Miz1 Is a Critical Repressor of cdkn1a during Skin Tumorigenesis

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Abstract

The transcription factor Miz1 forms repressive DNA-binding complexes with the Myc, Gfi-1 and Bcl-6 oncoproteins. Known target genes of these complexes encode the cyclin-dependent kinase inhibitors (CKIs) cdkn2b (p15Ink4b), cdkn1a (p21Cip1), and cdkn1c (p57Kip2). Whether Miz1-mediated repression is important for control of cell proliferation in vivo and for tumor formation is unknown. Here we show that deletion of the Miz1 POZ domain, which is critical for Miz1 function, restrains the development of skin tumors in a model of chemically-induced, Ras-dependent tumorigenesis. While the stem cell compartment appears unaffected, interfollicular keratinocytes lacking functional Miz1 exhibit a reduced proliferation and an accelerated differentiation of the epidermis in response to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Tumorigenesis, proliferation and normal differentiation are restored in animals lacking cdkn1a, but not in those lacking cdkn2b. Our data demonstrate that Miz1-mediated attenuation of cell cycle arrest pathways via repression of cdkn1a has a critical role during tumorigenesis in the skin.

Introduction

Miz1 (Myc-interacting zinc finger protein 1; Zbtb17) is a zinc finger transcription factor that forms a complex with the Myc oncoprotein [1]. In addition to 13 zinc fingers that are clustered in the central and C-terminal part of the protein, Miz1 bears a POZ (poxvirus zinc finger protein) domain at the N-terminus [2]. POZ domains are found in a variety of different transcription factors and can confer hetero- or homodimerization as well as tetramerisation [3,4]. The POZ domain of Miz1 forms tetramers [3] and is required for a stable association of Miz1 with chromatin [5,6]. In addition, Miz1 lacking the POZ domain can also interact with other proteins. As a result, the POZ domain is required both for transcriptional activation and repression by Miz1.

Among the proteins which interact with Miz1 independent of the POZ domain is Myc, which binds to Miz1 between the zinc finger domains 12 and 13 [1]. This complex represses the transcription of genes including cdkn2b (encoding p15Ink4b), cdkn1a (encoding p21Cip1), cdkn1c (encoding p57Kip2) and mxd4 (encoding Mad4) [7–10]. In the absence of Myc, Miz1 activates transcription of a number of genes including Bcl-2 [11], clusterin, several integrins and other proteins involved in cell adhesion [12], in a concerted manner with other transcription factors. For example, Miz1 synergizes with the Smad complex during the TGF-β-mediated activation of cdkn2b expression [7,8]. Similarly, increased transcription of cdkn1a depends on Miz1 in response to DNA damage [9] as well as in models of cellular differentiation [13–15]. Miz1 also forms repressive complexes with the Bcl-6 and Gfi-1 oncoproteins. Both complexes are capable of repressing expression of cdkn1a, and, in the case of Gfi-1, also of cdkn2b. These observations suggest that Miz1 functions as a general mediator of repression in association with several transcription factors [13,16,17].

The constitutive knockout of Miz1 is lethal at embryonic day E7.5 [18]. We previously analysed the function of Miz1 in keratinocytes using a conditional Cre/lox knockout model, in which the Cre recombinase is targeted via the keratin 14 promoter to the basal layer of both intra- and interfollicular epidermis [19], where Miz1 is predominantly expressed [12]. In this model, loxP sites flank exons 3 and 4, which encode the POZ domain [2], and deletion of these exons results in expression of a truncated Miz1 protein lacking the POZ domain [20]. Consistent with the biochemical model described above, keratinocytes lacking the Miz1 POZ domain show an attenuated expression of Miz1 target genes in response to TGF-β [20]. Furthermore, animals homozygous for this deletion exhibit an impaired morphogenesis of hair follicles with irregular order and extended length of the follicle, formation of epidermal cysts, delayed catagen during the hair cycle, loss of zig-zag hairs, as well as the occurrence of pigment incontinence in older animals [20].

The model described above suggests that Miz1 has a repressive function in highly proliferative and tumor tissues that express high levels of Myc, which may not be revealed during normal development [21]. Recent work by Trumpp and colleagues demonstrated that endogenous Myc is required for the formation of skin papillomas. The critical function of endogenous Myc in this context is to repress expression of cdkn1a, since deletion of c-myc leads to a loss of tumor formation as well as elevated levels of p21Cip1, and co-deletion of cdkn1a fully restores tumor formation [22]. We now used the conditional Miz1-POZ domain knockout model to test the role of Miz1 in proliferation, differentiation and...
tumorigenesis in keratinocytes. We report here that the deletion of the Miz1 POZ domain leads to increased differentiation and reduced proliferation of keratinocytes when skin is challenged by the tumor promoter agent 12-O-tetradecanoylphorbol-13-acetate (TPA) as well as strongly decreased papilloma formation. These alterations are dependent on an altered regulation of cdkn1a expression. Our findings show that Miz1 is part of a repressor complex that is critical for restraining p21^Cip1 expression in response to stimuli that enhance proliferation and promote skin carcinogenesis.

**Results**

The number of label retaining cells and the distribution of stem cell markers are unaffected in Miz1^ΔPOZ mice

The homozygous deletion of the Miz1 POZ domain in keratinocytes, using a mouse strain that expresses Cre recombinase under the control of the keratin 14 promoter (hereafter called Miz1^ΔPOZ mice; corresponding control animals do not express Cre recombinase; see also Material and Methods), revealed a complex skin phenotype [20]. To assess whether a defect of the stem cell compartment, located at the bulge region of the hair follicle, can account for the observed phenotypes in Miz1^ΔPOZ mice, we visualized label-retaining cells (LRCs) [23] by injecting BrdU on day 10 post partum (P10) and analysed the number and location of LRCs on P24. No significant morphological difference in number and location of BrdU positive cells of the bulge region was detected comparing control and Miz1^ΔPOZ animals (Figure 1 A and B and Figure S1). To test whether enhanced proliferation has an impact on LRCs, we applied 12-O-tetradecanoylphorbol-13-acetate (TPA), a known enhancer of keratinocyte proliferation [24], once per day over five days. Again, no significant difference has an impact on LRCs, we applied 12-O-tetradecanoylphorbol-13-acetate (TPA) and Figure S1). To test whether enhanced proliferation in number and location of BrdU positive cells of the bulge region was observed in control and Miz1^ΔPOZ animals (Figure 1 C and D and Figure S1).

In addition, immunohistochemical stainings for the stem cell markers K15 (Figure 1 E–H) and CD34 (Figure 1 I–L) [25,26] revealed no difference in the number and location of labelled cells between control and Miz1^ΔPOZ animals, irrespective of TPA or control treatment. Our data indicate that the deletion of the Miz1^ΔPOZ domain has little effect on the location, number and proliferation of stem cells in the bulge region of Miz1^ΔPOZ mice.

Alterations of differentiation and proliferation after TPA treatment are dependent on p21^Cip1

Since Miz1, together with Myc, regulates the expression of genes encoding cyclin dependent kinase inhibitors like cdkn2b (encoding p15^Ink4b) or cdkn2a (encoding p21^Cip1), we next asked whether proliferation, differentiation and apoptosis of interfollicular keratinocytes are affected when a functional Miz1 protein is missing. The epidermis of control and Miz1^ΔPOZ mice showed no difference in the expression pattern of the differentiation markers keratin 1 (Figure 2 A and C), loricrin (Figure 2 E and G) or filaggrin (Figure S2F and H). Additionally, the number and location of cells positive for the proliferation marker Ki67 was unaltered (Figure 2 I, K and M). When mice were treated with TPA, the thickness of the epidermis increased as expected (Figure S2A–E), and the expression of the suprabasal differentiation markers keratin 1 and loricrin, but not filaggrin, was undetectable in large areas of the epidermis from control animals (Figure 2 B, F and Figure S2F and G). In contrast, thickening of the epidermis was slightly but significantly reduced in Miz1^ΔPOZ mice under TPA treatment (Figure S2E) and all three markers of differentiation remained prominent throughout the epidermis of Miz1^ΔPOZ mice (Fig. 2 D, H and Figure S2H and I). Furthermore, skin from Miz1^ΔPOZ mice exhibited keratin 1 staining in lower suprabasal cell layers, relative to control animals, where keratin 1 expression was mostly restricted to superficial epidermal cell layers (Figure 2 B and D). We conclude that treatment with TPA delays the differentiation of keratinocytes in control, but not in Miz1^ΔPOZ mice.

Consistent with these observations, application of TPA over five days significantly enhanced the number of Ki67 positive cells in the epidermis of control animals, but to a much lesser extent in the epidermis of Miz1^ΔPOZ animals (Figure 2 J, L and M). In addition, while a considerable number of Ki67 positive cells were located in suprabasal cell layers in control mice, this was not observed in Miz1^ΔPOZ mice (Figure 2 J, L), strongly indicating that the absence of the Miz1 POZ domain prevents cell cycle entry in response to TPA. Taken together, our findings show that a decrease of cell proliferation and an earlier onset of increased differentiation attenuate the effect of TPA in the epidermis of Miz1^ΔPOZ mice.

To genetically test whether one of the Miz1 regulated cyclin dependent kinase inhibitors, p15^Ink4b or p21^Cip1, have a role in restricting proliferation and promoting differentiation of keratinocytes in Miz1^ΔPOZ mice, we generated Miz1^ΔPOZ mice that lack either cdkn2b or cdkn1a. TPA treatment of Miz1^ΔPOZ^ cdkn2b^−/− mice revealed no difference to Miz1^ΔPOZ^ cdkn2b^+/− mice in regard to differentiation and proliferation of interfollicular keratinocytes, indicating that p15^Ink4b is not required for restraining proliferation of Miz1^ΔPOZ keratinocytes (Figure 3E, Figure S3A–D, E). In line with these findings we didn’t observe changes in p15^Ink4b expression by quantitative RT-PCR (data not shown). In contrast, keratinocyte proliferation was induced by TPA to the same extent in Miz1^ΔPOZ^ cdkn1a^−/− animals as in control animals (Figure 3A–D and F, Figure S3F). In addition, the extended focal ablation of differentiation markers that was observed in control animals also occurred in Miz1^ΔPOZ^ cdkn1a^−/− mice, in contrast to Miz1^ΔPOZ mice (Figure S4). These genetic data show that the impact of Miz1 on keratinocyte proliferation and differentiation depends on p21^Cip1.

To determine the biochemical basis of these observations, we analysed p21^Cip1 expression by immunoblot analysis of skin from control and knockout animals with a cdkn1a^−/+ background (Fig. 3I). Without TPA treatment, expression of p21^Cip1 was below the limit of detection in the skin from control animals but gave a clear signal in skin from Miz1^ΔPOZ animals. TPA treatment induced the expression of p21^Cip1 in control animals and led to a further increase in p21^Cip1 expression in Miz1^ΔPOZ mice. Under both conditions p21^Cip1 expression was increased in Miz1^ΔPOZ animals compared to control animals, demonstrating directly that the Miz1 POZ domain restrains expression of p21^Cip1 in vivo.

To rule out the possibility that the increased p21^Cip1 expression was an indirect effect of an altered signal transduction in Miz1^ΔPOZ animals, we first analysed Myc levels and found by immunoblot analysis of skin from control animals with a cdkn1a^−/+ background (Fig. 3I). Without TPA treatment, expression of p21^Cip1 was below the limit of detection in the skin from control animals but gave a clear signal in skin from Miz1^ΔPOZ animals. TPA treatment induced the expression of p21^Cip1 in control animals and led to a further increase in p21^Cip1 expression in Miz1^ΔPOZ mice. Under both conditions p21^Cip1 expression was increased in Miz1^ΔPOZ animals compared to control animals, demonstrating directly that the Miz1 POZ domain restrains expression of p21^Cip1 in vivo.

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To confirm that *cdkn1a* is a direct target gene of Miz1 and Myc in keratinocytes, we performed chromatin immunoprecipitation (ChIP) assays with antibodies directed against Miz1 and Myc, respectively. Since the isolation of primary keratinocytes takes several hours under harsh conditions and since it is almost impossible to obtain sufficient primary keratinocytes for efficient chromatin isolation, we used the murine keratinocyte cell line PAM212 [27], which responds to TPA similarly as primary keratinocytes [28]. Using chromatin isolated from these cells, ChIP assays revealed that both Miz1 and Myc were bound to the core promoter region of *cdkn1a*, but not to a control region located about 20 Mb downstream of *cdkn1a*. The binding of Miz1 to the *cdkn1a* promoter was not altered under TPA treatment (Figure 3G and H).

Taken together our data show that (a) *cdkn1a* is a direct target gene of Myc and Miz1 in murine keratinocytes, (b) that the POZ domain of Miz1 is critical for repressing p21Cip1 expression in vivo and (c) that elevated levels of p21Cip1 restrain TPA-stimulated keratinocyte proliferation in *Miz1* DPOZ mice.

Reduced skin tumorigenesis in Miz1ΔPOZ mice

The reduced proliferation response to TPA treatment in the skin of *Miz1ΔPOZ* animals led us to ask whether Miz1 plays a role in skin tumorigenesis. We applied the well-established two-stage skin carcinogenesis protocol using 7,2-dimethylbenz(a)anthracene (DMBA) as tumor initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as tumor promoter [29]. Tumors initially emerged between weeks 8 and 9 of the TPA treatment both in control (see Materials and Methods) and in *Miz1ΔPOZ* animals, indicating that the principal time course of tumor development is similar in both mouse strains (Figure 4A). However, whereas 50% of the control animals developed tumors between weeks 10 and 11 of TPA treatment (n = 23), it took 15–16 weeks until 50% of the *Miz1ΔPOZ* animals exhibited tumors (Figure 4A; n = 26; p<0.001). To exclude that the tumors observed in *Miz1ΔPOZ* mice developed from keratinocytes that have escaped Cre recombination, we isolated DNA from tumor samples and genotyped them by PCR. In all 45 tumors tested we could confirm efficient Cre-mediated recombination (Figure S6I). Since skin papillomas in this animal model are usually monoclonal [29,30], a recombinant band indicates that the tumor has descended from a recombined keratinocyte. The non-recombined bands almost certainly come from cells of epidermal (melanocytes, dendritic cells) and/or dermal (fibroblasts, dendritic cells and many others) origin, in which the Cre recombinase is not active. This indicates that the tumors have not grown from escaper clones, but from cells lacking the Miz1 POZ domain. The gross morphology of tumors of comparable size from control and *Miz1ΔPOZ* animals was identical. No difference in the pattern of outfoldings was observed. In both genotypes, the thicknesses of the epidermis and of the cornified layer, and the amount of keratohyalin granules were increased compared to the interfollicular epidermis. Finally, no spread of epidermal cells into the dermal compartment occurred (Figure S6A–H).

The decreased tumorigenesis in *Miz1ΔPOZ* mice was further reflected by a reduced tumor burden per mouse, since the number of tumors was significantly lower in *Miz1ΔPOZ* compared to control animals (*Miz1ΔPOZ*; n = 3.38±4.30 tumors per mouse measured in 26 mice; control: n = 8.35±5.16 tumors per mouse measured in 23 mice; p<0.001; Figure 4B). Furthermore, tumors at the end of TPA treatment were significantly smaller in *Miz1ΔPOZ* mice than in control mice (1.94±1.64 mm vs 2.93±1.73 mm; Figure 4E and H). To exclude that the reduced tumor size is caused by increased apoptosis, we performed a...
Figure 2. Epidermal differentiation and proliferation is altered upon TPA treatment. Immunohistochemical staining revealed no difference in the expression of the differentiation markers keratin 1 or loricin (A, C and E, G) in the epidermis of untreated control (Ctrl) or Miz1ΔPOZ mice. When Ctrl animals were treated with TPA, focal areas were observed lacking these differentiation markers (B, F). In contrast, such foci did not
occur in the skin of Miz1ΔPOZ mice (D, H). Immunohistochemistry for the proliferation marker Ki67 revealed positive cells in the basal cell layer of untreated skin in both genotypes (I, K) and the labelling index was not significantly different (M, −TPA; n = 5 for each genotype). After TPA treatment, the Ki67 labelling index in Ctrl animals was about twice as high as in Miz1ΔPOZ animals (M, +TPA; n = 5 for each genotype; Ctrl vs Miz1ΔPOZ for ± TPA: p = 0.0001). In addition, Ki67 positive cells were scattered through the suprabasal cell layers of the epidermis in Ctrl but not in Miz1ΔPOZ animals (J, L).

TUNEL assay. TUNEL positive cells were rarely found in the tumors of both genotypes and were almost absent in the interfollicular epidermis (Figure S7A), indicating that the tumor size in Miz1ΔPOZ mice is not affected by increased programed cell death. Finally, when TPA treatment was finished after 20 weeks and mice were subsequently observed for further 17 weeks, tumor diameter increased about threefold in control animals but remained constant in Miz1ΔPOZ mice (Figure S7B and C). We conclude that tumor development and growth is strongly reduced in Miz1ΔPOZ mice.

Strikingly, immunohistochemistry of papillomas revealed low levels of p21<sup>wt</sup> in keratinocytes from 19 out of 21 tumors from control animals, but high p21<sup>WT</sup> levels in keratinocytes from 14 out of 15 tumors of Miz1ΔPOZ mice (Figure 4F and G; Figure S8A). Additionally, increase of p21<sup>WT</sup> in papillomas from Miz1ΔPOZ mice was observed by immunoblot analysis (Figure S8B). To test the impact of p21<sup>WT</sup> genetically, we monitored tumor development in a cohort of Miz1ΔPOZ<sup>cdkn1a<sup>-/-</sup></sup> mice. In these experiments, we noted that the tumor burden per mouse in cdkn1a<sup>-/-</sup> control animals (Figure 4D) was lower than in cdkn1a<sup>+/-</sup> control animals (Figure 4B), most likely due to subtle differences in the overall genetic background of the animals used in the two experiments (see Materials and Methods) or possibly to a general lower tumor incidence in p21<sup>WT</sup> deficient animals [31,32]. Importantly, Miz1ΔPOZ<sup>cdkn1a<sup>-/-</sup></sup> animals developed tumors with a time course that was indistinguishable from control cdkn1a<sup>-/-</sup> animals (Figure 4C; p = 0.6993). Furthermore, the difference of tumor burden between cdkn1a<sup>-/-</sup>-Miz1ΔPOZ mice and their corresponding control mice was smaller (5.04±7.03 vs 6.27±5.74 tumors per mouse, measured in 22 control and 26 cdkn1a<sup>-/-</sup>-Miz1ΔPOZ mice; p=0.5139) than in an cdkn1a<sup>-/-</sup> background (8.35 vs 3.38 tumors per mouse, see above). Finally, there was no difference in the tumor size between control and Miz1ΔPOZ mice in a cdkn1a<sup>-/-</sup> background (Figure 4D), in contrast to control and Miz1ΔPOZ mice with a cdkn1a<sup>+/+</sup> background (Figure 4H). Taken together, we conclude that the reduced tumorigenicity observed in Miz1ΔPOZ mice depends on the upregulation of p21<sup>CIP1</sup> expression.

Discussion

One well characterized function of Miz1 is the regulation of the cyclin dependent kinase inhibitor genes <i>cdha2b</i> (encoding p15<sup>ink4b</sup>), <i>cdkn1a</i> (encoding p21<sup>CIP1</sup>), and <i>cdkn1c</i> (encoding p57<sup>kip2</sup>) [33], although a number of other genes are now known to be expressed in a Miz1-dependent manner [6,11,12]. The current model proposes that Miz1, complexed with nucleoplasmin, binds to the core promoter of its target genes to enhance gene expression. Transactivation is blocked when the Myc/Max complex binds to Miz1, thereby releasing nucleoplasmin [34,35]. In this model, Miz1 has a dual role in expression of its target genes. In the absence of Myc, Miz1 contributes to target gene activation. However, Miz1 also serves to recruit Myc leading to the formation of a repressive complex. This suggests that abrogation of Miz1 function will enhance target gene expression in conditions of high Myc expression. The allele of Miz1 used here lacks the POZ domain, which is necessary both for the transactivating properties of Miz1 [1,9] and for its stable association with chromatin [6,21]. We observed fewer and smaller tumors in Miz1ΔPOZ animals. A detailed analysis revealed that a reduced proliferation of keratinocytes in response to the tumor promoter, TPA, can account for the reduction of tumor growth in these animals, while changes in the stem cell compartment could not be uncovered. Interestingly, proliferation was completely restored in a <i>cdkn1a</i> null background, strongly suggesting that the increase in p21<sup>CIP1</sup> expression that is observed in Miz1ΔPOZ mice is responsible for the observed phenotype. This notion is further supported 1) by an immunoblot analysis revealing elevated p21<sup>CIP1</sup> levels in the skin from Miz1ΔPOZ mice compared to their wildtype counterparts (Fig. 3I), 2) by immunohistochemical data showing that p21<sup>CIP1</sup> is consistently expressed in tumors of Miz1ΔPOZ mice, but is hardly detectable in control tumors (Fig. 4F, G) and 3) by the genetic experiment exhibiting a rescue of the reduced proliferation reflected by Ki67 positive cells (Figure 3A-D and F), a rescue of the tumor development (Figure 4C) and partial rescue of the tumor burden (Figure 4D) in Miz1ΔPOZ mice on a <i>cdkn1a</i> null background. In contrast, Miz1ΔPOZ mice with a <i>cdkn2b</i> null background exhibited only a small and statistically insignificant increase in Ki67 positive cells (Figure 3E; Figure S3A-E), suggesting that upregulation of p15<sup>ink4b</sup> is not involved in restraining proliferation of Miz1ΔPOZ keratinocytes in response to TPA treatment.

While our data show that Miz1 has a critical role in repressing <i>cdkn1a</i> expression during skin carcinogenesis, they do not directly address the mechanism by which Miz1 acts in this system. For example, Miz1 has been suggested to associate with p53 [36]. While deletion of the POZ domain does not affect levels of p53 [Figure S5A], it is possible that it enhances p53 function in more subtle ways, leading to enhanced p21<sup>CIP1</sup> expression. Furthermore, we do not know which of the several oncoproteins that can repress transcription via Miz1 (see Introduction) are functional during skin carcinogenesis. However, our data can account for the results obtained using tamoxifen inducible c-myc<sup>-/-</sup> mice [22]. In these mice, DMBA/TPA treatment led to elevated expression of p21<sup>CIP1</sup> and skin tumors could only be induced in the absence of p21<sup>CIP1</sup>, demonstrating that endogenous Myc has a critical function in repressing <i>cdkn1a</i> during skin tumor development. It should be noted, that a related model in which c-myc is deleted during development by a constitutively active Cre recombinase expressed under the keratin 5 promoter shows more severe phenotypes, suggesting that Myc has functions in addition to repressing p21<sup>CIP1</sup> during skin development [37].

Our data extend observations on Myc-induced lymphomagenesis in mice expressing a mutant allele of Myc that is selectively deficient in binding to Miz1 (MycV349D). Mice expressing this mutant display a reduced tumorigenesis, at least in part because binding of Myc to Miz1 is required to restrain expression of p15<sup>ink4b</sup> and of p57<sup>kip2</sup> in the lymphomas [38]. Importantly, lymphomas arising in these mice showed an accumulation of senescent cells, suggesting that binding of Myc to Miz1 may be required to antagonize senescence during tumorigenesis.

p21<sup>CIP1</sup> is a key player during the induction of senescence of human fibroblasts [39], keratinocytes [40], melanocytes [41] and mammary epithelial cells [42]. While we did not detect senescent cells in tumors that arose in either wild type or Miz1ΔPOZ
animals, a fraction of interfollicular and follicular keratinocytes and approximately 25% of the hair follicles stain positive for the senescence marker SA-ß-galactosidase in aged skin of Miz1ΔPOZ mice but not of control animals (Figure S9; [43]). We suggest, therefore, that repression via Miz1 may be more broadly involved in senescence suppression and the reduced tumorigenesis in Miz1ΔPOZ mice may reflect the need to overcome p21Cip1 mediated senescence during tumor formation [44,45]. While this remains to be formally demonstrated, the current data strongly support the view that the formation of a functional Miz1/Myc complex results be formally demonstrated, the current data strongly support the view that the formation of a functional Miz1/Myc complex results in p16 inactivation, while p21 remains essentially unchanged.

Materials and Methods

Transgenic mice

Miz1flox/flox mice [20] were crossed with K14Cre mice [19] to generate a conditional knockout of the POZ domain of Miz1 in murine basal epidermal cells as described elsewhere [20]. Mice were backcrossed 6 times on a 129S2/SvHsd background. Here, mice which are K14cre;Miz1flox/flox are designated Miz1ΔPOZ mice, while K14Cre_Miz1ΔPOZ mice were used as control animals designated Ctr. Miz1ΔPOZ mice were crossed on a cdkn2b−/− background (compare with Figure 2 M; one representative experiment of three independent experiments each). The experiment demonstrates that both transcription factors bind to the cdkn1a promoter in this cell type. Miz1 binds to the cdkn1a promoter in PAM212 keratinocytes, without and with TPA treatment (100 nM for 4 hours). Primers used either amplified genomic DNA comprising part of the cdkn1a promoter (p21) or a cdkn1a unrelated sequence of chromosome 17 (ctr). For details see Materials and Methods. The graphs show the mean value of 2–3 technical replicas.

Immunoblot analysis

Protein samples were extracted from dorsal skin and homogenized in RIPA buffer, containing 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; 150 mM NaCl; 10 mM Na2HPO4; 2 mM EDTA; 1% Apronitin; 50 mM NaF; 200 mM Na3VO4; pH 7.4. As a positive control for p21cip1, extracts from sciatic nerves were used [48]. Protein concentration was determined using the BCA-assay (Sigma). 20 μg per sample were separated on 10% polyacrylamide-SDS gels according to standard procedures. Proteins were blotted on nitrocellulose membranes and the blots were stained with antibodies against the following proteins: c-Myc (N-262, Santa Cruz; 1:400), p21 (C-19, Santa Cruz; 1:100), p-ERK (T202/Y204, Cell Signalling; 1:200), p53 (FL-393, Santa Cruz; 1:200), tubulin (YL1/2, Abcam; 1:2000) at 4°C overnight or for 72 hours in case of p53. Appropriate secondary peroxidase directed against c-Myc (N-262; Santa Cruz) and Miz1 (10E2, Staller et al. 2001). For the isotype control, IgG from mouse and rabbit serum (Sigma) was used. After the crosslink reversion, the chromatin was purified with the QIAquick PCR Purification Kit (QIAGEN). The promoter binding was detected by qPCR on a Mx3005p PCR machine (Stratagene/Agilent) with a QPCR SYBR Green (Thermo Scientific), using CTCAGCTCTAATCTGTTGTTCA as forward and CTGGGCTATTCTC-TACATTTAACCAACTATCAGAGCA as reverse primer, to detect the cdkn1a promoter sequence by amplifying genomic DNA between basepairs 29.230.454–29.230.529 of chromosome 17. Control primers were TACATTTAACCAACTATCAGAGCA as reverse primer, to detect the cdkn1a promoter sequence by amplifying genomic DNA between basepairs 29.230.454–29.230.529 of chromosome 17. Control primers were TACATTTAACCAACTATCAGAGCA as reverse primer, to detect the cdkn1a promoter sequence by amplifying genomic DNA between basepairs 29.230.454–29.230.529 of chromosome 17. Control primers were TACATTTAACCAACTATCAGAGCA as reverse primer, to detect the cdkn1a promoter sequence by amplifying genomic DNA between basepairs 29.230.454–29.230.529 of chromosome 17. Control primers were TACATTTAACCAACTATCAGAGCA as reverse primer, to detect the cdkn1a promoter sequence by amplifying genomic DNA between basepairs 29.230.454–29.230.529 of chromosome 17.

Histology

Skin samples were fixed in PBS buffered 3.7% formaldehyde and embedded in paraffin according to standard procedures. For immunohistochemistry, 3 μm sections were applied on silane-coated slides, preincubated with 10% goat serum (Sigma) and, if
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**Figure A**
- Graph showing the percentage of tumor-free mice over weeks of TPA promotion. Lines represent Ctr and Miz1ΔPOZ.

**Figure B**
- Graph showing tumors per mouse over weeks of TPA promotion. Lines represent Ctr and Miz1ΔPOZ.

**Figure C**
- Graph showing the percentage of tumor-free mice over weeks of TPA promotion for cdkn1a +/-. Lines represent Ctr and Miz1ΔPOZ.

**Figure D**
- Graph showing tumors per mouse over weeks of TPA promotion for cdkn1a +/-. Lines represent Ctr and Miz1ΔPOZ.

**Figure E**
- Images of mice showing Ctr and Miz1ΔPOZ groups.

**Figure F**
- Image of tumor tissue with p21 expression. 19 of 21 tumors.

**Figure G**
- Image of tumor tissue with p21 expression. 14 of 15 tumors.

**Figure H**
- Scatter plot showing tumor size in Ctr and Miz1ΔPOZ for cdkn1a +/-.

**Figure I**
- Scatter plot showing tumor size in Ctr and Miz1ΔPOZ for cdkn1a -/-.
necessary, further treated as outlined below. For Ki67, p21\(^{Cip1}\) and p-ERK staining, slides were microwaved in 10 mM citrate buffer pH 6 for 3×5 min. For bromodesoxyuridine (BrdU) staining, slides were incubated for 30 min in 2 N HCl/0.5% Triton X-100, for 3 min in borax buffer (0.5 M sodium diborate/0.5 M boric acid, pH 7.6) at RT and for 3 min in 0.025% trypsin in 0.05 M Tris/HCl, pH = 7.4. Primary antibodies were diluted in 10% goat serum (Dako) and incubated at 4°C overnight. Antibodies against the following antigens were used: Ki67 (Dako; 1:50), BrdU-Dianova; 1:100), p-ERK (T202/Y204, Cell Signaling; 1:100), CD34 (BD; 1:100), K15 (Abcam; 1:100), p21\(^{Cip1}\) (Abcam; 1:100), keratin 1 (Covance; 1:1000), loricrin (Covance; 1:1000). For visualization, appropriate secondary antibodies labelled either with FITC, TRITC (Molecular Probes) or with peroxidase were used. Slides were incubated 1 hour at room temperature and were subsequently covered with Mowiol. For documentation, a motorized BX61 microscope (Olympus) equipped with a F-View digital camera was used (Soft Imaging System, Münster, Germany).

The TUNEL assay was performed using the DeadEnd kit (Promega) according to manufacturer instructions. The staining for SA-β-galactosidase was performed as described by Dimri et al. [43]. To ascertain the percentage of positive hair follicles, about 100 follicles per sample were counted.

**Morphometric analysis**

The number of Ki67 positive cells per mm of skin and the ratio of suprabasal Ki67 positive cells were measured using the program Cell \(^{1}\) (Olympus). From 3 to 5 mice per condition (control, Miz1\(^{ΔPOZ}\), Miz1\(^{ΔPOZ}\)/cdkn1a\(^{f/f}\) and Miz1\(^{ΔPOZ}\)/cdkn2b\(^{f/f}\); TPA-treated and untreated) 15–25 pictures were taken. In each picture, the length of the epidermis was measured and the related Ki67 positive basal and suprabasal cells were counted. The amount of all Ki67 positive cells per mm of skin was calculated. In addition, the ratio of basal to superbasal Ki67 positive cells was determined.

**Statistical analysis**

Mean values and standard deviations of the morphometric and ChIP data were calculated with Excel (Microsoft). The statistical significance of the morphometric data, the Kaplan-Meier estimator and the average number of tumors per mouse was calculated using the Student’s t-test as implemented in the program GraphPad Prism (GraphPad Software).

**Supporting Information**

**Figure S1** Label-retaining cells (LRCs) in the bulge region. (A) Documentation of LRC number variability in bulge regions from ctr and Miz1\(^{ΔPOZ}\) animals, without and with TPA treatment. In (B), the percentage of LRCs (% BrdU positive cells) counted in the bulge region area are shown. 19 to 25 bulge regions per condition were evaluated for BrdU positive cells.

**Figure S2** TPA treated control and Miz1\(^{ΔPOZ}\) epidermis. HE-staining of control (A, B) and Miz1\(^{ΔPOZ}\) epidermis (C, D) under TPA treatment (B, D) or in untreated skin (A, C). The size of scale bar in A is 50 μm. The average epidermal thickness of TPA treated and untreated control and Miz1\(^{ΔPOZ}\) epidermis is shown in E. 100 single measurements per animal were done with 3 animals per condition. Fluorescence staining of filaggrin in control (F, G) and Miz1\(^{ΔPOZ}\) (H, I) skin with and without TPA treatment (+/−TPA). Filaggrin is equally expressed in Ctr and Miz1\(^{ΔPOZ}\) suprabasal epidermis, either with or without TPA treatment. Percentage of suprabasal Ki67 positive keratinocytes in untreated and TPA treated Ctr and Miz1\(^{ΔPOZ}\) skin (J). (TIF)

**Figure S3** TPA treated control and Miz1\(^{ΔPOZ}\) epidermis with a cdkn2b\(^{−/−}\) background. Fluorescence staining of Ki67 in Ctr (A, B) and Miz1\(^{ΔPOZ}\) (C, D) skin with a cdkn2b (encoding p15\(^{INK4b}\)) deficient background with and without TPA treatment (+/−TPA). The additional deletion of cdkn2b does not rescue the reduced proliferation in TPA treated Miz1\(^{ΔPOZ}\) skin compared to TPA treated Ctr skin. Quantification of suprabasal Ki67 positive keratinocytes in untreated and TPA treated Ctr and Miz1\(^{ΔPOZ}\) skin with either a p15\(^{INK4b}\) (E) or a p21\(^{Cip1}\) (F) deficient background. Under TPA treatment, suprabasal Ki67 positive cells are significantly reduced in Miz1\(^{ΔPOZ}\) skin compared to Ctr skin in mice with a cdkn2b\(^{−/−}\) background (E; p<0.0001), as observed in cdkn2b\(^{−/+}\) animals (compare with Figure S2 J). In contrast, a complete rescue was achieved in cdkn1a\(^{−/−}\) animals where no difference of Ki67 Miz1 suprabasal cells was observed between control and Miz1\(^{ΔPOZ}\) mice (F; p=0.9316). (TIF)

**Figure S4** Differentiation in cdkn2b or cdkn1a deficient Miz1\(^{ΔPOZ}\) epidermis. Fluorescence staining of keratin 1 (A–H) and loricrin (I–P) in control and Miz1\(^{ΔPOZ}\) skin with and without TPA treatment (+/−TPA) either with a cdkn2b (A-D) and I-L) or cdkn1a (E-H and M-P) deficient background. In cdkn2b\(^{−/−}\) mice and upon TPA treatment, keratin 1 expression is focally interrupted in Ctr skin while Miz1\(^{ΔPOZ}\) skin shows continuous keratin 1 expression (B, D). With a cdkn1a deficient background, Ctr and Miz1\(^{ΔPOZ}\) skin both show a focal interruption of keratin 1 expression after TPA (F, H). Also, with a cdkn2b\(^{−/−}\) background, loricrin expression is focally reduced in Ctr skin (J) but not in Miz1\(^{ΔPOZ}\) skin (L), while in cdkn1a\(^{−/−}\) skin, focal reduction of loricrin expression can be observed in both Ctr (N) and Miz1\(^{ΔPOZ}\) skin (P). The described expression patterns of keratin 1 and loricrin only occurred in TPA treated skin, whereas untreated skin did not show differences between Ctr and Miz1\(^{ΔPOZ}\) animals in regard to keratin 1 and loricrin expression, neither with a cdkn2b (A, C, I, K), nor with a cdkn1a deficient background (E, G, M, O). (TIF)

**Figure S5** ERK-phosphorylation, c-Myc and p53 expression in Miz1\(^{ΔPOZ}\) epidermis. Immunoblot of phosphorylated...
ERK (p-ERK), c-Myc and p53 in extracts of murine Control (Ctr) and Miz1ΔPOZ skin (A), untreated or treated with TPA (−/−TPA). α-tubulin was used as a loading control. The expression of p-ERK was also visualized in murine epidermis via immunohistochemistry in control (B, C) and Miz1ΔPOZ samples (D, E) both untreated (B, D) or TPA treated (C, E). Furthermore, p53 stained by immunohistochemistry in tumors did not reveal a difference between control (Ctr) and Miz1ΔPOZ papillomas (F, G), in contrast to p21\(^{\text{ip}}\) expression (see Figure 4F, G and Figure S8).

**Figure S6** Histology and genotyping of Miz1ΔPOZ papillomas. HE-staining of control (A, C, D, G) and control (ctr) and Ctr (TIF) animals. Animals 1–11 are Miz1ΔPOZ animals with a floxed Miz1 allele which express Crc recombinase expression was genotyped as a negative control. Animals 1–11 are Miz1ΔPOZ animals with a floxed Miz1 allele which express Crc recombinase. The lower band at 180 bp indicates the recombinant allele, while the upper band at 311 bp indicates the floxed allele. A floxed allele can also be detected in tumors from Miz1ΔPOZ animals due to the presence in the samples of other (non-keratinocyte) epidermal and dermal cell types that do not express Crc recombinase.

**Figure S7** Apoptosis and tumor growth in tissue from control (ctr) and Miz1ΔPOZ animals. (A) While there were essentially no TUNEL positive cells in the interfollicular skin, we occasionally observed TUNEL positive cells in tumors independent of the genotype, although most tumors from both genotypes lacked TUNEL positive cells. As a positive control for the assay we used either skin fixed in Carnoy’s solution, where most nuclei should be positive because of an acidic hydrolysis of the DNA (due to the acetic acid which is a component of this fixative) or thymus which usually exhibits a large number of apoptotic T-cells, predominately in the cortex. (B) Tumor development during 17 weeks after the last TPA treatment. Representative pictures of control (Ctr) and Miz1ΔPOZ papillomas 17 weeks after the last TPA treatment (B). Measurement of the tumor diameter (C) revealed an increased tumor-size in Ctr animals but not in Miz1ΔPOZ mouse (compare with Figure 4H).

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**Figure S8** p21-expression in Miz1ΔPOZ papillomas. Immunohistochemistry of control (Ctr) and Miz1ΔPOZ papillomas showing the expression of p21\(^{\text{ip}}\) (A). Each slide indicates a representative region of an individual tumor. In the majority of Miz1ΔPOZ papillomas, p21\(^{\text{ip}}\) was upregulated, whereas in most Ctr tumors p21\(^{\text{ip}}\) expression was not detectable. Expression of p21 protein in papillomas was also analyzed by immunoblot (B) in each of three (1–3) Ctr and Miz1ΔPOZ papillomas with p21\(^{\text{ip}}\). Ctr and Miz1ΔPOZ tumor samples with a p21\(^{\text{ip}}\)-background are negative controls. All Miz1ΔPOZ-tumors have an increased p21 expression compared to Ctr tumors, while in p21\(^{\text{ip}}\)-tumors, no p21 expression was detectable.

**Figure S9** Tumors of Miz1ΔPOZ mice are not positive for SA-β-galactosidase. Tumors from control (Ctr) (A) and Miz1ΔPOZ (B) animals after 20 weeks of TPA treatment were histochemically stained for SA-β-galactosidase, but were not positive independent of the genotype. In contrast, skin from one year old Miz1ΔPOZ mice displayed a focal staining which was absent in Ctr animals (C, D). In addition, about 25% of hair follicles stained positive for SA-β-galactosidase in Miz1ΔPOZ but not in Ctr animals (E). Arrowheads indicate sebaceous glands, which stain always positive for SA-β-galactosidase.

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**Author Contributions**

Conceived and designed the experiments: JH ME HPE. Performed the experiments: JH ASM EW. Analyzed the data: JH ME HPE ASW. Contributed reagents/materials/analysis tools: ME HPE. Wrote the paper: HPE ME.
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