Skin epidermis lacking the c-myc gene is resistant to Ras-driven tumorigenesis but can reacquire sensitivity upon additional loss of the p21<sup>Cip1</sup> gene

Thordur Oskarsson,1 Marieke Alida Gertruda Essers,1 Nicole Dubois,1 Sandra Offner,1 Christelle Dubey,1 Catherine Roger,1 Daniel Metzger,2 Pierre Chambon,3 Edith Hummler,4 Peter Beard,1 and Andreas Trumpp1,2,5

1Genetics and Stem Cell Laboratory, Swiss Institute for Experimental Cancer Research (ISREC) Ch. des Boveresses 155, 1066 Epalinges, Switzerland; 2Ecole Polytechnique Federale de Lausanne (EPFL), School of Life Sciences, 1015 Lausanne, Switzerland; 3Institut de Genetique et de Biologie Moleculaire et Cellulaire and Institut Clinique de la Souris, 67404 Illkirch, France; 4Department de Pharmacologie et Moléculaire et Cellulaire and Institut Clinique de la Souris, 67404 Illkirch, France; 5Corresponding author.

The target gene(s) required for Myc-mediated tumorigenesis are still elusive. Here we show that while endogenous c-Myc is surprisingly dispensable for skin homeostasis and TPA-induced hyperplasia, c-Myc-deficient epidermis is resistant to Ras-mediated DMBA/TPA-induced tumorigenesis. This is mechanistically linked to p21<sup>Cip1</sup>, which is induced in tumors by the activated Ras-ERK pathway but repressed by c-Myc. Acute elimination of c-Myc in established tumors leads to the up-regulation of p21<sup>Cip1</sup>, and epidermis lacking both p21<sup>Cip1</sup> and c-Myc reacquires normal sensitivity to DMBA/TPA-induced tumorigenesis. This identifies c-Myc-mediated repression of p21<sup>Cip1</sup> as a key step for Ras-driven epidermal tumorigenesis.

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The bHLH-LZ family members c-Myc and Max form heterodimers that either activate or repress two different sets of target genes. c-Myc has been suggested to have a role in controlling various cellular processes including cell division, cellular growth, stem cell differentiation, apoptosis/survival, angiogenesis, and immortalization [for review, see Grandori et al. 2000; Adhikary and Eilers 2005; Murphy et al. 2005]. Its in vivo function has recently been examined by genetic loss-of-function studies in mice, which show that hematopoietic progenitor cells, but not stem cells, require c-Myc to maintain cellular proliferation. In addition, c-Myc is required for development of the embryonic hematopoietic system and establishment of the intestinal crypts in young mice. In contrast, c-Myc is dispensable for maintenance of the adult intestinal mucosa [de Alboran et al. 2001; Trumpp et al. 2001; Wilson et al. 2004; Bettes et al. 2005; Murphy et al. 2005; Wilson and Trumpp 2006; N. Dubois and A. Trumpp, unpubl.].

In the mammalian skin epidermis, c-Myc is expressed in the basal layer and the hair bulb, as well as in the bulge region correlating with the proliferative and stem cell-containing portion of the skin epidermis [Hurlin et al. 1995; Bull et al. 2001]. Ectopic overexpression of c-Myc using the involucrin or keratin-5 promoter leads to hyperplasia and eventual tumorigenesis including invasive squamous cell carcinomas (SCCs) [Pelengaris et al. 1999; Rounbehler et al. 2001].

However, overexpression of c-Myc using the keratin-14 promoter results in an initial phase of hyperplasia but ultimately leads to a keratinocyte-migration defect, increased size of sebaceous glands, and depletion of label-retaining cells accompanied by partial epidermal loss in areas of mechanical stress. This phenotype is thought to be caused by promotion of epidermal stem cells to differentiate into interfollicular epidermis and sebaceous glands at the expense of hair-follicle differentiation and stem-cell maintenance [Arnold and Watt 2001; Waikel et al. 2001; Murphy et al. 2005].

On its own, c-Myc is unable to transform normal cultured cells. However, c-Myc has been shown to cooperate with the Ras oncoprotein to transform immortalized cells [Land et al. 1983]. The nature of this cooperation is not well understood, but c-Myc is thought to counteract cell-cycle arrest induced by ectopic Ras expression in primary cells. Conversely, Ras has been shown to stabilize the labile c-Myc protein [Sears 2004] and is thought to inhibit c-Myc-induced apoptosis through activation of the PI3-kinase pathway [Kaufmann-Zeh et al. 1997]. In this study we demonstrate that c-Myc is essential for cell cycle progression of cultured fibroblasts and keratinocytes but, in contrast, is dispensable for epidermal homeostasis in vivo. Most importantly, we show that c-Myc is required for H-Ras-induced epidermal tumorigenesis and that this is mechanistically linked to the activity of c-Myc to repress the CDK inhibitor p21<sup>Cip1</sup>.

Results and Discussion

To study whether the role of c-Myc is similar in cultured fibroblastic and epithelial cell types, we prepared 3T9 fibroblasts and normal keratinocytes from mice homozygous for the conditional c-myc<sup>flox</sup> allele [Trumpp et al. 2001]. Inducible conversion of the c-myc<sup>flox</sup>-null allele into the c-myc<sup>AOR</sup>-null allele [Fig. 1A] was achieved via an inducible MSCV-based retroviral system carrying a 4-hydroxy-tamoxifen (4OH)-inducible Cre recombinase [Supplemental Fig. [S] 1; Indra et al. 1999]. Addition of 4OH to the medium of cells carrying this construct efficiently leads to the disappearance of detectable c-Myc protein in 3T9 fibroblasts and normal keratinocytes within 24 h [Figs. S2, S3]. Upon loss of c-Myc expression,
both cell types ceased proliferation, accumulated in a Ki67\(^{+}\) (GO) stage, and acquired a flat-cell phenotype with an altered F-actin distribution [Figs. S2, S3]. In addition, 96 h post-c-myc deletion, keratinocytes turn on the senescence-associated β-galactosidase [SA-β-gal] activity [Dimri et al. 1995], indicating that c-Myc activity is essential to prevent premature senescence in these cells [Fig. S4]. In summary, these data show that similar to what we and others have suggested for cultured fibroblasts [de Alboran et al. 2001; Holzel et al. 2001; Trumpf et al. 2001; Guney et al. 2006; Prathapam et al. 2006], c-Myc activity in keratinocytes is essential for inhibiting premature senescence and maintaining cells in a productive cell cycle.

To address whether c-Myc is required for keratinocyte proliferation in vivo, we used the K5CreER\(^T\) mouse strain to conditionally eliminate the c-myc\(^{\text{loxp}}\) gene in the basal layer of the epidermis, hair follicle outer root sheath, sebaceous glands, and the hair follicle bulge region [Indra et al. 1999]. After weaning, K5CreER\(^T\), c-myc\(^{\text{loxp/lox}}\) or K5CreER\(^T\), c-myc\(^{\text{SOPR/lox}}\) mice [mutant] and c-myc\(^{\text{loxp/lox}}\), c-myc\(^{\text{SOPR/lox}}\) or K5CreER\(^T\), c-myc\(^{\text{loxp/lox}}\) mice [control] were injected with tamoxifen for 5 consecutive days. Assessment of the recombination efficiency in the epidermis was determined by Southern blot analysis [Fig. 1B] and real-time Taqman PCR, and revealed 81.3% ± 11% recombination at 3 wk and 88.3% ± 3% 20 wk after tamoxifen induction, suggesting that no selection against c-myc-deficient cells occurred in the mouse epidermis over time. In addition, as epidermal stem cells are the only epidermal cells with a long half-life, it is likely that most of these cells exhibit c-myc deletion as well.

To determine the effects of c-myc deletion in keratinocytes, the dorsal skin, whisker area [mechanically stressed area], and tail epidermis of mutant mice were analyzed in greater detail. The skin in these areas exhibited normal overall epidermal morphology and thickness, and the hair follicles and sebaceous glands also appeared normal [Fig. 1C,D, data not shown]. In addition, epithelial proliferation as assessed by expression of Ki67 [Fig. 1E,F] and incorporation of BrdU [4-h pulse] [Fig. 1G,H] was similar in control and mutant epidermis. Furthermore, the expression patterns of the keratin markers K14 and K1 were normal, suggesting normal differentiation of mutant epidermis [Fig. 1I–N]. c-Myc has recently been reported to be important for cellular growth in the developing mouse epidermis [Zanet et al. 2005]. However, in our study the number of basal cells per millimeter of epidermis was found to be similar between mutants and controls, indicating no changes in epidermal cell size in the absence of c-Myc function [Fig. S5].

To further address the role of c-Myc during epidermal proliferation in response to stress conditions, skin was treated with 12-O-tetradecanoylphorbol-13-acetate [TPA]. This phorbol ester induces a robust proliferative response leading to substantial epidermal hyperplasia in a matter of days, known to be associated with high expression of c-Myc [Kennard et al. 1995]. Surprisingly, control and mutant skin developed comparable epidermal thickening [Fig. 2A–D] and a similar increase in BrdU incorporation [Fig. 2E–J], suggesting that endogenous c-Myc is not required for TPA-induced epidermal hyperplasia. In summary, these data suggest that c-Myc is not required for proliferation, growth, and differentiation of the adult mouse epidermis and is therefore dispensable for skin epidermal homeostasis and TPA-induced hyperplasia. This result is surprising considering the expression of this gene in the basal layer, bulge, and hair bulb [Hurlin et al. 1995; Bull et al. 2001], and with respect to our findings showing that c-Myc is essential to maintain proliferation of cultured keratinocytes. A recent study on c-Myc-deficient epidermis using a noninducible K5Cre transgenic line showed decrease in cellularity presumably caused by premature keratinocyte differentiation and a defect in cell growth [Zanet et al. 2005]. The reason for this apparent discrepancy is unclear, however, one possible explanation may be the use of a noninducible transgene that already eliminates c-Myc during embryogenesis [Zanet et al. 2005] rather than in the adult epidermis. In support of this possibility, we have recently demonstrated distinct roles for c-Myc during development and adult homeostasis in the intestinal epithelium [Bettess et al. 2005].
Tumors of the skin are the most frequently diagnosed tumors worldwide, and amplification of the c-myc gene is often found in such tumors, especially in those derived from squamous keratinocytes (Boukamp 2005). Transgenic mice overexpressing c-Myc develop spontaneous papillomas and SCCs (Pelengaris et al. 1999; Rounbehler et al. 2001). Alternatively, papilloma formation can be chemically induced by the two-stage carcinogenesis protocol (for review, see Kemp 2005). Application of the carcinogen 7,2-dimethylbenzanthracene (DMBA) to the skin surface is thought to result in mutations in long-lived keratinocytes, probably stem cells (Perez-Losada and Balmain 2003). In response to applications of the tumor promoter TPA, initiated cells proliferate, leading to clonal expansion and papilloma formation. The development of these tumors is dependent on oncogenic Ras activity, and ~90% of these tumors have been shown to select for an A→T182 mutation in the Harvey-Ras (H-Ras) gene (Quintanilla et al. 1986). In this respect, it is important to note that Ras and Myc comprise the first example of oncogene cooperation during the cellular transformation process (Land et al. 1983). In addition, recent findings suggest that the Ras-ERK pathway can stabilize the labile c-Myc protein (Sears 2004; Adhikary and Eilers 2005), thus we hypothesized that a functional endogenous c-myc gene might be necessary for Ras-driven tumorigenesis. To address this, adult control and mutant mice were first injected with tamoxifen to induce c-myc^flox excision followed by treatment with DMBA/TPA [see Materials and Methods for details]. Consistent with previous studies (for review, see Kemp 2005) papillomas became evident 5–6 wk after TPA promotion. On average, ~30 papillomas/mouse developed in controls [Fig. 3A–C], of which the vast majority contained the A→T182 mutation in H-Ras [see below]. Although the onset of tumor formation was similar in mutant mice [Fig. 3A], the number of tumors was strongly reduced [Fig. 3B]. Indeed, in some mutants, tumor formation was negligible [Fig. 3C, arrowhead]. The tumors that formed in mutant mice were very similar with respect to size, histology, and proliferative activity as estimated by BrdU incorporation compared with those formed in control mice [Fig. 3; data not shown]. In addition, the percentage of the A→T182 mutation in the H-Ras gene was found to be comparable [83% in tumors on control mice [10/12] vs. 86% in tumors on mutants [31/36]] [Fig. 4F]. However, subsequent genotyping of the papillomas grown on mutant skin by Taqman real-time PCR showed that none of these tumors (n = 24) contained the recombined c-myc^ORFrec-null allele [Figs. 1A, 3D]. Thus, tumors grown on the mutant epidermis have originated from cells that escaped recombination and for Ras-driven tumorigenesis. To address this, adult control and mutant mice were first injected with tamoxifen to induce c-myc^flox excision followed by treatment with DMBA/TPA [see Materials and Methods for details]. Consistent with previous studies (for review, see Kemp 2005) papillomas became evident 5–6 wk after TPA promotion. On average, ~30 papillomas/mouse developed in controls [Fig. 3A–C], of which the vast majority contained the A→T182 mutation in H-Ras [see below]. Although the onset of tumor formation was similar in mutant mice [Fig. 3A], the number of tumors was strongly reduced [Fig. 3B]. Indeed, in some mutants, tumor formation was negligible [Fig. 3C, arrowhead]. The tumors that formed in mutant mice were very similar with respect to size, histology, and proliferative activity as estimated by BrdU incorporation compared with those formed in control mice [Fig. 3; data not shown]. In addition, the percentage of the A→T182 mutation in the H-Ras gene was found to be comparable [83% in tumors on control mice [10/12] vs. 86% in tumors on mutants [31/36]] [Fig. 4F]. However, subsequent genotyping of the papillomas grown on mutant skin by Taqman real-time PCR showed that none of these tumors (n = 24) contained the recombined c-myc^ORFrec-null allele [Figs. 1A, 3D]. Thus, tumors grown on the mutant epidermis have originated from cells that escaped recombination and

![Figure 2. TPA-induced epidermal hyperplasia occurs to a similar extent in control and c-Myc-deficient skin. (A,D) Hematoxilin and Eosin staining of dorsal skin taken from the same individuals either from treated or untreated area of the back skin. Thickness of epidermis (Epi) is indicated. (E,I) Immunohistochemical analysis of BrdU incorporation. Arrows indicate examples of BrdU-positive cells; arrows in I and H indicate BrdU-positive suprabasal cells. Original magnification: 20×.](image)

![Figure 3. c-Myc mutant skin is resistant to chemical carcinogenesis. (A) Kinetics of tumor incidence in control and c-Myc-deficient (mutants) epidermis. (B) Tumor number per mouse. Mutants, n = 17; controls, n = 9. (C) Typical examples of control and mutant mice after 11 wk of TPA promotion. Arrows point to typical papillomas, arrowhead points to tumor-free skin. (D) Analysis of Cre-mediated recombination at the c-myc locus in tumors developed in mutant mice. Real-time Taqman PCR analysis detecting the c-myc^ORFrec allele on DNA isolated from tumors and epidermis isolated from mutant mice. DeltaCt indicates the additional number of PCR cycles needed to detect the product in relation to the control with 90% deletion, which was set to 1. Samples with a relative c-myc^ORFrec content of 0%, 45%, and 90% [as determined by Southern blot] were used as references. (T1–T24) DNA from 24 tumors. The line dividing the graph indicates a c-myc^ORFrec content of 1.4%.](image)
strongly suggest that tumors specifically selected against c-Myc deficient cells. Hence, the endogenous c-myc gene is essential for Ras-driven epithelial tumorigenesis triggered by the DMBA/TPA protocol.

One explanation for the resistance of c-Myc-deficient epidermis to Ras-induced tumorigenesis could be that (mutant) tumor-initiating cells show increased apoptosis in response to DMBA. Such a mechanism was suggested for the tumor resistance observed in C/EBPβ and Stat3-deficient mice that show increased apoptosis of bulge cells in response to DMBA (Zhu et al. 2002; Chan et al. 2004). However, while treatment with DMBA increased the number of apoptotic cells in the epithelium, c-Myc mutants showed decreased apoptosis compared with controls excluding this possibility as a possible mechanism for the observed tumor resistance [Fig. S6A]. Recent evidence has shown that c-Myc activity induces neoangiogenesis through induction of VEGF and repression of thrombospondin-1 (Pelengaris et al. 1999; Watsnick et al. 2003), raising the possibility that c-Myc is necessary to mount the angiogenic response needed to initiate and promote tumorigenesis. However, while DMBA/TPA treatment leads to significant induction of angiogenesis (CD31 expression) in the dermis and in the papillomas, no significant difference was observed between mutant and control skin [Fig. S6B]. In summary, these data suggest that neither reduced angiogenesis nor increased apoptosis can explain the resistance of c-Myc mutant skin to DMBA/TPA-induced papilloma formation.

Ectopic Ras expression induces proliferation by induction of CyclinD1 and repression of p27Kip1 [Pruit and Der 2001]. However, this cell cycle induction is coupled to the up-regulation of cell cycle inhibitors such as p15INK4b, p16INK4a, and p21Cip1, which under certain conditions can lead to cell cycle arrest and senescence [Serrano et al. 1997; Dajee et al. 2003; Braig et al. 2005; Collado et al. 2005]. In human fibroblasts and keratinocytes, p21Cip1 has been reported to be a key inducer of cellular senescence in vitro [Brown et al. 1997; Sayama et al. 1999]. Since the p21Cip1 promoter is bound and repressed by the c-Myc/Miz-1 complex [Herold et al. 2002; Seoane et al. 2002], we hypothesized that c-Myc activity in tumors might be critical to prevent the expression of p21Cip1. To address this possibility, we initiated tumor formation in nondeleted K5::CreERト; c-myc−/− mice and subsequently induced Cre activity by tamoxifen. Immunohistochemical analysis revealed strong up-regulation of the p21Cip1 protein in tumors following acute deletion of c-myc [Fig. 4A,B]. Interestingly, p21Cip1 up-regulation is tumor-specific and is not observed in acutely deleted hyperplastic epidermis [in between papillomas] [Fig. 4C,D] or untreated skin [data not shown]. This phenomenon correlates well with the differential activity of the Ras–ERK pathway. We found that the already high ERK phosphorylation in TPA-induced epithelium is further increased in papillomas [Fig. 4E], which is consistent with the presence of the mutated dominant active H-Ras allele in tumors but not hyperplastic epidermis [Fig. 4F].

To genetically test the hypothesis that p21Cip1 is a key target that needs to be repressed by c-Myc during papillomagenesis, K5::CreERト; c-myc−/− mice were bred onto a p21Cip1-deficient background [Brugarolas et al. 1995] and double-mutant mice were treated with DMBA/TPA. Interestingly, homozygous [but not heterozygous] loss of p21Cip1 restored normal tumor frequency in female epidermis lacking c-Myc [Fig. 5A,B]. In agreement, the majority of double-mutant tumors lacked detectable c-Myc expression [Fig. 5C–F]. These data show that while c-Myc-deficient tumors were never observed on a wild-type background, they do form in the additional absence of a functional p21Cip1 gene. Collectively, these data provide genetic evidence that the key function for the oncogene c-Myc during epidermal tumorigenesis is the repression of the CDK inhibitor p21Cip1. This seems to be a critical step only during tumorigenesis and not during homeostasis, since hyperactivation of the Ras–ERK pathway (which induces p21Cip1) occurred only during the transition from TPA-induced hyperplasia to papillomagenesis. Uninhibited p21Cip1 activity is known to efficiently block proliferation and/or senescence, thus providing an explanation for the resistance of c-Myc-deficient epidermis to Ras-driven tumorigenesis.

A similar role for c-Myc has been observed in a CML cell line, in which a Ras-p21-mediated cell cycle arrest could be rescued by ectopic Myc expression [Delgado et al. 2000; Vaque et al. 2005]. A role for p21Cip1 in skin tumorigenesis has been established both in vitro and in vivo. Whereas primary mouse keratinocytes isolated from p21Cip1-deficient mice are effectively transformed by Ras, control cells are not [Missero et al. 1996]. Importantly, two-stage carcinogenesis studies on p21Cip1-deficient mice suggest a suppressive role for p21Cip1 during...
vent premature senescence at least in cultured cells consistent with observations in fibroblasts (Guney et al. 2006). Future experiments will need to examine the specific roles of c-Myc and p21\(^{Cip}\) in the senescence and cell cycle programs during the transition from hyperplastic epidermis to papillomagenesis. In any event, given that the c-Myc-p21\(^{Cip}\) and c-Myc-CDK4 pathways are surprisingly dispensable for normal epithelial homeostasis, targeting these pathways with specific compounds may only inhibit tumor growth but not normal epidermises.

**Materials and methods**

**Mice**

KSCreER\(^T\); c-myc\(^{floxed}\)/\(^{lox}\) or KSCreER\(^T\); c-myc\(^{floxed/KO}\) conditional knock-out animals on a wild-type or p21\(^{−/−}\)-deficient background were generated using previously described alleles (Brugarolas et al. 1995; Indra et al. 1999; Trumpp et al. 2001). Three- to five-week-old mice were injected intraperitoneally with 1 mg of tamoxifen (dissolved in sunflower oil; Sigma) once a day for 5 or 10 d (consecutive). All experiments were approved by the Schweizer-Bundesamt für Veterinärwesen authorization number 1728.

**DMBA/TPA treatment**

TPA (Sigma) was applied on shaved back skin. Applications were 1–4 × 6.5 ug/mouse, and mice were sacrificed 1 d after the last application. For the two-stage carcinogenesis protocol, DMBA (Fluka) was applied in acetone on shaved dorsal skin 32 ug/mouse. Single DMBA treatment was followed by TPA applications (12.5 ug in acetone) two times per week.

**Antibodies**

Immunohistochemistry: Ki67 (Novocastra, 1:50), Keratin 14 (Covance, FITC-155L), Keratin1 (Covance, PRB-165P), p21 (Santa Cruz, sc-6246), anti-BrdU (Oxford Biotechnology), and c-Myc (Upstate Biotechnologies, FITC-155L), Keratin1 (Covance, PRB-165P), p21 (Santa Cruz, sc-6246), anti-BrdU (Oxford Biotechnology), and c-Myc (Upstate Biotechnologies, 06-340). Western blot analysis: phosphospecific p44/42 MAPK-Thr202/Tyr204 and p44/42 MAPK antibody (Cell Signaling).

Detailed methods are published as supplemental information.

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Thordur Oskarsson, Marieke Alida Gertruda Essers, Nicole Dubois, et al.

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