC40 Reverse GTCTGAGCTAGCACCTGAGGCTGGCTCACTATAACT

Miniarms were cloned in the β-globin-lacZ vector p1229 or in pGL3 basic Luc (Promega). After cloning the vector was linearized and was electroporated together with BAC RP23-239F8 in the modified DH10B bacterial strain SW102 using a Biorad GenePulser. Prior electroporation, SW102 were grown at 32°C

and induced to express RED recombination proteins at 42°C for 15', as previously described (Liu et al. 2003).

Cloning C38 and C40 elements

The following sequence of mouse C38 element was clone in TK-pGL3-luc (Ohno et al. 1999) in SacI/NheI:

AAAAATCCTGTGATTTGGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTG
GTGTGTGTGTGTGTGTGTCTTCAGGCAAAGTGGGTGAAATGATGTGTTTGGGAGATGAATG
TTCTTGTACTAGGCAAGACATAGCTTGCAGGAGAACATCTGGAGCCGGCCCATTCTGTATG
GGATGGGGCCAAGACGTGTTTATGCCTAAGCGCCTGTTAGAACACGTCTAATCTTAAAAT
GATTTATAAGGTTTTAGAGGATTCCTCTTCACATATTATTTCCTTATATAACCGGCATATGT
ACTGGCAGCTTCATTAAACAATACAAAGCAATGGTGGTTATCCATATG

The following sequence of mouse C40 element was clone in TK-pGL3-luc (Ohno et al. 1999) in SmaI:

GTGCGTTGCATATAAAGCAGAGTCTGAAGGCAGGGAGACATGACGCTGCTTGTCAGGTAT
GAGAGAGACAAGACCAGAGGCGCCGGGAAGCTTGACTACCGTGGAGTGTTCAGTTGAGA
CTTGTCTCTGCCCCATGGCCTGCAGCGTTTACGTAGAAATTGGGGATGCTTTTACCTGTTCC
AAAGCCTTACCTGATCAGACTTGCCCTGGACGGAGTTTGGGAAAAAAGTGTTTGAAGTAA
ACAGGAAAATTACTCACTGAGGCGCTGTGCTTTACTGGCTCTGGCAAAGCTTTGATTTCTT
TTCCCCCCTTTTCAGTTTTGGGACAAGAGACT

Oligonucleotide primers for Real Time RT-PCR on mouse samples.

ß-actin

CTAAGGCCAACCGTGAAAAGAT

GCCTGGATGGCTACGTACATG

p63

CATGAGCTGAGCCGTGAGTTC

GGCTGTTCCCTTCTACTCGAA

Irf6

CAGCTCTCTCCCCATGACTGA

CCCATACTCCTTCCCACGATAC

Cebpa

GCGAGCACGAGACGTCTATAGA

GCCAGGAACTCGTCGTTGAA

Cebpb

ACAAGGTGCTGGAGCTGAC

CTGCTCCACCTTCTTCTGC

Pou3f1

TGGGCACCCTCTACGGTAAC

GGCCTCGAAACGGCAGAT

Pou2f3

CCTGAGCCAAGGACCTACCA

GAAGCCATGTCCCCAGACA

Oligonucleotide primers for ChIP analysis on mouse genomic DNA

-20kb from p63 P1 promoter

ATAAGAAAGAGAGCAGGGCATGA

TGCTTGGTCAAATTAGGATCCA

p63 P1 promoter

GGGCTCTAAAACTGTGGCAGA

CTGGGCACCCCCTGTAAAG

p63 intron 1

TGCTGCCGAAGAGAGCATTTA

AGCATTGATAACCACTCCAAGGA

p63 P2 promoter

AGCCCAGGTGGAAGTTGATG

GGGCGGGACTCTTCACTTTAC

C38

ACATAGCTTGCAGGAGAACATCTG

TCTTGGCCCCATCCCATAC

C40

TGTTCCAAAGCCTTACCTGATCA

TTTTCCCAAACTCCGTCCAG

Oligonucleotide primers for ChIP analysis on human genomic DNA

C38

CAACAAACCCTTATTCTGCATGAG

CATGTTTGAACAGGCACTAACACA

C40

CGTTCCAAAGCCTAACCTGATCA

TTTTCCCAAACTCCAACCTG

Irf6 positive control

GACGCGCTCTTGCACAGA

TTGGAATCGGGAGATTTTTCC

Oligonucleotide primers for site-directed mutagenesis (mutant bps are underlined)

C38p63BS_1 AGGCAAGACATAGCCCGCAGGAGAACATCT

C40p63BS_1 GAGACATGACGCTGCCCGTCAGGTATGAGAG

C40p63BS_2 GGTATGAGAGAGACCCGACCAGAGGCGCCG

C40p63BS_3 TGTTCAGTTGAGACCCGTCTCTGCCCCATG

siRNA sequences

p63 UCACAACAGUCCUGUACAAUUUCAU (Stealth RNAi, Invitrogen)

Cebpa CCUGAGAGCUCCUUGGUCAUU (siRNA, Qiagen)

Cebpb GAAAAGAGGCGUAUGUAUAUU (siRNA, Qiagen)

Pou2f3 GGAAAUGAUCGAAAUGGCCUAGAUU (Stealth RNAi, Invitrogen)

Pou3f1 GAGAGCCACUUUCUCAAGUGUCCA (Stealth RNAi, Invitrogen)

References

Antonini D, Rossi B, Han R, Minichiello A, Di Palma T, Corrado M, Banfi S, Zannini M, Brissette JL, Missero C. 2006. An autoregulatory loop directs the tissue-specific expression of p63 through a long-range evolutionarily conserved enhancer. *Mol Cell Biol* **26**(8): 3308-3318.

Ernst J, Kheradpour P, Mikkelsen TS, Shoresh N, Ward LD, Epstein CB, Zhang X, Wang L, Issner R, Coyne M et al. 2011. Mapping and analysis of chromatin state dynamics in nine human cell types. *Nature* **473**(7345): 43-49.

Kouwenhoven EN, van Heeringen SJ, Tena JJ, Oti M, Dutilh BE, Alonso ME, de la Calle-Mustienes E, Smeenk L, Rinne T, Parsaulian L et al. 2010. Genome-wide profiling of p63 DNA-binding sites identifies an element that regulates gene expression during limb development in the 7q21 SHFM1 locus. *PLoS Genet* **6**(8): e1001065.

Liu P, Jenkins NA, Copeland NG. 2003. A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res* **13**(3): 476-484.

McDade SS, Henry AE, Pivato GP, Kozarewa I, Mitsopoulos C, Fenwick K, Assiotis I, Hakas J, Zvelebil M, Orr N et al. 2012. Genome-wide analysis of p63 binding sites identifies AP-2 factors as co-regulators of epidermal differentiation. *Nucleic acids research* **40**(15): 7190-7206.

Ohno M, Zannini M, Levy O, Carrasco N, di Lauro R. 1999. The paired-domain transcription factor Pax8 binds to the upstream enhancer of the rat sodium/iodide symporter gene and participates in both thyroid-specific and cyclic-AMP-dependent transcription. *Mol Cell Biol* **19**(3): 2051-2060.

Sabo PJ, Kuehn MS, Thurman R, Johnson BE, Johnson EM, Cao H, Yu M, Rosenzweig E, Goldy J, Haydock A et al. 2006. Genome-scale mapping of DNase I sensitivity in vivo using tiling DNA microarrays. *Nat Methods* **3**(7): 511-518.