Supplementary Information

Loss of DLX3 tumor suppressive function promotes progression of SCC through EGFR-ERBB2 pathway

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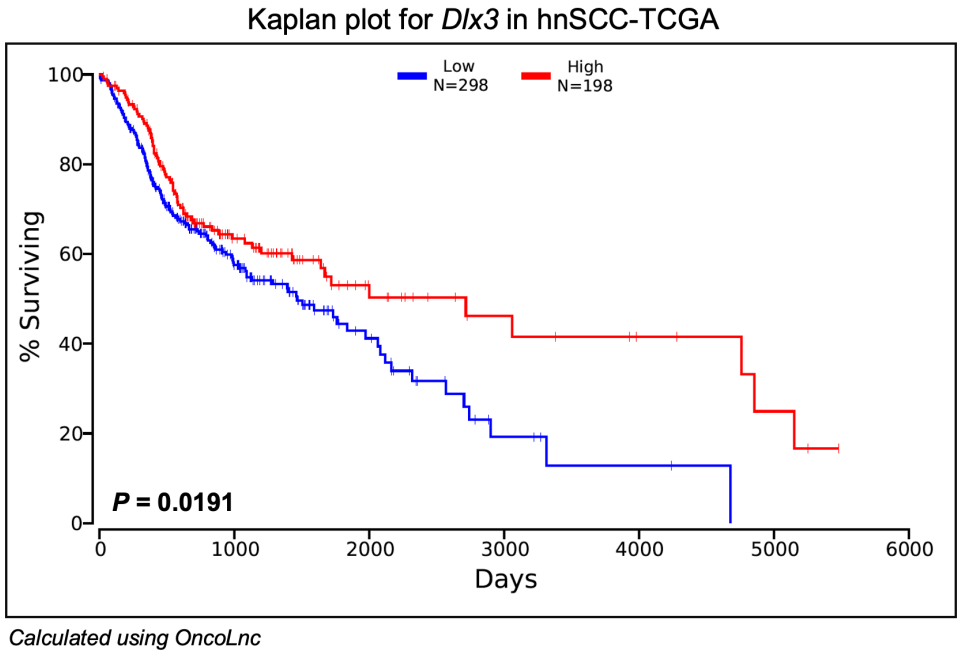
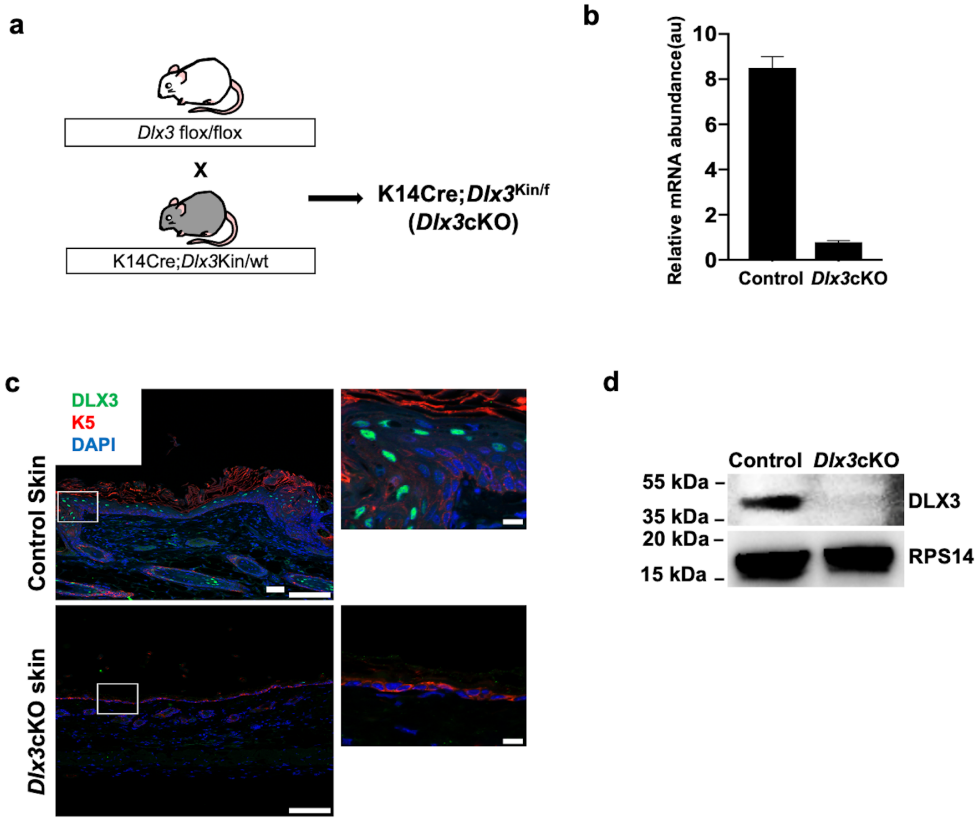
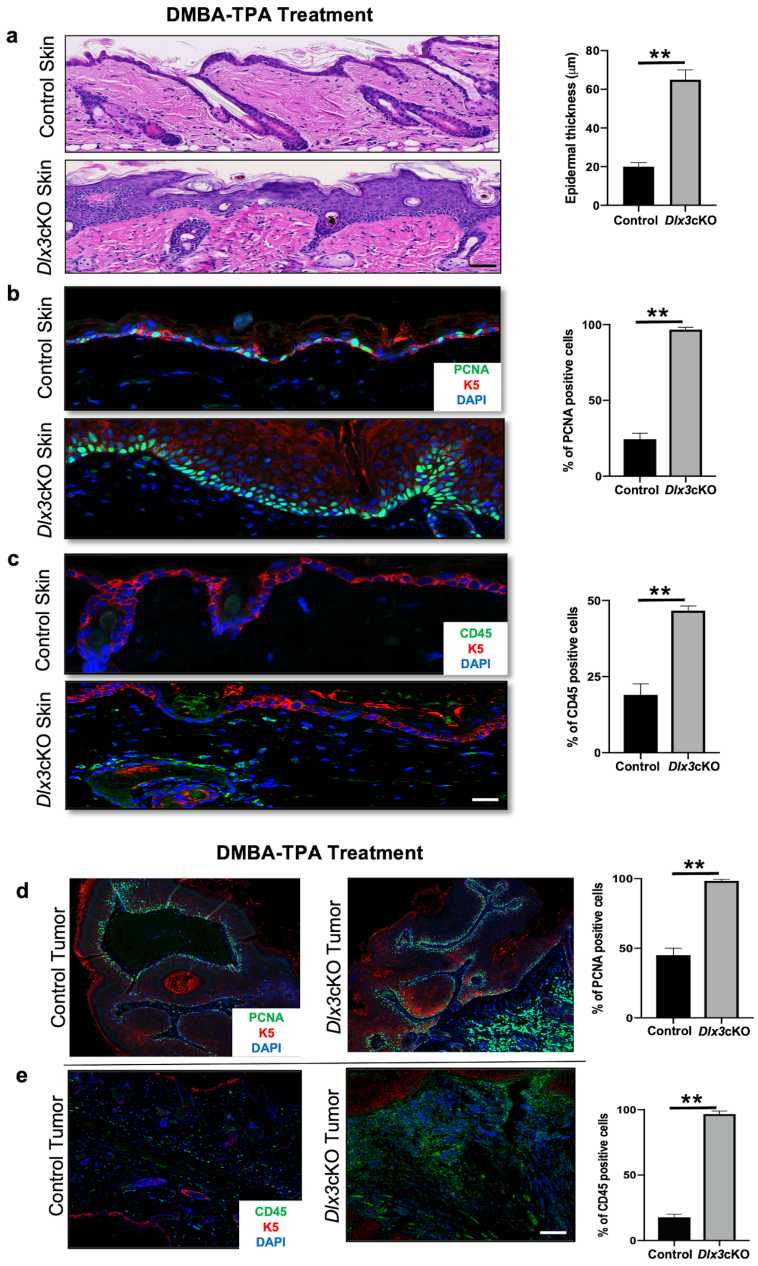


Supplementary Figure S1. Dlx3 mRNA levels correlates with poor prognosis in hnSCC. Kaplan-Meier plot for Dlx3 in hnSCC patients (No. of patients=497). (Source data: http://www.oncolnc.org/). The logrank P-value is indicated on the graph

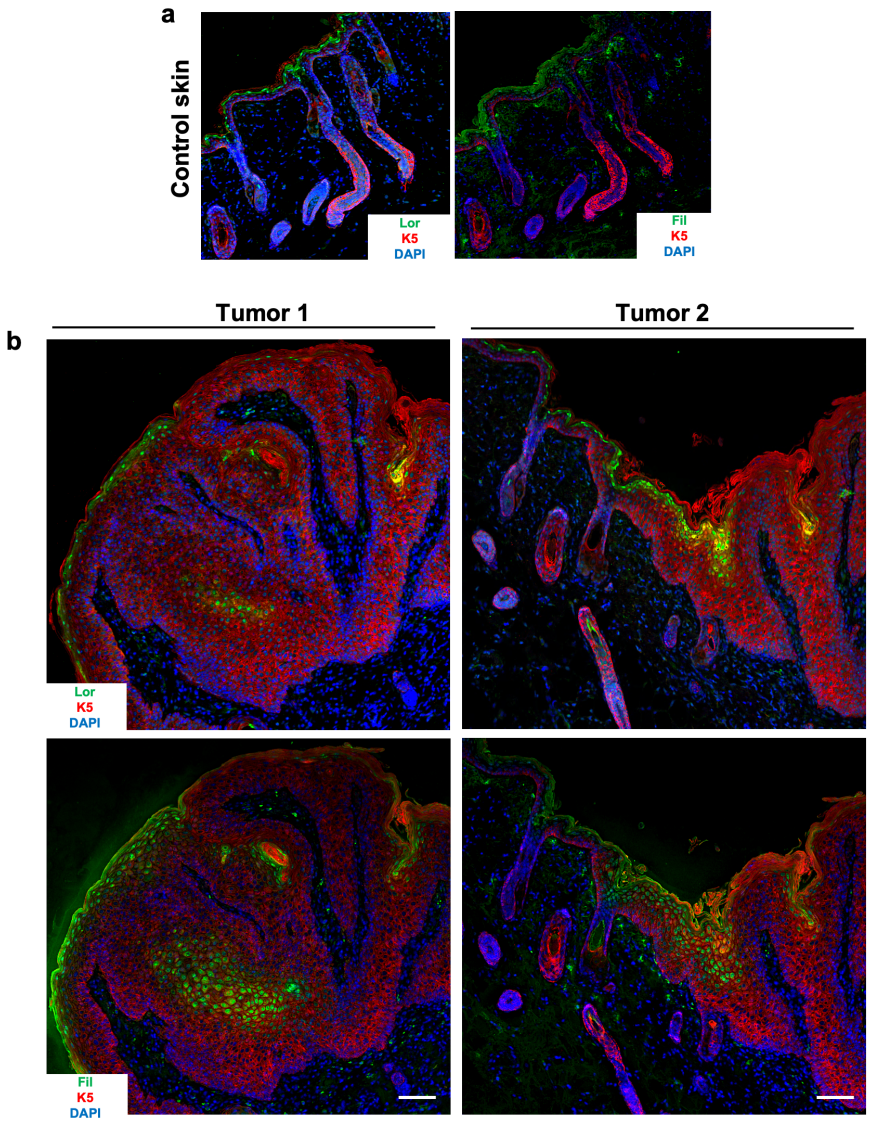


Supplementary Figure S2. K14Cre-mediated conditional deletion of *Dlx3*. (a) Schematic representation of mouse genotypes used to create skin keratinocyte-specific induction of *Dlx3* deletion. Homozygous *Dlx3*$^{f/f}$ mouse were crossed with K14Cre;Kin mouse where one *Dlx3* allele was replaced by LacZ knock-in. **(b)** Total RNAs were quantified from the epidermis of both control and *Dlx3*cKO mice by real-time RT-PCR analysis, and the knockdown levels of *Dlx3* was determined by the relative Ct method (P-value <0.005). **(c)** The specificity of Cre mediated deletion was determined by immunohistochemistry on skin sections from wild-type and *Dlx3*cKO littermates. DLX3 (green) expression is shown in the suprabasal cells in the control skin only. Images are DAPI stained to denote nuclear staining. Scale bar: 100µm and 10µm. **(d)** The specific deletion of DLX3 expression was detected in the *Dlx3*cKO epidermal extracts of P12 skin by Western blot.

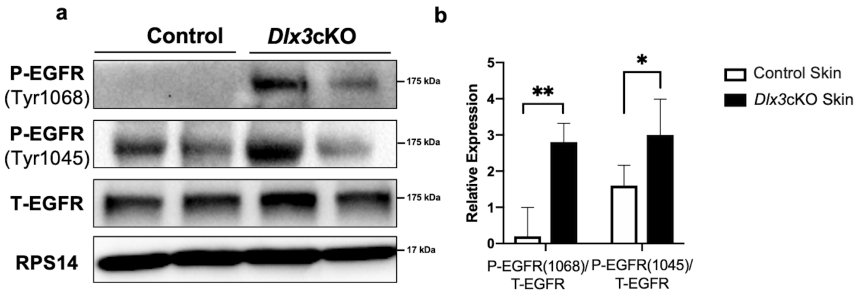


Supplementary Figure S3. *Dlx3* deficiency enhances DMBA/TPA induced epidermal hyperproliferation and inflammation. Epidermal thickness; Means of epidermal thickness was calculated based on 10–15 random site measurements. (a) HE-stained skin sections from the backs of the mice. Sections were used for evaluation of epidermal thickness. Columns in (a) are group means ±SEM (n=5, n=5 for control skin, *Dlx3*cKO skin respectively). (b) PCNA immunofluorescence staining reveals higher cell proliferation in *Dlx3*cKO skin. Skin sections were stained with antibodies to detect PCNA expression and quantitated. (c) Confocal images of skins

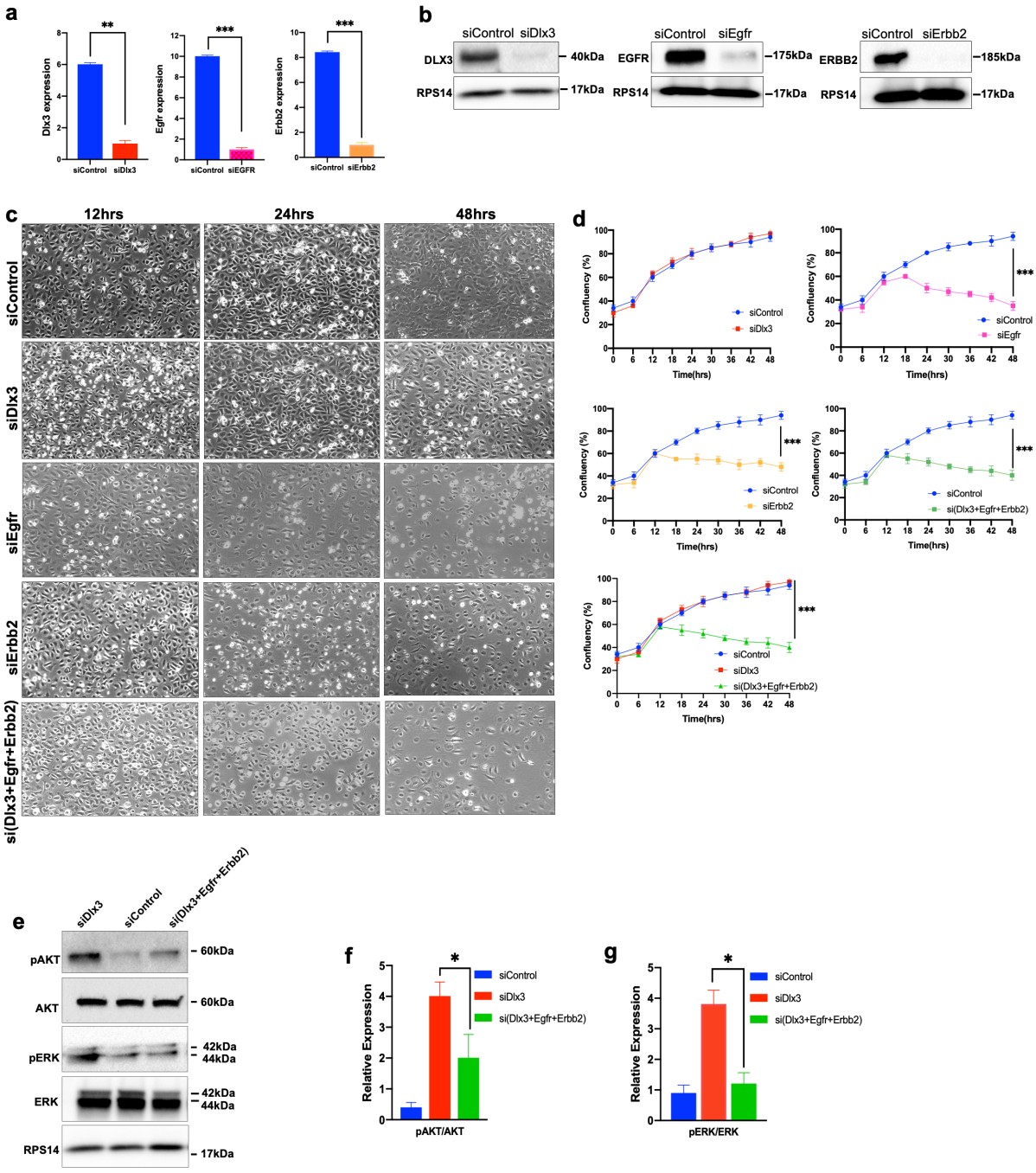
sections indicated mice stained for expression of CD45, cytokeratin 5 and counter stained with DAPI. (d) Immunofluorescence analysis of PCNA in a representative control mouse-derived papilloma (left panel) and *Dlx3*cKO mouse-derived papilloma (right panel). The numbers of PCNA positive epidermal cells were counted from at least 100 cells in five separate fields for each section respectively (n = 3). **P-value < 0.01. (e) Images of control mouse-derived papilloma (left panel) and *Dlx3*cKO mouse-derived papilloma (right panel) stained with antibodies for CD45. Scale bars = 100μm



Supplementary Figure S4. Representative images of control skin, and papilloma from *Dlx3*cKO mice. Dual immunofluorescence staining was performed for K5 (red), Loricrin (green) and Filaggrin (green). The nuclei were counterstained with DAPI (blue). Scale bars: 100μm.



Supplementary Figure S5. Intracellular domain of EGFR correspond to different signal pathways and functions. The levels of total EGFR, pEGFR (Y1068), pEGFR (Y1045), and RPS14 were assessed by immunoblot analysis (a) and quantitated by ImageJ (b). Data were normalized to total EGFR, expressed as the fold change (mean ± SEM; n = 4) and analyzed by paired t test. *P < 0.05; **P < 0.01 (versus Control).



Supplementary Figure S6. Suppressing downstream mediators of DLX3 function in skin keratinocytes inhibits proliferation in vitro. (a) mRNA expression of Dlx3, Egfr and Erbb2 in mouse primary keratinocytes treated with si-target gene and si-Control for 48 hours. (b) Immunoblot images showing knockdown of DLX3, EGFR, and ERBB2 expression in mouse primary keratinocytes treated by si-target gene and si-Control for 48 hours. (c) Phase contrast

images show in primary keratinocytes at hours 12, 24, and 48 after siRNA transfection. The cells were treated with siControl, siDlx3, siEgfr, siErbb2, and combination of all the three siRNAs **(d)** Proliferation assays with primary mouse keratinocytes were quantified every 6 hours for 48 hours, n = 3 per group. Cell proliferation was monitored by analyzing the occupied area (% confluence) of cell images over time. Data expressed as mean ± standard error of the mean. ***P<0.005 **(e)** Immunoblot images showing loss of AKT and ERK1/2 expression in Dlx3 knockdown keratinocytes transfected with si-Egfr and si-Erbb2 for 48 hours. RPS14 was used as a loading control. **(f)** Quantification of pAKT and **(g)** pERK1/2 protein (n=3). Data expressed as mean ± standard error of the mean *P<0.05.

Supplementary Table 1: List of antibodies used for immunofluorescence studies

Primary antibodies	supplier	species	type	dilutions	reference
DLX-3	Abcam	Rabbit	Monoclonal	1:500	ab178428
KRT-13	Abcam	Rabbit	Monoclonal	1:400	ab92551
KRT-10	BioLegend	Rabbit	Polyclonal	1:500	PRB-159P
KRT-5	LSBio	Guinea pig	Polyclonal	1:500	LS-C22715-100
PCNA	Cell Signaling Technology	Rabbit	Monoclonal	1:100	13110
Loricrin	BioLegend	Rabbit	Polyclonal	1:1000	PRB-145P
Filaggrin	BioLegend	Rabbit	Polyclonal	1:500	PRB-417P
Secondary Antibodies (ThermoFisher Scientific)		**species**	**type**	**dilutions**	**reference**
Alexa Flour 488		Goat	Polyclonal	1:250	A-11008
Alexa Fluor 546		Goat	Polyclonal	1:250	A-11010

Supplementary Table 2: List of antibodies used in western blotting

Antibody name	Company	Dilution
DLX3	Morasso lab; [10]	1:1000
EGFR	Cell Signaling (2232)	1:2000
phospho-EGFR, Tyr845	Cell Signaling (2231)	1:1000
phospho-EGFR, Tyr1045	Cell Signaling (2237)	1:1000
phospho-EGFR, Tyr1068	Cell Signaling (3777)	1:1000
phospho-EGFR, Tyr1173	Cell Signaling (2244)	1:1000
ERK	Cell Signaling (9102)	1:2000
phospho-ERK	Cell Signaling (4370)	1:1000
ERBB2	Cell Signaling (2165)	1:2000
phospho-ERBB2	Cell Signaling (2241)	1:1000
AKT	Cell Signaling (4691)	1:2000
phospho-AKT	Cell Signaling (4060)	1:1000
RPS14	Bethyl A304-031	1:1000