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TRP63/TP63 loss accelerates skin tumorigenesis through activation of Wnt/ β -catenin signaling

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Cutaneous Squamous Cell Carcinoma (cSCC) is the second most common skin cancer arising from the epidermis. Approximately 700,000 new cSCC cases are diagnosed every year in the United States [1]. It is commonly believed that the transcription factor *TRP63* (mouse)/TP63 (human) plays an oncogenic role in cSCCs. Contradicting the notion that expression of TP63 is causally linked to cSCC development or progression is the observation that complete or focal loss of TP63 expression occurs in a subset of cSCCs [2]. To address these conflicting findings, we performed immunostaining for TP63 and the epithelial marker KRT14 on archival human cSCCs. We observed focal (Suppl. Fig. S1A, B) or complete (Fig. 1A) TP63 loss in the epithelial compartment of 64% of well-differentiated cSCCs (n = 25) and 79% of moderately/poorly differentiated cSCCs (n = 29). Thus, loss of TP63 expression occurs in a large subset of both early- and late-stage human cSCCs. Unfortunately, as transcriptome data of human cSCCs are not publicly available, we were unable to determine the prognostic value of our findings. However, we did find that low TP63 expression correlated with an overall shorter patient survival time in other types of SCCs (Suppl. Fig. S2A–D), suggesting that loss of TP63 expression is a general marker of tumor malignancy.

To address the functional consequences of *TRP63*/TP63 loss in cSCCs, we generated inducible epidermal-specific *Trp63* knockout mice (*Krt14.CrePR1/Trp63^{fl/fl}*) (Fig. 1B). In these mice, topical application of RU486 leads to ablation of all isoforms of *Trp63* in

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Conflict of interest

The authors state no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jdermsci.2018.05.011>.

epidermal keratinocytes (Suppl. Fig. S3A–C). Next, we subjected control (*Krt14.CrePR1/Trp63^{fl/+}*) and *Trp63*-ablated mice (*Krt14.CrePR1/Trp63^{fl/fl}*) to the two-stage skin carcinogenesis protocol [3]. *Trp63* was ablated by topically treating mice with RU486 for 10 days. Subsequently, mice received a single application of the tumor initiator 7,12-Dimethylbenz(a)anthracene (DMBA) and weekly applications of the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA). *Trp63*-ablated mice displayed an earlier onset of tumor formation (Fig. 1C) and developed significantly more tumors (Fig. 1D,E) than control mice. Further, although cSCCs developed in both control and experimental mice, histopathological analysis revealed that 62.5% of *Trp63*-ablated tumors were poorly differentiated cSCCs, compared to only 12.5% of control tumors (Suppl. Fig. S4A, B). In contrast to control cSCCs, *TRP63* expression was essentially absent from cSCCs obtained from *Trp63*-ablated mice (Fig. 1F,G, Fig. 2H). *Trp63*-ablated cSCCs showed a significantly higher number of proliferative cells than control tumors (Fig. 1F,G). In addition, we observed dramatically increased expression of KRT13 (Fig. 1F,G), a marker for mouse cSCC progression [4], suggesting that *Trp63*-ablated cSCCs have a more aggressive phenotype. Taken together, these data demonstrate that loss of *Trp63* from mouse epidermis accelerates cSCC initiation and progression.

To determine the relevance of these findings for human cSCC development and progression, we generated orthotopic human cSCCs using a grafting system (Fig. 2A). Specifically, we transduced immortalized human keratinocytes (HaCaT) with a retrovirus expressing a constitutively active form of HRAS (*HRAS^{V12}*), one of the most commonly mutated (3–30%) genes in cSCCs [5]. Subsequently, we introduced doxycycline-inducible TP63 or non-silencing (NS) shRNAs. Of three TP63 shRNAs tested, TP63 shRNA3 was the most effective at downregulating TP63 at the RNA and protein levels (Fig. 2B,C). Grafting of HaCaT-*HRAS^{V12}*-NS shRNA (control) and HaCaT-*HRAS^{V12}*-TP63 shRNA3 (experimental) cells onto the back of athymic nude mice led to tumor development in all mice (Suppl. Fig. S4C). However, after two months, 67% of the control tumors had completely regressed whereas all experimental mice had developed cSCCs (Fig. 2D, Suppl. Fig. S4A). Further, TP63 expression was lost in experimental cSCCs, and these tumors were more proliferative and less differentiated than control tumors, as judged by immunofluorescence analyses of BrdU and KRT1 expression (Fig. 2E,H). Collectively, these data demonstrate that loss of TP63 leads to accelerated initiation and progression of cSCCs in both the mouse and the human systems.

We next addressed the mechanism by which *TRP63*/TP63 loss leads to cSCC development and progression. It has been reported that TP63 can repress Wnt/ β -catenin signaling in SCC cell lines and that activation of this pathway is required for cSCC tumorigenesis [6–8]. Interestingly, we observed nuclear β -catenin localization and higher levels of Axin2, markers for active Wnt/ β -catenin signaling, in both mouse and human *Trp63*/TP63-deficient cSCCs, but not in control cSCCs (Fig. 2G,H). These findings support our hypothesis that loss of *TRP63*/TP63 leads to activation of Wnt/ β -catenin signaling and consequently to accelerated skin tumorigenesis.

Taken together, data from our mouse and human model systems demonstrate that genetic inactivation of *Trp63*/TP63 accelerates cSCC initiation and progression, thus establishing a

tumor suppressive function for this gene. Interestingly, this effect appears to be dosage-dependent, as heterozygous *Trp63*-null mice do not show an increased susceptibility to skin carcinogenesis [9]. We also found that loss of TRP63/TP63 leads to activation of the canonical Wnt pathway, a pathway previously implicated in cSCC development and progression. Recently, overexpression of the *Np63a* isoform of *Trp63* was found to promote skin tumorigenesis [10]. How can we reconcile the notion that both overexpression of *Np63a* and loss of all *Trp63* isoforms can facilitate skin tumorigenesis? A likely explanation lies in the non-physiological levels of *Np63a* overexpression in the mice used by Devos et al. [10]. These high expression levels led to a severe epidermal and hair follicle phenotype prior to initiating tumorigenesis experiments. In contrast, our *Trp63*-ablated mice did not have an observable skin phenotype (data not shown). Further, the consequences of *Np63a* overexpression on the expression of other TRP63 isoforms were not determined. Future studies aimed at understanding the isoform-specific roles of TP63 are warranted to address the controversy surrounding the role of TP63 in cSCCs and other cancers.

In summary, our study is the first to demonstrate that loss of TP63/TRP63, as observed in human cSCCs, directly facilitates cSCC development and progression, and thus provides novel insights into the function of TP63/TRP63 in skin cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Ablation of *Trp63* from the epidermis promotes tumorigenesis.

(A) Immunostaining with antibodies against TP63 (green) and KRT14 (red) on sections of human cSCCs. Scale bar = 25 mm (B) Schematic illustrating the generation of control and *Trp63*-ablated mice. (C) Graph depicting survival in control and *Trp63*-ablated mice (n = 8/group) (log-rank test, **: P < 0.01). (D) Representative photographs of skin tumors on the back of control and *Trp63*-ablated mice at the end of study. (E) Graph depicting the number of tumors per mouse (Mann-Whitney U test, **: P < 0.01) (n = 8/group). (F) Immunostaining with antibodies against *TRP63* (green) and KRT14 (red) (I), BrdU (green) and KRT14 (red) (II), and KRT13 (green) and DAPI (blue) (III), on well-differentiated cSCCs from control and *Trp63*-ablated mice. Scale bar = 25 mm. (G) Quantification of F (two-tailed unpaired Student t-test, **: P < 0.01; Mean ± SD).

Fig. 2. Reduced TP63 expression leads to the initiation and progression of human cSCCs.

(A) Experimental approach for generating human cSCCs on the back of athymic nude mice. Scale bar = 25 mm. (B) Transformed keratinocytes (HaCaT-HRAS^{V12}) were transduced with a recombinant lentivirus encoding *TP63* shRNAs (TP63 shRNA1–3) or a non-silencing shRNA as a control (NS shRNA), and TP63 levels were analyzed by qRT-PCR, (two-tailed unpaired Student t-test, *: $P < 0.05$; **: $P < 0.01$; Mean $\pm$ SD) and (C) Western blot analysis. Quantification of relative TP63 levels after normalizing to GAPDH is shown at the bottom. (D) Representative photographs of cSCCs on the back of athymic nude mice grafted with either HaCaT-HRAS^{V12}-NS shRNA (Control) or HaCaT-HRAS^{V12}-TP63 shRNA3 (experimental) cells ($n = 6$ /group). Mice were fed with a doxycycline-containing diet (625 mg/kg) for the duration of the study. (E) Immunostaining with antibodies against TP63

(green) and KRT14 (red) (I), BrdU (green) and KRT14 (red) (II), and KRT1 (green) and DAPI (blue) (III), on well-differentiated cSCCs from mice grafted with HaCaT-HRAS^{V12}-NS shRNA and HaCaT-HRAS^{V12}-TP63 shRNA3 cells. (F) Quantification of E (two-tailed unpaired Student t-test, **: $P < 0.01$; Mean $\pm$ SD. (G) Immunohistochemistry for β -catenin on (I) control and *Trp63*-ablated cSCCs and (II) HaCaT-HRAS^{V12}-NS shRNA and HaCaT-HRAS^{V12}-TP63 shRNA3 cSCCs. Scale bar = 25 μ m. (arrows indicate nuclear β -catenin expression) (H) Western blot analysis for TP63/*TRP63* and Axin2 on lysates of control and *Trp63*-ablated cSCCs, and HaCaT-HRAS^{V12}-NS shRNA and HaCaT-HRAS^{V12}-TP63 shRNA3 cSCCs. Quantification of relative TP63/TRP63 and Axin2 levels after normalizing to β -actin is shown at the bottom.