

Id2 Is Dispensable for Myc-Induced Epidermal Neoplasia

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We have previously described a transgenic mouse model of epidermal neoplasia wherein expression of a switchable form of c-Myc, MycER^{TAM}, is targeted to the postmitotic suprabasal keratinocytes of murine epidermis via the involucrin promoter. Sustained activation of c-MycER^{TAM} results in a progressive neoplastic phenotype characterized by aberrant ectopic proliferation and delayed differentiation of suprabasal keratinocytes, culminating in papillomatosis. Transcription of the *Id2* gene is regulated by Myc family proteins. Moreover, *Id2* is implicated as a pivotal determinant of cell fate in multiple lineages and has a demonstrated role in mediating Myc-dependent cell proliferation in vitro through its interaction with retinoblastoma protein. Using *Id2* nullizygous mice, we assessed in vivo the requirement for *Id2* in mediating Myc-induced papilloma formation in skin. We show that absence of *Id2* has no discernible impact on any measurable attribute of Myc function or on the timing or extent of eventual tumor formation. Thus, our data argue against any essential role for *Id2* in mediating Myc action in vivo.

Deregulated expression of *myc* family oncogenes occurs in a wide range of human cancers and is often associated with aggressive, poorly differentiated tumors (28). As members of the bHLH-Zip transcription factor family, Myc proteins are thought to exert their biological effects by modulating, either positively or negatively, expression of downstream target genes (24). Indeed, recent advances in expression array and serial analysis of gene expression technologies have indicated that Myc proteins regulate a bewilderingly diverse variety of genes which are variously implicated in almost every aspect of cellular activity, including proliferation and differentiation, growth and apoptosis, metabolism, intracellular transport and organization, and interactions with the somatic cell environment (6, 10, 27, 31, 41). The breadth of potential Myc target genes, together with the highly contingent nature of the pathways and processes they oversee, makes it a great challenge to determine which attributes of Myc proteins are most critical for their tumorigenic properties.

The *Id2* gene was recently identified as a direct transcriptional target of both N-Myc and c-Myc (23). Using a conditional form of N-Myc (N-MycER), in which N-Myc activity is rendered dependent on the presence of the ligand 4-hydroxy-tamoxifen (4-OHT) by fusion with the estrogen receptor ligand binding domain (25), activation of N-Myc was demonstrated to rapidly induce expression of *Id2* at both mRNA and protein levels. Induction of *Id2* by N-Myc (and by c-Myc) is dependent on the presence of two Myc consensus E boxes located within the *Id2* 5' regulatory region. Consistent with this, chromatin immunoprecipitation analysis has indicated binding of c-Myc/Max heterodimers to the *Id2* promoter (23). Moreover, transforming growth factor β -mediated inhibition of *Id2* expression is associated with displacement of Myc/Max heterodimers from, and enhanced binding of Mad4/Max heterodimers to,

the *Id2* promoter and correlates with proliferative arrest in both mouse mammary epithelial cells and human keratinocytes (42). Intriguingly, the combination of deregulated Myc and constitutively activated Ras fails to transform *Id2*-deficient mouse embryonic fibroblasts in vitro (23; D. J. Murphy and M. A. Israel, unpublished results), intimating that *Id2* may mediate a Myc-dependent function(s) necessary for cell transformation. An additional study revealed a close correlation between N-Myc amplification and *Id2* expression in neuroblastomas, implicating *Id2* as a potential prognostic indicator for this disease (21), although more recent analyses have questioned this particular finding (40, 44, 45).

Id2 is one of four related and evolutionarily conserved helix-loop-helix (HLH) proteins that dimerize with the various basic region HLH (bHLH) transcription factors whose members modulate diverse aspects of cell differentiation, proliferation, and survival (29, 30, 48). Because the Id proteins lack a DNA binding basic motif, their heterodimerization with bHLH proteins prevents the latter from binding E-box DNA elements. Consequently, Id proteins act as endogenous negative regulators of bHLH function (3, 17). Indeed, Id proteins are widely expressed during embryogenesis and appear to be intimately involved in regulating cellular differentiation in a spectrum of lineages (15, 16, 37).

Several in vitro studies have indicated that Id proteins promote cell proliferation. Thus, antisense blockade of *Id1*, *Id2*, and *Id3* expression significantly extends the G₁ phase of the cell cycle (1, 11), while both *Id2* and *Id4* bind the retinoblastoma gene (*Rb*) product Rb and related proteins, p107 and p130, antagonizing their ability to repress E2F-dependent transcription and cell cycle progression (13, 22; previously discussed in reference 29). Moreover, *Id1* (and probably other Id proteins) blocks the ability of the ubiquitous bHLH protein E2A to induce expression of the cell cycle inhibitor p21 (35), while *Id4* blocks E protein-mediated induction of *p21^{CIP1}*, *p15^{INK4b}*, and *p16^{INK4a}* promoter activity (33). In addition, *Id1*, *Id2*, and *Id3* can all inhibit Ets domain transcription factors

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(47), and Id1 specifically inhibits induction of p16^{INK4a} by Ets1 and Ets2 (32). The demonstrated role of Id proteins in promoting cell proliferation in vitro is corroborated by the general observation that Id proteins are more highly expressed in rapidly dividing cells and absent from most terminally differentiated adult tissues. Furthermore, Id genes are frequently expressed at high levels in many human tumors while enforced transgenic expression of Id proteins is tumorigenic in several tissues, including pancreas, colon, and thymus (14, 39, 43). Taken together, these various observations show that the Id proteins are excellent candidates for coordinating the often mutually exclusive processes of differentiation and proliferation.

All four *Id* genes have been germ line ablated individually in mice, and while each alone exhibits a relatively mild phenotype, the combined loss of any two of the *Id1*, *Id2*, and *Id3* genes results in embryonic lethality (26; previously discussed in references 29 and 39). Deletion of *Id2* alone results in a dramatic reduction in NK cell number and the absence of secondary lymphoid organs. In addition, *Id2*-null mice, while indistinguishable from their wild-type (wt) littermates at birth, exhibit stunted growth by adulthood and, furthermore, mammary gland maturation is defective in pregnant *Id2*-null females. Intriguingly, deletion of *Id2* partially rescues *Rb*-null embryos, which, instead of aborting by embryonic day 14.5, survive to term. However, such *Id2/Rb* double nullizygous pups are stillborn, most likely as a consequence of reduced skeletal muscle development leading to inadequate respiratory muscle function postpartum (23). Although this might suggest that *Rb* functions in part during embryogenesis to regulate *Id2* and that the lethality of *Rb*-null embryos at embryonic day 14.5 is in part due to unrestrained *Id2* activity, recent studies indicating that the lethality of *Rb*-null mice is principally a placental defect (7, 46) have complicated interpretation. Nonetheless, in vitro bromodeoxyuridine incorporation experiments have shown that the inability of *Myc* to prevent cell cycle exit of *Id2*-null mouse embryonic fibroblasts upon serum withdrawal is corrected when *Rb* is also deleted, suggesting that *Myc* requires *Id2* to overcome the *Rb*-mediated cell cycle arrest (23).

Previously, we reported the development of an acutely inducible mouse model of epidermal hyperplasia in which the 4-OHT-dependent switchable c-MycER^{TAM} protein is targeted to suprabasal keratinocytes of the murine epidermis via the involucrin promoter. Topical application of 4-OHT to the dorsal skin of such *Inv-MycER^{TAM}* mice activates MycER^{TAM} function, triggering rapid entry of normally postmitotic immediate suprabasal keratinocytes into the cell cycle. This leads to progressive suprabasal hyperplasia associated with delayed keratinocyte differentiation, profound angiogenesis in the adjacent dermal compartment, and, eventually, papilloma formation. The rapidity, reproducibility, and pleiotropism of *Myc* action in this in vivo model make it an excellent system with which to assess the role of *Id2* as an obligate downstream *Myc* effector. Here, we present data demonstrating that the absence of *Id2* has no discernible influence on any measured aspect of *Myc* function in murine epidermis.

MATERIALS AND METHODS

Mice, genotyping, treatment, and sample collection. All mice were housed, fed, and treated in accordance with protocols approved by the committee for

animal research at the University of California, San Francisco. *Inv-MycER^{TAM}* mice, on a mixed C57Bl6/CBA background, were bred to *Id2* heterozygous mice, on a 129 background, and backcrossed to yield *Inv-MycER^{TAM}/Id2*-null and *Inv-MycER^{TAM}/Id2*-wt offspring. Genotyping was performed on tail snip DNA by PCR with the following primer sets: *MycS3* (forward, AGGGTCAAGTTGGA CAGTGTCAGAGTC); *MycER^{TAM}* (reverse, CCAAAGTTGGCAGCCCTC ATGTC); *ID2-105* (forward, AGGCGCCAGTCTGCTTCTTGTAAC); *ID2-3* (wt) (reverse, CAAACTGTAGCCCTCTGAG); and *NEO-8* (*Id2* null) (reverse, TAGCCTGAAGAACGAGATCAGCAG).

Two- to four-month-old mice were shaved to reveal an ~2-cm² area of dorsal skin and topically treated daily with 200 μ l of 4-OHT (5 mg/ml) in ethanol or 200 μ l of ethanol alone. Two to three mice of each genotype were euthanized with CO₂, followed by cervical dislocation, at each indicated time point. Skin tissue was harvested from the treatment area, bisected, either frozen fresh in OCT (TissueTek) or fixed in 4% neutral buffered paraformaldehyde (PFA), dehydrated, and embedded in paraffin.

In situ hybridization. Antisense RNA probes were transcribed in vitro from linearized plasmid template DNA in the presence of ³⁵S-UTP. To probe for *Id2* expression, a 235-bp *XbaI-SalI* fragment representing 3' untranslated sequence, subcloned into pBluescript SK (Stratagene), was linearized with *BglII* and transcribed with T7 RNA polymerase. To probe for *Id4* expression, a 443-bp *EcoRI* fragment representing 3' untranslated sequence, subcloned into pCRII (Invitrogen), was linearized with *NotI* and transcribed with SP6 RNA polymerase. *ODC-1* probe was generated against an ~530-bp *HindIII-NcoI* fragment from the 3' end of the *ODC-1* cDNA, subcloned into pBluescript SK, linearized with *HindIII*, and transcribed with T7 RNA polymerase. Probes were hydrolyzed to ~300 bp (*Id2* probe excepted) in 2 \times sodium carbonate buffer (1 M NaHCO₃-1 M Na₂CO₃ [pH 10]), neutralized with acetic acid (to a final concentration of 0.3%), precipitated in ethanol, and resuspended in 10 mM dithiothreitol.

Ten-micrometer-thick frozen tissue sections were thawed, air dried, and pre-treated at room temperature (RT) for hybridization as follows: 20 min in 4% PFA, 4 min in 3 \times phosphate-buffered saline (PBS), two times for 4 min in 1 \times PBS, air dried briefly, 20 min in proteinase K solution (100 mM Tris [pH 8.0]-50 mM EDTA-1 μ g of proteinase K/ml), 5 min in 4% PFA, 4 min in 3 \times PBS, two times for 4 min in 1 \times PBS, 15 s in diethyl pyrocarbonate-treated distilled water, air dried briefly, 10 min in 200 ml of 0.1 M triethanolamine containing 500 μ l of acetic anhydride, rinsed twice in diethyl pyrocarbonate-treated distilled water, and air dried prior to hybridization.

Riboprobes (10⁶ dpm/slide) were heat denatured at 65°C for 5 min and mixed with hybridization solution (50% [vol/vol] formamide, 10% [wt/vol] dextran sulfate, 2 \times Denhardt's solution, 5 \times SSC [1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 10 mM β -mercaptoethanol, 250 μ g of yeast tRNA/ml, 500 μ g of single-stranded DNA/ml; ~100 μ l/slide) preheated to 55°C. Probe was added directly to sample slides, which were then covered with Parafilm and incubated overnight at 55°C in a humid chamber. After hybridization, Parafilm strips were removed and slides were washed in the following solutions before being dehydrated through graded ethanol and air dried: 30 min at 55°C in 50% formamide-2 \times SSC-1 mM EDTA-10 mM β -mercaptoethanol, 30 min at RT in 2 \times SSC, 1 h at RT in 2 \times SSC-1 mM EDTA-20 μ g of RNase A/ml-1 U of RNase T1/ml, two times for 30 min at 55°C in 2 \times SSC, and 5 min at RT in 0.2 \times SSC. Radiolabeled slides were dipped in photographic emulsion (Kodak) mixed at a ratio of 1:1 with 1% glycerol, dried, and stored for 4 to 10 days. Slides were then immersed in developing solution (Kodak no. 146 4593) for 5 min at 16°C, rinsed in water, and fixed (Kodak fixer no. 197 1746) and then dehydrated through graded ethanol and mounted with coverslips. Labeled sections were analyzed by dark-field imaging using a Leica DM RXA microscope.

Immunohistochemistry. The primary antibodies used were anti-K10 (1:2,000 dilution) and anti-K14 (1:10,000 dilution; Covance PRB-155p and PRB-159p), anti-PCNA (1:100 dilution; Biogenex-mu206-uc), CD-31 (1:50 dilution; Pharmingen 01951D), and anti-Id1 (1:50 dilution) and anti-Id3 (1:50 dilution; Santa Cruz sc488 and sc490). Immunohistochemistry and immunofluorescence analyses were performed on 5- μ m-thick paraffin-embedded sections as previously described (34), with the exception of CD31 staining, which was performed on 10- μ m-thick frozen sections. Briefly, paraffin-embedded slides were deparaffinized and rehydrated through graded ethanols. For MCM-7, PCNA, and K14, antigen retrieval was performed by microwaving on low power for 5 min in 10 mM sodium citrate (pH 6.0). Sections were incubated in blocking solution (4% bovine serum albumin-3% preimmune goat serum-2% fish gelatin) for 1 h at RT, and primary antibodies were added in blocking buffer and incubated overnight at 4°C. Secondary antibodies were diluted 1:200 in blocking solution and incubated at RT for 1 h. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Pierce) was used to identify K10 and K14 staining, HRP-conjugated goat anti-mouse immunoglobulin M (Vector) was used for PCNA staining, Alexa

488-conjugated goat anti-rabbit was used to detect anti-Id1 and Id3 antibodies, and Alexa 488-conjugated goat anti-rat (Molecular Probes) was used to detect CD-31. Alexa 488-stained sections were counterstained with propidium iodide (1 μ g/ml) in fluorescent mounting medium (Dako) and analyzed by fluorescence microscopy. HRP-labeled sections were incubated with ABC reagent (Vectastain) and developed with diacylbutyrate (DAB). DAB-stained slides were counterstained lightly with hematoxylin, dehydrated through graded ethanols, mounted with coverslips, and analyzed by light microscopy.

RESULTS

Id2 is expressed in c-Myc-induced epidermal hyperplasia.

We first investigated whether Id2 is expressed in skin epidermis following c-Myc activation. Myc-induced papillomatous epidermal lesions were elicited by topical treatment of shaved dorsal *Inv-MycER^{TAM}* mouse skin (2 cm²) daily with 1 mg of 4-OHT/day for a period of 2 weeks, as described previously (34). Littermate transgene-negative mice were treated similarly to serve as controls. At the end of the 2-week treatment, *Inv-MycER^{TAM}* skin was visibly thickened and flaking, as described previously (34). Mice were then euthanized, and the treated area of skin was harvested. Half of the sample was fixed with 4% PFA and paraffin-embedded, while the other half was snap frozen.

Histological analysis of *Inv-MycER^{TAM}* skin revealed the epidermis of 4-OHT-treated mice to be several layers thicker than that of transgene-negative control samples. Immunohistochemistry confirmed the marked accumulation of less differentiated suprabasal keratinocytes. Expression of *Id2* was then assayed by in situ hybridization, while adjacent sections were probed for expression of the well-characterized Myc target gene *ODC-1* to confirm Myc transcriptional activity (Fig. 1). As a negative control, we used antisense riboprobe for *Id4*, which is not expressed in skin (16). *ODC-1* expression was detectable in all nucleated keratinocytes, with essentially uniform expression across the epidermal strata. *Id2* was also detected throughout the epidermis, although in this case a gradient of expression was apparent, with highest expression of *Id2* in the inner (i.e., basal and immediately suprabasal) layers. This gradient in expression is consonant with that of the proliferation markers PCNA and Ki67 (34; I. Flores, D. J. Murphy, L. Brown Swigart, and L. Evan, unpublished data). Thus, the pattern of *Id2* expression correlates with the proliferative state of keratinocytes rather better than with Myc activity, as assessed by *ODC-1* expression. *Id4* expression was undetectable anywhere within either normal or hyperplastic epidermis.

Id2 is dispensable for development and full progression of Myc-induced papillomas. To determine the extent to which Id2 expression might be required for any aspect of the Myc-induced papilloma phenotype, *Inv-MycER^{TAM}* mice were crossed with *Id2*-deficient mice and then backcrossed to yield either *Inv-MycER^{TAM}/Id2*-null or *Inv-MycER^{TAM}/Id2*-wt offspring. Two- to four-month-old mice of appropriate genotypes were then treated topically with 4-OHT as before, and littermate or age-matched cohorts were sacrificed after 7, 14, or 22 days of sustained Myc^{TAM} activation. We could discern no difference between *Id2*-deficient animals and *Id2*-wt controls with regard to either the rate or extent of Myc-induced tumor formation (Fig. 2). After 7 days of sustained Myc^{TAM} activation, the epidermis of both *Inv-MycER^{TAM}/Id2*-null and *Inv-MycER^{TAM}/Id2*-wt mice presented five to six layers of nucle-

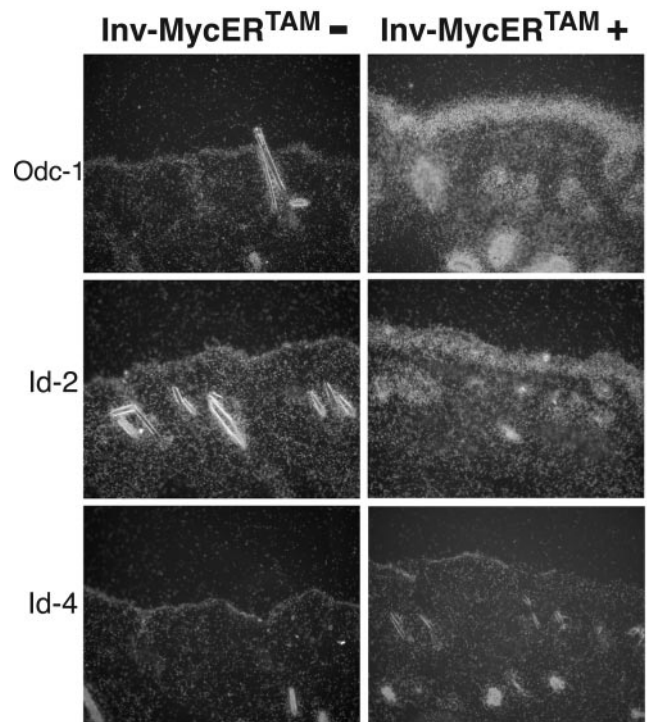


FIG. 1. *Id2* is expressed in hyperplastic *Inv-MycER^{TAM}* epidermis. Frozen sections of dorsal epidermis from wt (left panels) and *Inv-MycER^{TAM}* (right panels) mice treated for 2 weeks with 4-OHT were hybridized with ³⁵S-labeled antisense riboprobe specific for *ODC-1*, *Id2*, or *Id4* mRNA. Dark-field microscopy revealed the presence of *ODC-1* transcripts throughout all layers of the hyperplastic epidermis and a gradient of *Id2* transcripts, highest in the inner (less differentiated) layers of the hyperplastic epidermis. *Id4* transcripts by contrast were absent from normal and hyperplastic epidermis. Staining of hair cells was nonspecific.

ated keratinocytes in place of the normal one to two layers. Epidermal thickness roughly doubled over the second week of Myc activation and was accompanied by progressive dysplasia, with papillomatosis clearly evident by the onset of the third week of Myc activation.

Id2 is not required for Myc-induced keratinocyte proliferation. Although absence of *Id2* had no overall effect on Myc-induced papillomatosis, it remained possible that lack of *Id2* might compromise specific aspects of Myc function that are then compensated for in some way. For this reason, we assessed separately the effects of *Id2* loss on Myc-induced keratinocyte proliferation and apoptosis.

In normal epidermis, proliferating keratinocytes are present only within the basal layer: as cells detach from the basal lamina and migrate into the suprabasal compartment, they drop out of cycle and differentiate (8). In contrast, Myc-induced epidermal hyperplasia in *Inv-MycER^{TAM}* mice is characterized by the presence of proliferating keratinocytes several layers into the expanded suprabasal compartment. To assess whether the absence of *Id2* affected Myc-induced keratinocyte proliferation, we used antibodies specific for the cell proliferation marker PCNA to analyze immunohistochemically the proliferative status of skin cells (Fig. 3). As expected, activation of Myc in the suprabasal compartment of murine epidermis

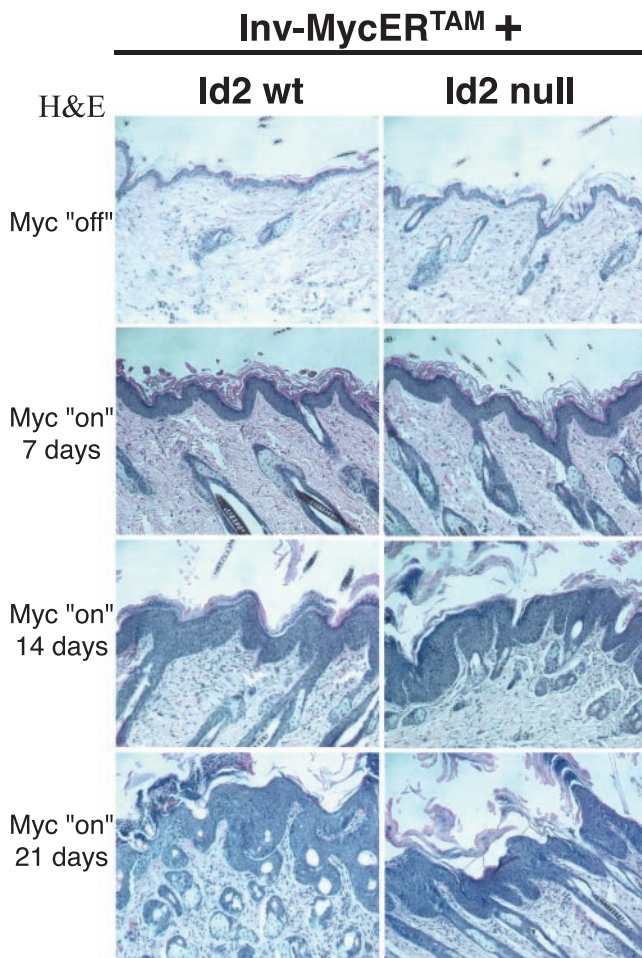


FIG. 2. Id2 is not required for the Myc-induced papillomatous phenotype. Hematoxylin and eosin (H&E) staining of *Inv-MycER^{TAM}/Id2-wt* and *Inv-MycER^{TAM}/Id2-null* epidermis after 7, 14, or 22 days of Myc activation. *Inv-MycER^{TAM}*-positive mice treated for 22 days with ethanol (top panels) and *Inv-MycER^{TAM}*-negative mice treated with 4-OHT (data not shown) comprised negative controls.

resulted in the progressive accumulation of cycling suprabasal keratinocytes in *Inv-MycER^{TAM}/Id2-wt* mice. Within 7 days of onset of sustained MycER^{TAM} activation, PCNA expression was clearly evident in the immediately suprabasal cell layers. By 3 weeks, PCNA expression extended all the way through the hyperplastic suprabasal skin to the granular layer. Essentially identical staining was observed using antibodies specific for the DNA replication licensing factor MCM-7 (data not shown). We could discern no difference in the onset or prevalence of Myc-induced suprabasal keratinocyte proliferation in the absence of Id2 nor on the polarity of accumulation (from the basal layer outwards).

Negligible apoptosis accompanies Myc-induced epidermal hyperplasia in *Id2-wt* mice (34; Flores et al., unpublished). We observed a similar absence of detectable keratinocyte apoptosis in the absence of Id2 (data not shown).

Id2 expression is not required for c-Myc to delay keratinocyte differentiation. The augmented proliferation and hyperplasia of suprabasal keratinocytes induced by activation of MycER^{TAM} is accompanied by accumulation of less differen-

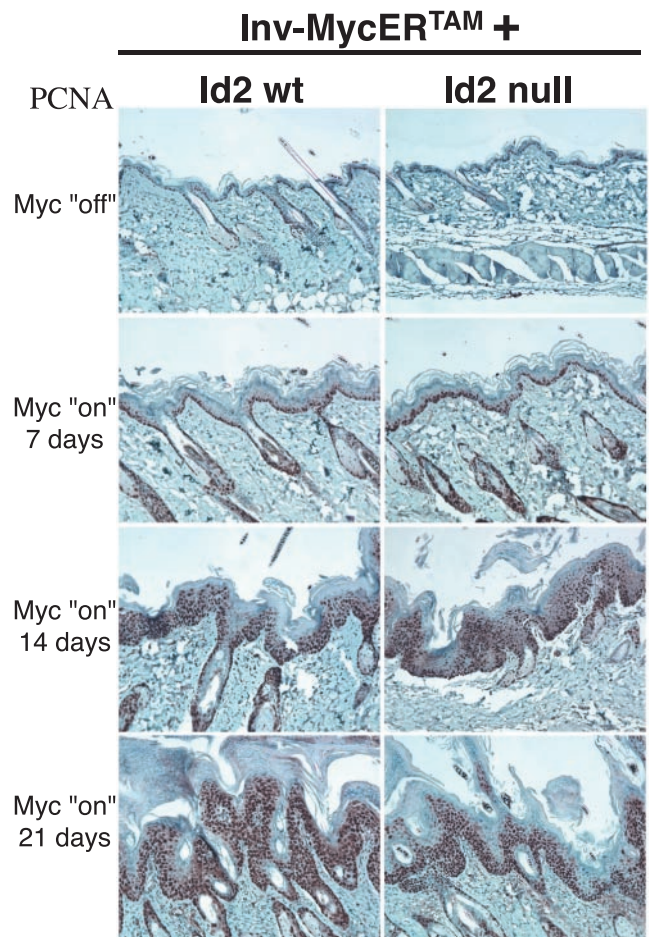


FIG. 3. Myc-induced keratinocyte proliferation is unaffected by deletion of *Id2*. *Inv-MycER^{TAM}/Id2-wt* and *Inv-MycER^{TAM}/Id2-null* mice were treated with 4-OHT for 7, 14, or 22 days or with ethanol for 22 days. Expression of PCNA, indicative of cycling cells, is normally restricted to the basal layer of murine epidermis (top panels). DAB staining of keratinocyte nuclei reveals PCNA expression gradually extending through all layers of the suprabasal epidermis upon prolonged activation of MycER^{TAM} with 4-OHT.

tiated suprabasal cells. This is manifested by the persistent expression of basal markers such as keratin K14 and p63 out into the suprabasal layers together with a delayed or reduced expression of suprabasal cell markers such as involucrin and keratins K1 and K10 (34; Flores et al., unpublished). To explore any influence of Id2 absence on this aspect of Myc action, we examined the effects of Myc activation in *Id2-wt* and *Id2-null* mice immunohistochemically by using basal and suprabasal epidermal markers.

In normal epidermis, K10 is expressed exclusively in the spinous and granular layers (5). After 3 weeks of sustained suprabasal MycER^{TAM} activation, expression of K10 becomes progressively lost from the inner suprabasal layers, shifting outwards into the greatly thickened spinous and granular layers (Fig. 4A). In contrast, expression of the basal marker K14 progressively extends out from the basal compartment into the suprabasal one in a manner reciprocal to that of K10 (Fig. 4B). Such apposition was identical in *Id2-null* mice. The absence of Id2 altered neither the timing nor the extent of these Myc-

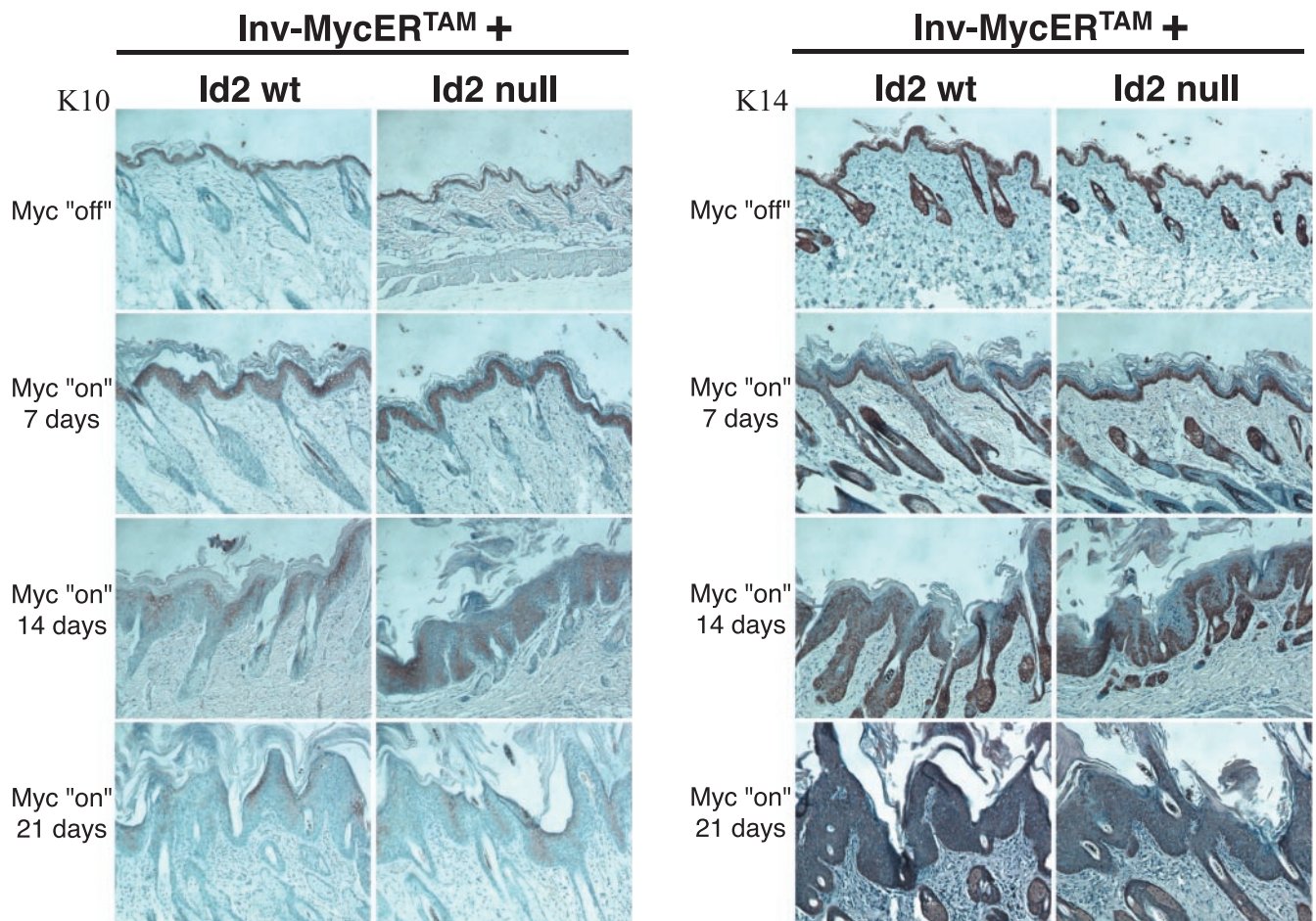


FIG. 4. Deletion of *Id2* does not alter the delayed differentiation of suprabasal keratinocytes, induced by prolonged Myc activation. (A) Expression of keratin K10 in *Inv-MycER^{TAM}/Id2-wt* and *Inv-MycER^{TAM}/Id2-null* skin treated with 4-OHT for 7, 14, or 22 days. K10 expression normally initiates in the immediately suprabasal cell layer (top panels) but is delayed in onset and reduced in levels of expression after 14 days and especially after 22 days of continuous Myc activation. (B) Expression of keratin K14 in *Inv-MycER^{TAM}/Id2-wt* and *Inv-MycER^{TAM}/Id2-null* skin treated with 4-OHT for 7, 14, or 22 days. Keratin K14 identifies basal keratinocytes and keratinocyte stem cells. Because K14 is a relatively stable protein, DAB staining extends into the immediately suprabasal layer, but K14 is nonetheless lost in the outer layers of early hyperplastic epidermis (compare Myc "off" with Myc "on" at 7 days). Prolonged activation of Myc results in the retention of K14 protein throughout the late hyperplastic epidermis.

induced effects, challenging the notion that *Id2* is a key mediator of Myc's ability to disrupt differentiation.

***Id2* is dispensable for Myc-induced angiogenesis.** Although double knockout *Id1/Id3* mice die in utero, *Id1*-heterozygous/*Id3*-null and *Id1*-null/*Id3*-heterozygous mice are viable and, moreover, exhibit profound resistance to tumor xenografts because of a defect in tumor angiogenesis (26). *Id1*, *Id2*, and *Id3* are all expressed in the endothelial cells lining the vasculature, with the exception that *Id2* is absent from central nervous system vasculature (2, 4). Although *Id2*-deficient mice are viable, with no apparent deficit in vasculature, there remained the possibility that *Id2* status might affect the profound angiogenesis that suprabasal activation of c-Myc elicits in adjacent dermis, most notably in areas of papilloma formation (34). We used the marker of vascular endothelium, CD31, to explore any role for *Id2* in Myc-induced skin angiogenesis (Fig. 5). In both *Inv-MycER^{TAM}/Id2-null* and *Inv-MycER^{TAM}/Id2-wt* mice, activation of Myc for 2 to 3 weeks induced similar robust dermal angiogenesis in the region of skin treated with 4-OHT.

Thus, *Id2* appears not to be required for Myc-dependent angiogenesis.

***Id1* and *Id3* are abundantly expressed in hyperplastic *Inv-MycER^{TAM}* epidermis.** Although there is no evidence that any other member of the *Id* family is regulated by Myc, it is possible that the lack of a discernible influence of the absence of *Id2* upon the Myc-induced papillomatous phenotype in *Id2*-null animals is due to functional redundancy with other expressed *Id* proteins. We therefore investigated whether other *Id* proteins are expressed in the hyperplastic epidermis of 4-OHT-treated *Inv-MycER^{TAM}* skin.

Myc was activated for 14 days in *Inv-MycER^{TAM}* mice, either *Id2-wt* or *Id2-null*, and sections were then assayed for expression of *Id1* and *Id3* by immunofluorescence. Abundant cytoplasmic *Id1* staining was observed in all nucleated layers from hyperplastic epidermis of both *Id2-wt* and *Id2-null* mice. *Id3* was also expressed in all nucleated keratinocytes in both *Id2-wt* and *Id2-null* mice, although in contrast to what was seen with *Id1*, *Id3* staining was predominantly nuclear (Fig. 6). These

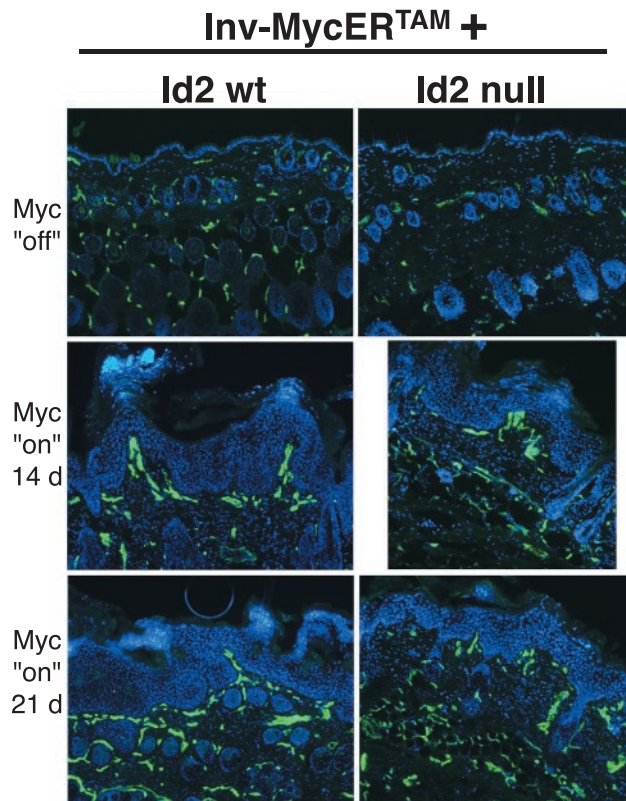


FIG. 5. *Id2* is dispensable for Myc induction of dermal angiogenesis. The endothelial cell marker CD31 (FITC labeled) was used to identify total vasculature in the dermis of *Inv-MycER^{TAM}/Id2-wt* and *Inv-MycER^{TAM}/Id2-null* skin treated with 4-OHT for 14 or 22 days. Control tissue consisted of transgene-negative *Id2-wt* and *Id2-null* skin treated with 4-OHT for 22 days. Prolonged Myc activation in suprabasal epidermis results in an increase in the complexity and epidermal proximity of the underlying dermal vasculature irrespective of *Id2* status.

results concur perfectly with those reported previously for expression of Id proteins in samples of human squamous cell carcinoma of the skin (19). The presence or absence of *Id2* had no significant effect on expression of either *Id1* or *Id3*. Thus, both *Id1* and *Id3* are expressed in Myc-induced hyperplastic epidermis. However, neither *Id1* nor *Id3* expression correlates with Myc-induced keratinocyte proliferation nor indeed with the differentiation status of *Inv-MycER^{TAM}* suprabasal keratinocytes. It is therefore unlikely that either *Id1* or *Id3* mediates Myc's tumorigenic function in this tissue.

DISCUSSION

We used a switchable MycER^{TAM} transgenic approach to investigate the potential role for *Id2* in Myc-dependent tumorigenesis in skin in vivo. Sustained activation of MycER^{TAM} in the suprabasal keratinocytes of murine epidermis results in a complex neoplastic phenotype, characterized by progressive hyperplasia accompanied by anaplasia, dysplasia, and angiogenesis and culminating in papillomatosis. Despite the published evidence indicating that *Id2* is a pivotal proliferative effector of Myc (21, 23) and the abundance of *Id2* transcripts in

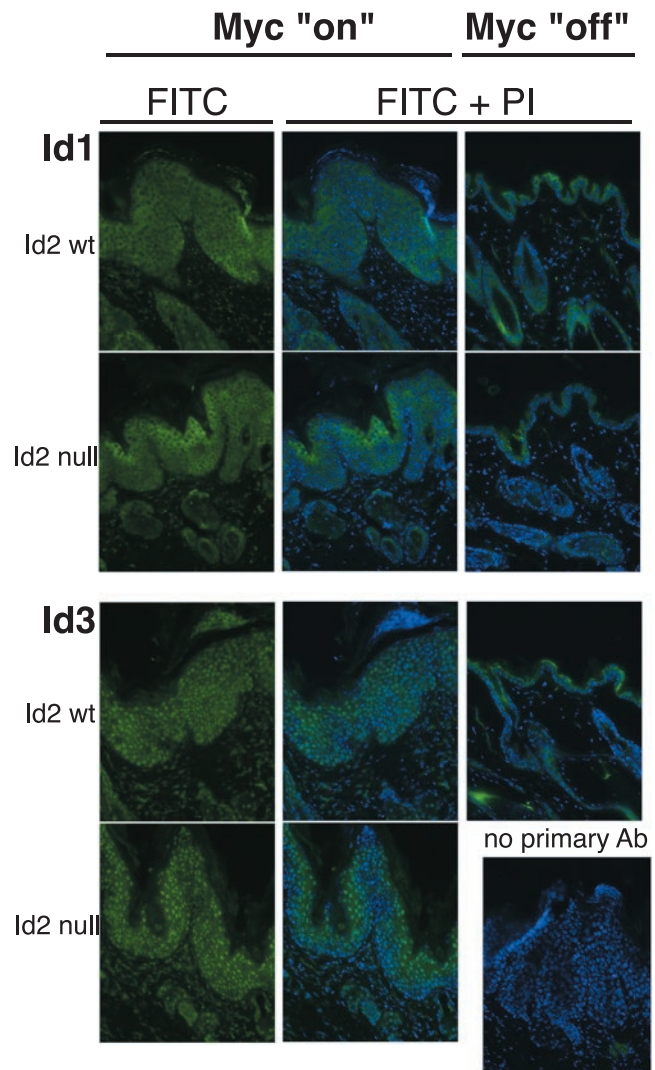


FIG. 6. *Id1* and *Id3* are abundantly expressed in *Inv-MycER^{TAM}*-induced epidermal hyperplasia. *Inv-MycER^{TAM}/Id2-wt* and *Inv-MycER^{TAM}/Id2-null* skin treated with 4-OHT for 14 days was stained for the presence of *Id1* protein (top set of panels) or *Id3* protein (bottom set of panels) by immunofluorescence. Alexa 488-conjugated secondary antibody (green), visible on the fluorescein isothiocyanate (FITC) channel, was used for detection, and sections were counterstained with propidium iodide (PI) (blue). Deletion of *Id2* does not appreciably influence expression of *Id1* or *Id3* in the hyperplastic epidermis. Ab, antibody.

the MycER^{TAM}-induced skin hyperplasia, we found that deletion of the *Id2* gene has no detectable influence on the extent or the kinetics of the complex neoplastic phenotype elicited by Myc in skin.

Substantial data exist to implicate Myc family proteins in the regulation of *Id2* transcription. The *Id2* promoter contains multiple E-box sequences which, by transient reporter analysis, are required both for induction by c-Myc and N-Myc and also for transforming growth factor β -dependent repression of *Id2*, demonstrably mediated in part by the Myc antagonist Mad-4. Direct binding of c-Myc/Max and Mad-4/Max complexes to the *Id2* promoter in vivo has also been demonstrated in both fi-

broblasts and immortalized keratinocytes by chromatin immunoprecipitation (23, 42). Moreover, while the initial report suggesting a close correlation between N-Myc amplification and Id2 expression in neuroblastoma (21) has recently been called into question (40, 44, 45), some correlation between c-Myc and *Id2* expressions appears to exist although Id2 still lacked prognostic value for neuroblastoma in this and other studies (40, 45). However, regulation of the *Id2* promoter is quite complex. *Id2* promoter activity is induced by a number of mitogenic signals and fluctuates in response to various tissue culture conditions, such as plating and refeeding of cells (30, 48). In addition to the potential Myc-binding E boxes, the *Id2* promoter also contains at least one *SP-1* site and is, furthermore, bound by Ets domain and ATF family transcription factors (9, 18) and activated by both BMP4 and STAT3 (12, 36). Given that the bulk of the present evidence suggests only a modest (i.e., approximately twofold) influence of Myc on *Id2* expression, it seems unlikely that Myc is the decisive arbiter of *Id2* transcription. Consistent with this, our own *in situ* analysis suggests that while Myc may indeed contribute to *Id2* transcription in hyperplastic keratinocytes, the principal determinant of *Id2* expression is the overall proliferative status of keratinocytes in which it is expressed.

Generally speaking, Id proteins are highly expressed in rapidly dividing and less differentiated cell populations. We found that *Id1*, *Id2*, and *Id3* are all expressed in the hyperplastic epidermis of 4-OHT-treated *Inv-MycER^{TAM}* skin, in close agreement with the results of a previous report demonstrating Id protein expression in human squamous cell carcinoma of the skin (19). It is possible that the extensive expression of Id1 and Id3 functionally compensates for loss of Id2 in the Myc-induced phenotype. All four Id proteins can bind the ubiquitously expressed E-box proteins E2A, HEB, and E2-2, disrupting their interaction with tissue-specific bHLH transcription factors (30). Expression of E2A has been demonstrated in keratinocytes, although tissue-specific interacting proteins that regulate epidermal lineage commitment have yet to be defined (19). Furthermore, Id proteins can bind tissue-specific bHLH proteins (e.g., MyoD, Neuro D, and Mash 1) directly, although they do so with differing affinities (21). Id1, Id2, and Id3 are each also capable of binding paired domain transcription factors, Pax-2, Pax-5, and Pax-8, as well as Ets domain proteins Elk-1 and Sap-1, although, again, they each exhibit varying affinities for these factors (38, 47). Thus, there is ample potential for degeneracy of function between Id family proteins that might compensate for loss of any one during development. Nonetheless, whereas *Id2* mRNA expression correlates well with the delay in differentiation of MycER^{TAM} keratinocytes, no such correlation is evident for Id1 or Id3. This absence of any such correlation between Id1 or Id3 expression and either keratinocyte proliferation or the Myc-induced disruption of keratinocyte differentiation strongly argues against any role for Id1 or Id3 in mediating Myc's tumorigenic properties in this tissue, either in the presence or absence of Id2.

By crossing our *Inv-MycER^{TAM}* mice into an *Id2*-null background, we were able to interrogate directly the potential role of Id2 as a mediator of Myc's oncogenic properties *in vivo*. Careful analysis of MycER^{TAM}-induced epidermal neoplasia throughout its progression revealed no obvious phenotypic deficit ascribable to the absence of Id2 expression. The rate

and extent of keratinocyte proliferation, delay in keratinocyte differentiation, dermal angiogenesis, and eventual papillomatosis elicited by Myc were all identical in the presence or absence of Id2. While it remains possible that Id2 may still mediate critical Myc functions in specific tissues or tumor types, our studies indicate that Id2 is not a universal requirement for any of the diverse aspects of Myc's biological function.

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