

# c-MYC-Induced Sebaceous Gland Differentiation Is Controlled by an Androgen Receptor/p53 Axis

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<http://dx.doi.org/10.1016/j.celrep.2013.01.013>

## SUMMARY

Although the sebaceous gland (SG) plays an important role in skin function, the mechanisms regulating SG differentiation and carcinoma formation are poorly understood. We previously reported that c-MYC overexpression stimulates SG differentiation. We now demonstrate roles for the androgen receptor (AR) and p53. MYC-induced SG differentiation was reduced in mice lacking a functional AR. High levels of MYC triggered a p53-dependent DNA damage response, leading to accumulation of proliferative SG progenitors and inhibition of AR signaling. Conversely, testosterone treatment or p53 deletion activated AR signaling and restored MYC-induced differentiation. Poorly differentiated human sebaceous carcinomas exhibited high p53 and low AR expression. Thus, the consequences of overactivating MYC in the SG depend on whether AR or p53 is activated, as they form a regulatory axis controlling proliferation and differentiation.

## INTRODUCTION

The sebaceous gland (SG) is part of the epidermis and produces the sebum that lubricates the skin surface. SGs are often associated with hair follicles (HFs), and loss of sebocyte function can lead to scarring alopecia, indicating a dependence of HFs on SGs (Sundberg et al., 2000). SG products also form the protective lipid barrier of the skin and thus function in skin immunity (Schneider and Paus, 2010), whereas specialized eyelid SGs (meibomian glands) provide a lipid film to prevent drying of the eye surface (McCulley and Shine, 2004). Sebaceous carcinomas, although rare, often recur locally, frequently metastasize, and have high mortality (Buitrago and Joseph, 2008). There is therefore considerable interest in elucidating SG biology.

*Myc* was first identified as the cellular homolog of the avian myelocytomatosis viral oncogene (*v-Myc*) (Sheiness et al., 1980) and originally designated *c-Myc* for cellular-Myc (Hayward

et al., 1981). MYC is a transcription factor that regulates sebocyte differentiation (Arnold and Watt, 2001; Honeycutt and Roop, 2004; Watt et al., 2008). *K14MycER* transgenic mice provide an experimental model with which to study MYC-regulated sebocyte differentiation. In these mice, human MYC is expressed under the control of the human keratin 14 (*K14*) promoter as a fusion protein with a mutant mouse estrogen receptor (ER)  $\alpha$  ligand-binding domain. The *MycER* transgene is constitutively expressed in the basal layer of the epidermis, but MYCER protein (henceforth MYC) is only activated upon topical activation of 4-hydroxytamoxifen (4OHT). Low levels of MYC activation promote SG expansion and differentiation, whereas high levels stimulate SG proliferation and inhibit differentiation (Berta et al., 2010). MYC is often associated with histone modifications marking active genes (Nascimento et al., 2011; Rahl et al., 2010) and may therefore serve to amplify the program of gene expression dictated by other transcription factors. These observations led us to explore potential factors that influence the outcome of MYC activation in the SG.

The *Ar* has been identified by chromatin immunoprecipitation (ChIP) as a MYC target gene in mouse epidermis (Nascimento et al., 2011), and MYC can promote androgen receptor (AR) activity and *AR* mRNA expression in human prostate (Nadiminty et al., 2012). In rats and humans, the AR is an early marker of sebocyte differentiation (Bayer-Garner et al., 1999; Rosenfield et al., 1998). In primary rat preputial sebocyte cultures, androgen inhibits proliferation (Deplewski and Rosenfield, 1999). In cultured human sebocytes, androgen can either promote or inhibit proliferation, depending on the type of androgen and the origin of the cells (Akamatsu et al., 1992). In vivo androgens can promote growth and development of the human sebaceous gland (Zouboulis, 2010), while poorly differentiated sebaceous carcinomas have reduced AR expression (Bayer-Garner et al., 1999). Nevertheless, testicular feminization (TFM) mice, which have a spontaneous loss of function *Ar* mutation (Gaspar et al., 1991), still form SGs (Markova et al., 2004), suggesting the AR is dispensable for morphogenesis.

Another candidate modulator of MYC activity is p53. p53 is mutated in two-thirds of sebaceous carcinomas (Kiyosaki et al., 2010). Oncogenic levels of MYC activity promote p53 activation indirectly because of DNA damage (Hong et al.,

2006; Murphy et al., 2008; Pusapati et al., 2006). In human sebaceous carcinomas that retain p53 expression, elevated p53 protein levels correlate with worse prognostic outcome (Hasebe et al., 1994; Hayashi et al., 1994; Izumi et al., 2008).

In the prostate, a mutually antagonistic relationship exists between the AR and p53. p53 can inhibit AR gene expression by direct association with the AR promoter (Alimirah et al., 2007) and by inhibiting AR protein activity (Shenk et al., 2001). Conversely, strong AR activity can inhibit p53 expression (Rokhlin et al., 2005) and p53 activity (Nantermet et al., 2004). Several of the p53 mutants identified in sebaceous carcinoma (Kiyosaki et al., 2010) are also found in prostate cancer and retain the ability to impair AR signaling, despite being mutations in the DNA-binding domain (Nesslinger et al., 2003).

Collectively, these observations prompted us to explore the role of AR and p53 in modulating the consequences of MYC activation in the sebaceous gland.

## RESULTS

### Markers of SG Differentiation

The SG is a sac-like structure, comprising an undifferentiated, proliferating, peripheral (basal) layer that gives rise to centrally located differentiating sebocytes. As they accumulate lipids, differentiating sebocytes increase in size (Montagna et al., 1963; Rosenfield, 1989), eventually bursting to release their contents into the sebaceous duct (SD), a thin, cornifying squamous epithelium that connects the SG to the infundibulum of the HF (Laurent et al., 1992; Schneider and Paus, 2010). Experimental evidence from DNA label retaining cells (Reichelt et al., 2004) and lineage tracing has established the existence of SG stem cells (SCs) in the upper HF (Jensen et al., 2009; Snippet et al., 2010). Their progeny travel around the basal layer to the lower sebaceous tip before transiting internally and upward toward the SD as they undergo terminal differentiation (Cui et al., 2003; Petersson et al., 2011) (Figure 1N).

In telogen back skin of wild-type (WT) mice (Figures 1A–1M), strong nuclear AR was observed in the lower SG, with weak cytoplasmic and nuclear AR in the upper SG and elsewhere in the epidermis (Figures 1A and 1B). Proliferative SG cells, marked by Ki67 and proliferating cell nuclear antigen (PCNA) expression, were enriched near the basal SG tip and had weak nuclear AR (Figures 1C and 1D). Nuclear AR is indicative of active AR signaling, and although the distribution of AR-positive cells in the SG was the same in the males and females, males tended to exhibit stronger nuclear staining than females (Figure 1B) (Azzi et al., 2006). Skin is also a local source of androgens (Chen et al., 2006, 2010). Antibody specificity was confirmed by staining skin of AR-TFM mice, which lack functional ARs (Figures S1N, S1O, and S1P).

Differentiating sebocytes expressed lipogenic enzymes, such as fatty acid synthase (FASN) and peroxisome proliferator activated receptor  $\gamma$  (PPAR $\gamma$ ) (Figures 1E and 1F). The largest sebocytes, located next to the SD, had low AR activity, expressed the Myc corepressor, B-lymphocyte induced maturation protein (BLIMP1) (Figure 1G), and were weakly positive for involucrin (IVL) (Lo Celso et al., 2008; Zouboulis et al., 1998) (Figure 1H). Although BLIMP1 is reported to be a sebocyte progenitor marker

(Horsley et al., 2006), we only observed BLIMP1 expression in differentiating sebocytes (Magnúsdóttir et al., 2007) and in keratinocytes of the SD, marked by IVL (Latham et al., 1989) (Figure 1H, see arrows). This is consistent with previous reports that BLIMP1 is a marker of terminal differentiation in all epidermal lineages (Chang et al., 2002; Lo Celso et al., 2008; Magnúsdóttir et al., 2007; Sellheyer and Krahl, 2010).

In wild-type mouse skin, MYC expression was higher in the interfollicular epidermis (IFE) and SG than HFs (Reichelt et al., 2004), consistent with the finding that MYC activation favors differentiation along these lineages (Arnold and Watt, 2001; Honnycutt and Roop, 2004) (Figure 1J). MYC was expressed by differentiating, AR-positive sebocytes (detected with a 1:50 antibody dilution) (Figure 1K); however, the SG cells with highest levels of MYC (detected with a 1:200 antibody dilution) were basal, proliferating cells with low levels of AR (Figures 1L and 1M; Figure S1). Antibody specificity was confirmed on *K14MycER* and epidermal *Myc* knockout mice (Figures S1B–S1G, S1L, and S1M).

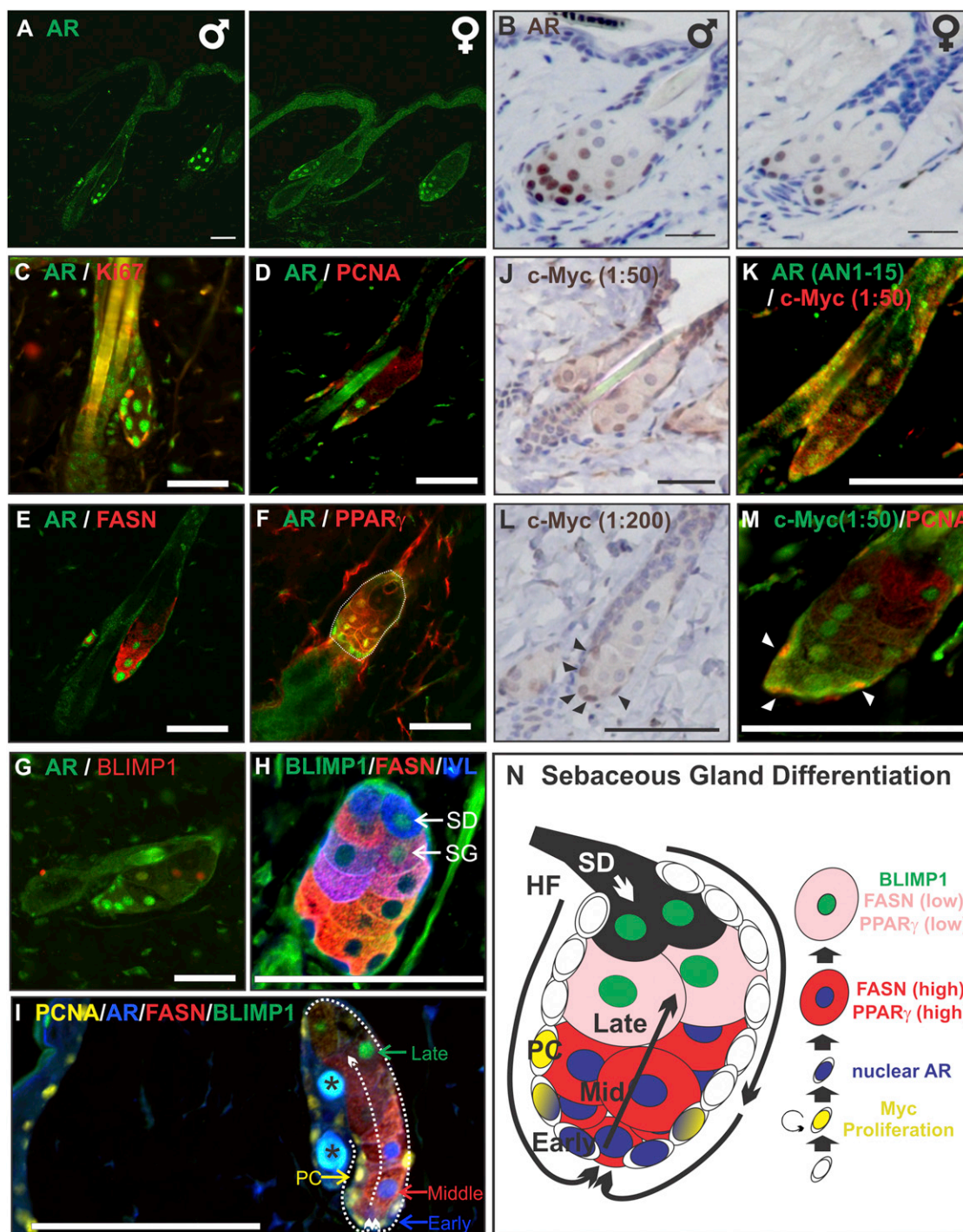
Figure 1N summarizes marker expression within the SG. Proliferative basal cells express the highest levels of MYC, whereas early differentiating sebocytes at the base of the SG have high levels of nuclear AR. Mid-differentiated cells exhibit high nuclear AR and express high levels of FASN and PPAR $\gamma$ . Late-stage differentiating sebocytes, in the upper part of the gland, exhibit low levels of AR activity, low expression of IVL, FASN, and PPAR $\gamma$ , and are BLIMP1+ve.

### Effect of MYC on AR Activity

We next examined the effect of MYC activation on proliferation, differentiation, and AR activity in the epidermis. *K14MycER* mice were treated once with a low (0.1 mg) or high (1.5 mg) dose of 4OHT or vehicle (acetone) and examined for up to 8 days (Figure S2A). Control *K14MycER* mice treated with acetone and WT mice treated with 1.5 mg 4OHT were indistinguishable from untreated WT mice (Figures 2A–2G) (Arnold and Watt, 2001; Berta et al., 2010). Although MYC activation is known to promote IFE thickening and to cause hair follicle abnormalities in *K14MycER* mice (Arnold and Watt, 2001), lineage tracing established that there was no relocation of cells from the IFE to the SG (data not shown).

Four days following low-dose 4OHT treatment, the SG of *K14MycER* mice was enlarged and the differentiation compartment expanded, as assessed by hematoxylin and eosin staining (H&E) staining (Figure S2B) and AR and FASN expression (Figures 2D–2I). On activation, the AR translocates to the nucleus, and this provides a readout of AR activity in vivo. A secondary readout is expression of FASN, which is an AR-responsive gene (Schirra et al., 2005). Differentiation was quantified (Figure 2C) by measuring the average cross-sectional area of the SG differentiation compartment (Figure S2B).

The SGs of *K14MycER* mice treated with 0.1 mg 4OHT enlarged by 4 days. This was not due to increased cell size (Figure 2J) but to an increased number of differentiating sebocytes (Figure 2K), including AR-positive cells (bracket, Figure 2I), which correlated with increased proliferation predominantly in basal layer cells, but also occasionally in differentiated cells. By 8 days, low-dose 4OHT-treated SGs were hyperplastic



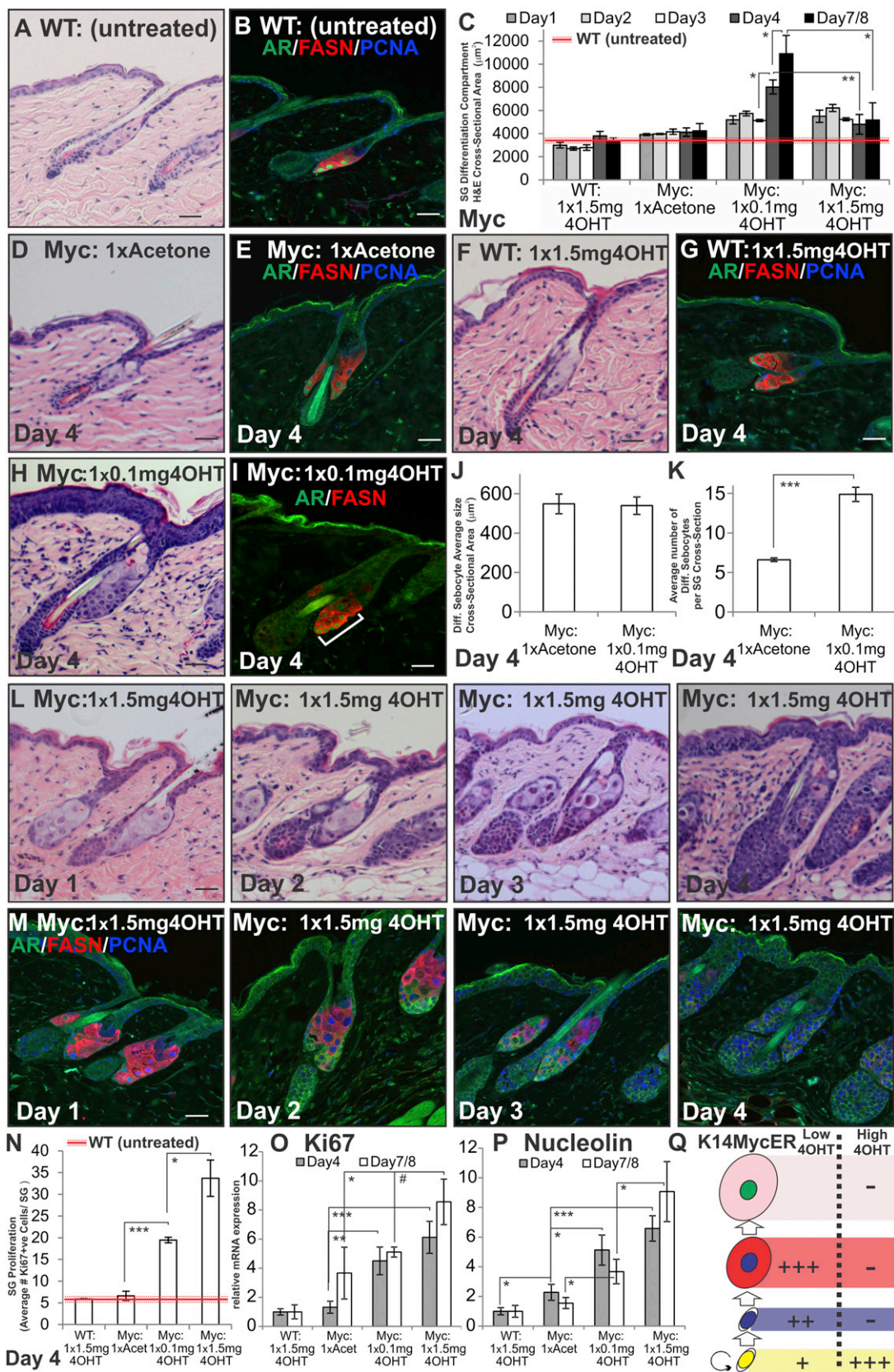
**Figure 1. Expression of the AR and Other Markers in the SG**

(A–M) Adult WT mouse telogen back skin labeled with antibodies to the markers indicated. (H) Arrows indicate a cell of either sebaceous duct (SD) or sebaceous gland lineage (SG). (J–M) c-MYC antibody dilution is indicated. SG is outlined by dashed line in (F). Arrowheads indicate basal c-MYC +ve sebocytes. (I) Asterisks indicate autofluorescent hair shaft.

(N) Schematic of SG showing undifferentiated sebocytes and early, mid, and late stages of terminal differentiation. Circular arrow indicates proliferating cells. HF, hair follicle; SD, sebaceous duct; PC, progenitor cell.

Scale bars, 40  $\mu$ m. See also Figure S1.





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(Figure 2C; Figures S4A and S4C), exhibiting weakened expression of FASN (Figures S4B and S4D) and increased proliferation (Figures S4F and S4H).

In contrast to the effect of a low dose of 4OHT, by 4 days of high-dose 4OHT treatment, the SGs of *K14MycER* mice were filled with undifferentiated, immature sebocytes that frequently lacked nuclear AR and exhibited reduced FASN expression, indicating a collapse in AR signaling (Figures 2L and 2M). In some cases cytoplasmic AR was also reduced (see Figure 6C). This correlated with proliferative expansion of undifferentiated sebocytes at the base of the gland (Figures 2L and 2M). The reduction in the differentiation compartment (Figure 2C) was also observed 8 days after 4OHT treatment, the longest period for which mice could be monitored (Berta et al., 2010). MYC activity remained elevated at 4 and 7–8 days after one dose of 4OHT, as assessed by qRT-PCR of *Nucleolin* (Figure 2P), an established MYC target gene (Berta et al., 2010). In the IFE, formation of the cornified envelope persisted (Figure 2L), indicating the negative effects of high-dose MYC activation on differentiation were most pronounced in the SG lineage.

Although MYC stimulates AR activity and AR expression in prostate (Nadiminty et al., 2012), *Ar* mRNA levels in the skin of *K14MycER* mice were not affected by low or high doses of 4OHT (Figures S2D, S2E, S2F, and S2G). In AR-luciferase assays in immortalized human sebocytes, MYC repressed AR activity in the presence of testosterone but stimulated activity in cells treated with testosterone and the anti-androgen Casodex (Figure S2H), demonstrating MYC can regulate AR activity in a context-dependent manner.

These data suggest that in response to a low dose of 4OHT there is an accumulation of AR-positive differentiated sebocytes, whereas the impairment in sebaceous differentiation upon high-dose 4OHT treatment may reflect the inability of sebocytes to exit the proliferative basal cell compartment and acquire AR activity (Figure 2Q).

### Effect of AR Activity on MYC-Induced SG Differentiation

To test the role of the AR in MYC-induced sebocyte differentiation, *K14MycER* mice were crossed onto the *AR-Shah* x *AR-TFM* strain background. Mice inheriting the *AR-Shah* reporter allele have functional ARs (Shah et al., 2004). Mice inheriting the *AR-TFM* mutation lack functional ARs (Gaspar et al., 1991). Their SGs are morphologically normal (Figure 3A), although sebum production was not assessed (Imperato-McGinley et al., 1993). *K14MycER AR-Shah* mice exhibited a similar response to low and high doses of 4OHT as the original *K14MycER* mice, although the SGs of control *K14MycER*

*AR-Shah/AR-TFM* mice were slightly larger than original control *K14MycER* mice.

Compared to *K14MycER AR-Shah* mice, *K14MycER AR-TFM* mice showed a less pronounced induction of differentiation upon low-dose 4OHT treatment (Figures 3A–3C). FASN expression was reduced (Figure 3D), and there were increased numbers of Ki67+ve cells within the SG (Figures 3E and 3F). Similar results were observed when AR signaling in *K14MycER* mice was inhibited with high doses of Casodex (Figures S3I–S3K). As predicted from the block in differentiation, loss of AR did not affect the response to high-dose 4OHT (Figures 3A–3C). Although AR signaling is reported to regulate MYC protein stability (Bernard et al., 2003), we did not observe a significant change in *Nucleolin* mRNA expression (Figure 3G). We conclude that AR activity is required for efficient MYC-induced SG differentiation (Figure 3H). In contrast, genetic ablation of the *Ar* did not alter the IFE response to MYC or MYC-induced changes in telogen HF s (Arnold and Watt, 2001).

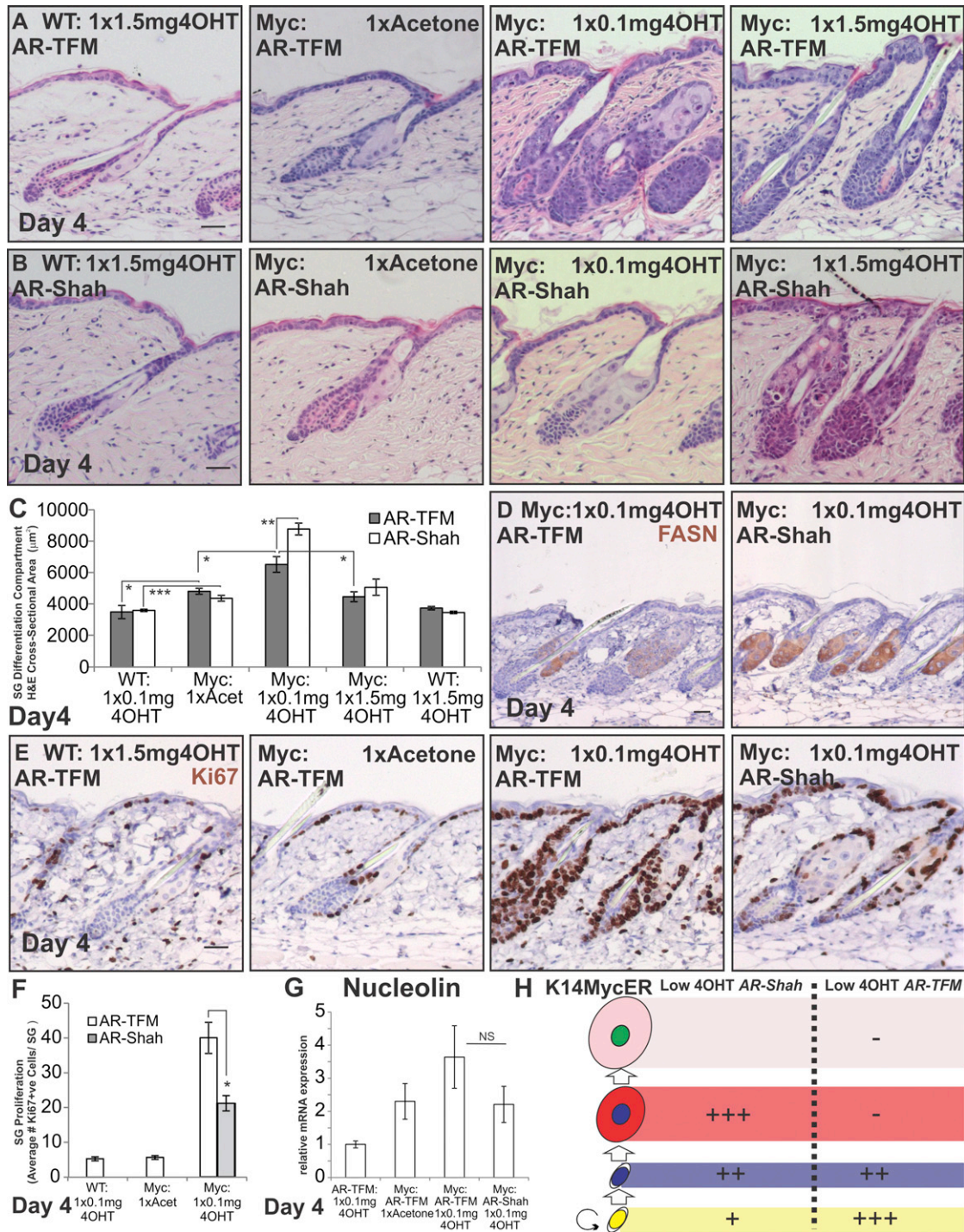
We next examined the effect of activating AR signaling with testosterone. Daily application of 2 mg testosterone had little effect on the number of differentiated sebocytes in WT 4OHT-treated and acetone-treated *K14MycER* mice of either gender, confirming that testosterone did not act directly on the MYCER fusion protein (Figure 4A). Testosterone did not enhance SG differentiation in *K14MycER* mice treated with a low dose of 4OHT for 4 days, suggesting that AR activity was already maximal in this condition (Figures S4G and S4H), although at 8 days SG hyperplasia was reduced (Figures S4E–S4H). In contrast, daily application of 2 mg testosterone to high-dose-treated *K14MycER* mice markedly stimulated differentiation and increased the SG differentiation compartment to the same extent as low-dose 4OHT (Figures 4A and 4D). Relocation of the AR to the nucleus and increased FASN expression confirmed testosterone restored AR activity (Figure 4B). Most sebocytes in skin treated with high-dose 4OHT and testosterone were AR and FASN+ve (Figures 4A and 4B), suggesting downregulation of AR activity might be required for later stages of maturation (Figure 1N). This was confirmed by examining the effect of Casodex on *K14MycER* mice treated with a low dose of 4OHT (Figures S3A–S3H).

The AR-dependent action of testosterone was confirmed by the competitive effect on SG differentiation of high doses of Casodex (Figure 4G) and by the inability of testosterone to rescue sebocyte differentiation in high-dose 4OHT *K14MycER AR-TFM* mice (Figure 4H). Testosterone increased levels of *Nucleolin* mRNA slightly, but the effect was not statistically significant (Figure 4F).

### Figure 2. Effects of MYC Activation on SG Proliferation and Differentiation

(A–Q) WT and *K14MycER* (*Myc*) mouse telogen back skin was untreated, acetone-treated (vehicle), or 4OHT-treated, as indicated. (A, D, F, H, and L) Skin sections were stained with H&E. (B, E, G, I, and M) Skin sections were stained with the antibodies shown. White bracket indicates expansion of AR-expressing sebocytes in (I). (C) Quantitation of SG differentiation compartment (average cross-sectional area occupied by differentiated sebocytes per SG) for the experiment described above. Red line represents average untreated WT measurement, and dashed red lines represent SEM. (J) Quantification of average size of individual differentiated sebocytes (cross-sectional area). (K) Average number of differentiated sebocytes per SG cross-section. (N) Quantitation of Ki67+ve sebocytes per SG. Red line represents untreated WT measurement. (O and P) qRT-PCR of *Ki67* and *Nucleolin* mRNA levels relative to *Gapdh*. (Q) Schematic summary of changes relative to WT SG of treating *K14MycER* mice with low or high dose 4OHT. See Figure 1N for stages in sebocytes differentiation. Increases in cell number are represented by +, ++, or +++, according to the strength of the effect. Reduction in cell number is represented by –. Three to nine mice were examined per condition. Error bars represent SEM #p < 0.06, \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.005. Scale bars 40  $\mu$ m. See also Figure S2.

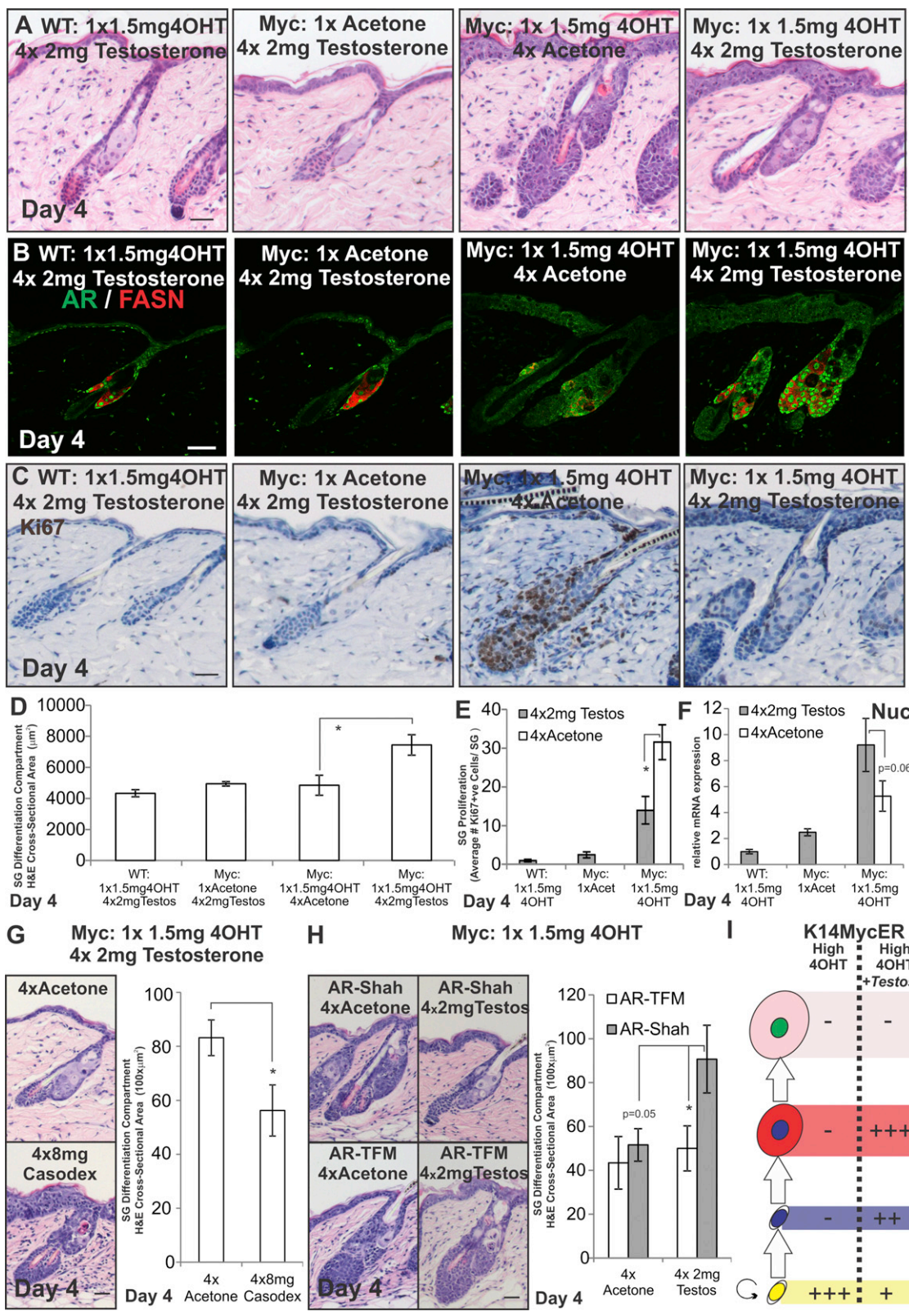




**Figure 3. Effect of Loss of AR Function during MYC Activation**

(A–H) WT and *K14MycER* mice (*Myc*) were crossed with *AR-Shah* or *AR-TFM* mice and treated as indicated. (A and B) Skin sections were stained with H&E. (C) Quantitation of size of SG differentiation compartment. (D and E) Immunostaining (brown) for antibodies indicated. (F) Quantitation of Ki67+ve sebocytes. (G) qRT-PCR of *Nucleolin* mRNA levels relative to *Gapdh*. (H) Schematic summary of the effects of AR loss of function. See Figure 1N for stages in sebocyte differentiation. Increases in cell number are represented by +, ++, or +++, according to the strength of the effect. Reduction in cell number is represented by –. Three to seven mice were examined per condition. Error bars represent SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.005$ . Scale bars, 40  $\mu\text{m}$ . See also Figure S3.





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The differentiation promoting effects of testosterone in *K14MycER* mice treated with a high dose of 4OHT are summarized in Figure 4I.

### Role of p53 in MYC-Induced Sebocyte Differentiation

Oncogenic levels of MYC can activate p53 via DNA damage (Hong et al., 2006; Murphy et al., 2008; Pusapati et al., 2006). p53 can inhibit *AR* gene expression (Alimirah et al., 2007) and activity (Shenk et al., 2001) and block lipogenesis (Hallenborg et al., 2009). We therefore investigated whether p53 modulated the *K14MycER* sebaceous gland phenotype.

MYC activation induced DNA damage throughout the epidermis, as detected by  $\gamma$ -H2AX expression (Pusapati et al., 2006) (Figure 5A). When MYC was activated with high-dose 4OHT, accumulation of active p53 was observed with an antibody to nuclear p53 (Figures 5B and 5G). Antibody specificity was confirmed on *p53null* mice (Figure S5A). However, the number of cells expressing the apoptotic marker cleaved caspase-3 was very low even when high levels of MYC were induced (Figure 5C). The induction of p53 was confirmed by qRT-PCR (Figure 5D); in contrast, levels of total p63 and p73 were not significantly changed (Figures 5E and 5F). Given that p53 was induced within 4 days of MYC activation, it is likely to be wild-type and not mutant.

When *K14MycER* mice were treated with 4OHT and testosterone, nuclear p53 protein (but not mRNA) levels were reduced throughout the epidermis (Figures 5H–5J). p53 activity was higher in *K14MycER AR-TFM* mice treated with a low dose of 4OHT than in *K14MycER AR-Shah* mice (Figures 5K and 5L), confirming that AR functions to repress p53 activity (Nantermet et al., 2004).

To examine whether p53 activation in *K14MycER* mice treated with a high dose of 4OHT contributed to the inhibition of SG differentiation, mice were crossed onto a *p53null* background. In the SG, the effect was most profound and restored differentiation, with increased numbers of cells expressing nuclear AR and FASN, relative to mice that were heterozygous for p53 (Figures 6A–6C). IVL showed some restoration in expression, but BLIMP1 expression was not restored (Figure S5B). Genetic ablation of p53 partially reduced MYC-induced IFE hyperproliferative changes but did not alter MYC-induced changes in telogen HFs (data not shown).

p53 deletion did not increase *Ar* (Figure 6D) or *Nucleolin* mRNA expression (Figure 6E) but increased MYC-dependent induction of *Fasn* and *Ppar $\gamma$*  (Figures 6F and 6G). Consistent with the stimulation of differentiation, there was reduced expression of *Ki67* (Figure 6H) and of *Keratin 7* (K7), a sebaceous lineage marker (Zouboulis et al., 1999) that is expressed in the SG basal layer (Ju et al., 2011) (Figure 6I). Conversely, when p53 activation in

4OHT-treated *K14MycER* mice was enhanced by daily application of camptothecin (a topoisomerase inhibitor that causes DNA breaks and activates p53 [Rudolf et al., 2011]) (Figures S5C–S5F), AR activity and proliferation in the SG lineage were reduced resulting in a reduction in gland size (Figure S5F). Testosterone coapplication partially antagonized the effects of camptothecin on the SG (Figures S5C–S5F).

We were able to generate a single *K14MycER p53null AR-TFM* mouse. This mouse exhibited reduced SG differentiation and enhanced proliferation compared to *K14MycER p53null* controls, with proliferation persisting in FASN+ve sebocytes. Sebocytes were also detected in the IFE (Figure 6J, see arrowhead).

Collectively, these experiments suggest that p53 activation resulting from DNA damage induced by high levels of MYC, contributes to the inhibition of SG differentiation and disruption of AR signaling (Figure 6K). Therefore, AR and p53 form an axis of mutual antagonism controlling the outcome of MYC activation in the SG. For further details of all prior results, please refer to the Extended Results.

### AR and p53 Expression in Human Sebaceous Tumors

To place our observations into the context of human pathophysiology, we examined expression of MYC, Ki67, AR, and p53 in human sebaceous tumors. Human SGs differ from mouse back skin SGs because they are larger and have a multilobed structure. In this regard the human SG resembles the murine preputial gland (Figures 7A and 7B). In both human scalp SGs and mouse preputial glands, we observed significant overlap in the expression of endogenous MYC and the AR, as well as MYC and the proliferative Ki67 basal compartment. We also observed active p53 in basal cells at low frequency (Figures 7A and 7B).

Differentiation status in tumors was assessed in H&E-stained sections and correlated inversely with the number of Ki67+ve cells, with one exception (Figure 7B). MYC was present in all tumors but often at very low levels, and the level of MYC expression exhibited no correlation with proliferation, differentiation status, or tumor type (Figure 7B).

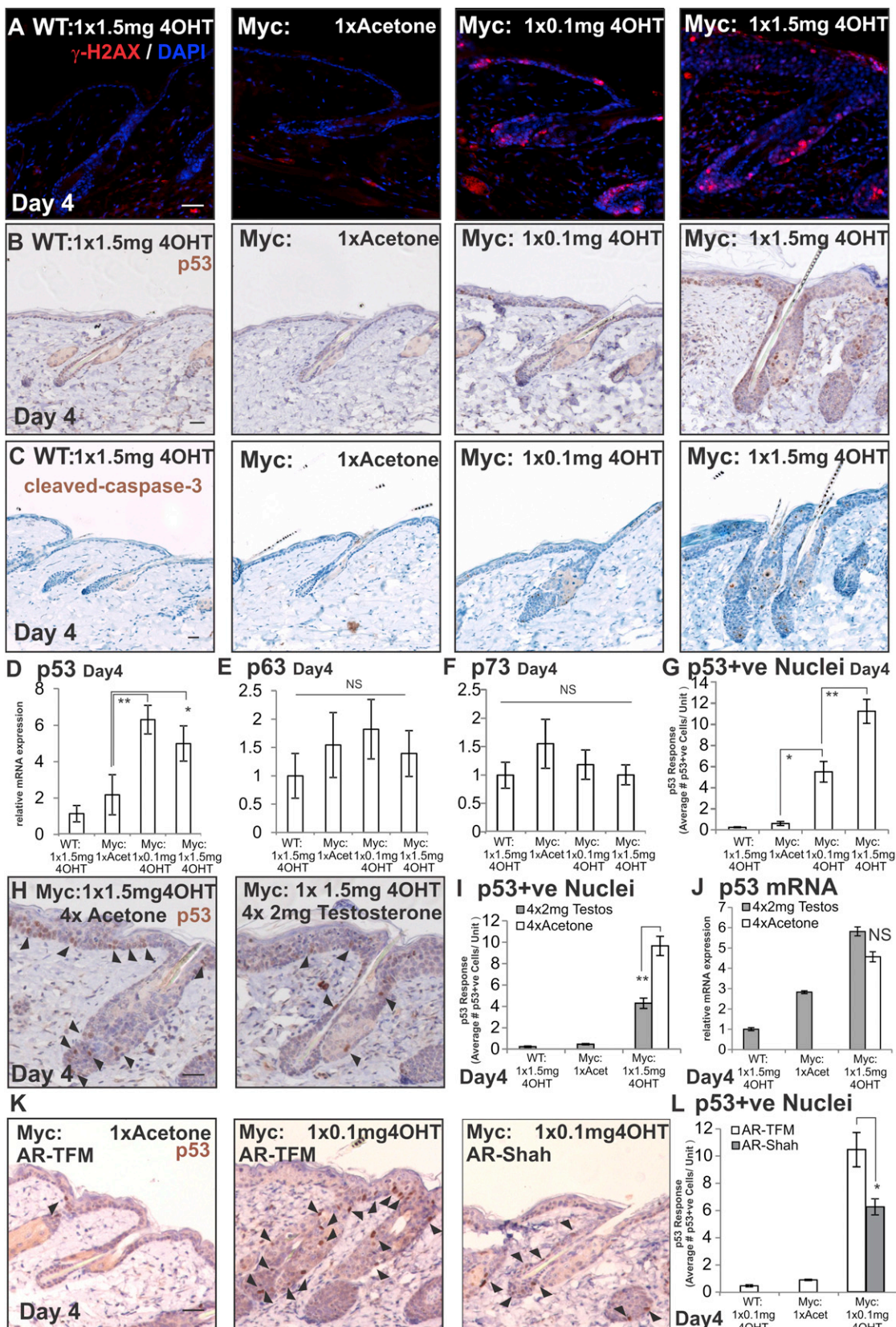
Although p53 is often mutated in sebaceous carcinoma (Kiyosaki et al., 2010), many of these transcriptionally inactive mutations can inhibit AR activity (Nesslinger et al., 2003). Tumors exhibiting reduced differentiation had a higher proportion of p53+ve sebocytes compared to AR+ve sebocytes, whereas those with increased differentiation had an equal or higher proportion of AR+ve sebocytes compared to p53+ve sebocytes. Three specimens exhibited multiple neoplasms within a single section, with distinct regions of adenoma (A) and carcinoma (C). These confirmed that the differences between high and low differentiation status tumors were not due to patient-specific

### Figure 4. Testosterone Modulation of AR Activity during MYC Activation

(A–I) WT, *K14MycER* (Myc<sup>+</sup>), *K14MycER AR-Shah*, and *K14MycER AR-TFM* mice were treated as indicated. (A) Skin sections were stained with H&E. (B and C) Skin sections were stained with the antibodies shown. (D) Quantitation of the SG differentiation compartment. (E) Quantitation of Ki67+ve sebocytes per SG. (F) qRT-PCR of *Nucleolin* (Nuc) mRNA levels relative to *Gapdh*. (G and H) Skin sections were stained with H&E. Quantitation of SG differentiation compartment size is shown. (I) Schematic summary of the effect of testosterone on *K14MycER* mice treated with high-dose 4OHT. See Figure 1N for stages in sebocytes differentiation. Increases in cell number are represented by +, ++, or +++, according to the strength of the effect. Reduction in cell number is represented by –.

Three to six mice were examined per condition. Error bars represent SEM. \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.005. Scale bars, 40  $\mu$ m. See also Figures S3 and S4.





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effects (Figures 7B and 7C). Our results suggest that mutual antagonism of AR and p53 may contribute to progression of sebaceous carcinoma.

## DISCUSSION

Our findings support a model whereby when MYC is overactivated with a low dose of 4OHT, the AR inhibits proliferation and stimulates the onset of differentiation. However, greater MYC activity induces a p53 response as a result of DNA damage, consistent with previous observations (Pusapati et al., 2006). The p53 response (increased nuclear p53 protein and its downstream consequences) inhibits AR activity and without the AR to trigger the onset of differentiation, cells continue proliferating in response to MYC and accumulate as undifferentiated sebocytes. The nonapoptotic p53 response could reflect the reported ability of MYC to inhibit p53-mediated apoptosis (Ceballos et al., 2000).

The AR can promote or retard sebocyte proliferation, depending on androgen type and the body site from which sebocytes are derived (Akamatsu et al., 1992). The phenotype of *AR-TFM* mice highlights the antiproliferative role of the AR in murine back skin following MYC activation but shows that the AR is not required for SG differentiation during normal skin homeostasis (Bayer-Garner et al., 1999; Rosenfield et al., 1998). In rodent meibomian sebocytes, the AR functions early in differentiation to initiate lipogenesis and lipid metabolism via transcriptional regulation of genes, such as *Fasn* and *Ppar $\delta$*  (Schirra et al., 2005, 2006). Placing the AR upstream of the lipogenic program is consistent with our observations because peak AR expression was observed just before the onset of differentiation and then persisted during the first accumulation of lipids, marked by *FASN* and *PPAR $\gamma$* . Downregulation of AR activity late in sebocyte differentiation may play a role in the final stages of sebocyte maturation because low doses of the anti-androgen Casodex facilitated lipid accumulation (Figure S3D). The AR also appears to have a role in repressing p53 activation (Figures 5H, 5I, 5K, and 5L; Figures S4I and S4J and S5C–S5G), as previously reported in prostate (Nantermet et al., 2004).

Loss of p53, like loss of AR, did not affect SG homeostasis, that is, the normal balance between proliferation and differentiation, although p53 is expressed in undifferentiated human and mouse sebocytes (Figures 7A and 7B). p53 deletion restored SG differentiation in high-dose 4OHT-treated *K14MycER* mice, and this rescue was diminished in the absence of AR signaling, highlighting the antagonistic effects of AR and p53. Additional MYC-induced differentiation factors may be inhibited by p53, such as *PPAR $\gamma$*  (Figure 6G), which can bypass the requirement for androgens in normal SG differentiation (Rosenfield et al., 1999). Although p53 activation in *K14MycER* mice did not induce apoptosis, epidermal *Setd8* deletion triggers apoptotic p53 acti-

vation, resulting in loss of the IFE and SGs (Driskell et al., 2011a). Increasing p53 activity with camptothecin (Figures S5E and S5G) resulted in an apoptotic phenotype that was similar to the early stages of epidermal *Setd8* deletion (Driskell et al., 2011a), demonstrating that different thresholds of p53 activation can have distinct outcomes.

Our observation that AR and p53 are mutually antagonistic in regulating MYC-induced sebocyte differentiation is relevant to human sebaceous carcinoma. As in the mouse model, increased numbers of p53-positive sebocytes correlated fewer AR-positive sebocytes, reduced differentiation, and poor prognosis in the tumors (Figure 7) (Bayer-Garner et al., 1999; Hasebe et al., 1994; Hayashi et al., 1994; Izumi et al., 2008). MYC was detected in all the tumors we examined and did not correlate with Ki67 expression, consistent with a role of MYC in regulating both proliferation and differentiation. Although in contrast to *K14MycER* mice, p53 is often mutated in sebaceous carcinoma (Kiyosaki et al., 2010) as a result of DNA damage, and these mutations are still capable of inhibiting AR activity when tested in prostate cells (Nesslinger et al., 2003). It is possible therefore that within p53-positive sebaceous carcinomas, p53 accumulation impairs AR signaling without inhibiting proliferation (Figures 5, 6, and 7). p53 could interfere posttranslationally with AR activity by disrupting AR homodimerization and DNA binding (Shenk et al., 2001). Alternatively, because AR and p53 protein stability are both regulated by murine double minute 2 (MDM2), AR downregulation in tumors could be due to activation of MDM2 in response to increased p53 (Kulikov et al., 2010; Lin et al., 2002). Finally, p53 could indirectly affect AR signaling by reducing local androgen synthesis in the skin (Chen et al., 2006, 2010; Hallenborg et al., 2009). The observation that p53 deletion confers resistance to spontaneous and chemically induced epidermal papillomas and squamous cell carcinomas (Greenhalgh et al., 1996) suggests that p53 activation also influences differentiation in the interfollicular epidermis.

In conclusion, our study identifies an AR/p53 axis that determines the outcome of MYC overactivation in the SG. When MYC activity is moderately elevated, the AR functions to prevent p53 activation, terminate proliferation, and promote the onset of differentiation. However, in response to high MYC activity, p53 can block AR signaling and thereby inhibit differentiation, leading to expansion of undifferentiated sebocytes. Our observations help to explain how MYC, an oncogene, can trigger SG differentiation and how activation of p53 can facilitate proliferation of undifferentiated cells in human sebaceous carcinomas via downregulation of the AR.

## EXPERIMENTAL PROCEDURES

### Transgenic Mice

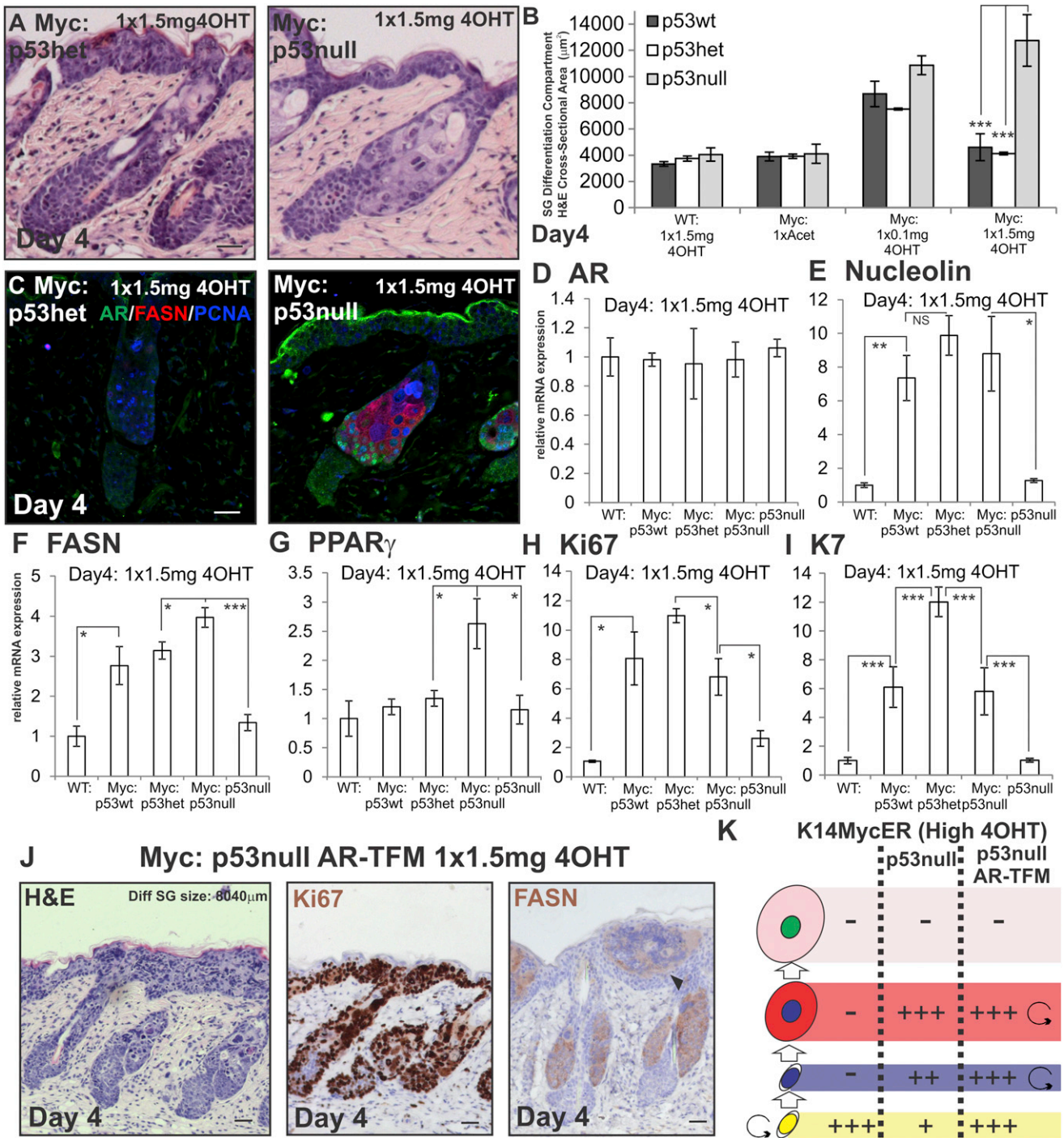
*K14MycER* transgenic mouse founder line 2184C.1 (Arnold and Watt, 2001) was used for this study and maintained on a C57/Bl6 x CBA F1 background.

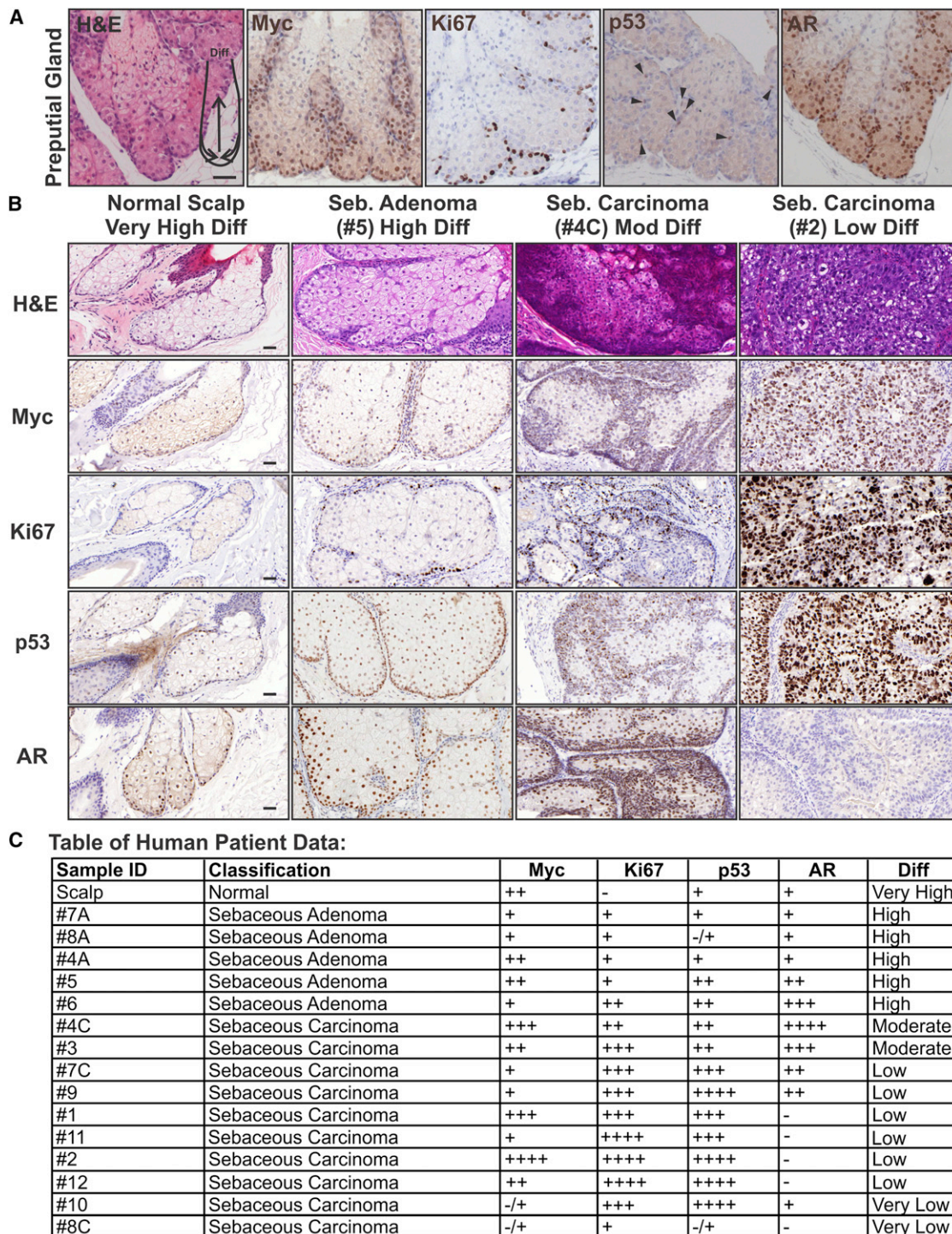
### Figure 5. Effect of MYC and AR Activation on p53 Expression, DNA Double Strand Breaks, and Apoptosis

(A–L) WT and *K14MycER* (Myc) mice were treated as indicated. (A) Skin sections were labeled with antibodies to  $\gamma$ -H2AX with DAPI nuclear counterstain. (B, H, and K) Skin sections were labeled with antibodies to p53 (arrowheads indicate positive nuclei). (C) Skin sections were labeled with antibodies to cleaved caspase 3. (D–F) qRT-PCR of mRNA levels relative to *Gapdh*. (G, I, and L) Quantitation of p53+ve nuclei per unit area of skin (HF, SG, and overlying IFE). (J) qRT-PCR of *p53* mRNA levels relative to *Gapdh*.

Three to nine mice were examined per condition. Error bars represent SEM. \* $p < 0.05$ , \*\* $p < 0.01$ . Scale bars, 40  $\mu$ m.







**Figure 7. p53 and AR Expression in Human Sebaceous Tumors**

(A) Mouse preputial gland sections were stained with H&E or the antibodies shown. Diagram on H&E indicates differentiation within each sebaceous lobe. Arrowheads indicate p53+ve cells.

(B) Representative tumor sections are stained with H&E or the antibodies shown. Scale bars 40  $\mu$ m.

(C) Summary of immunostaining and differentiation status of individual tumors. Staining was scored as follows. – negative, –/+ almost entirely negative, + to ++++ increasing proportion of positive cells, with ++++ indicating almost every cell positive.



The other strains of mice used in crosses are described in the [Extended Experimental Procedures](#). Mice were treated once with 100  $\mu$ l acetone or 0.1 or 1.5 mg 4OHT (Sigma-Aldrich H6278; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 100  $\mu$ l acetone. 4OHT was applied to clipped lower back skin, and mice were analyzed 1–8 days later. In some experiments, mice additionally received daily doses of 100  $\mu$ l of acetone and/or 2 mg testosterone (Testos; Sigma-Aldrich T1500) and/or 2, 4, or 8 mg Casodex (bicalutamide anti-androgen; Sigma-Aldrich B9061) in 100  $\mu$ l acetone. See [Figure S2A](#) for treatment illustration and the [Extended Experimental Procedures](#).

All experiments were performed on a minimum of three mice per condition, with the exception of *K14MycER p53null AR-TFM* mice. Experiments were subject to Cancer Research UK ethical review and performed under the terms of a UK Government Home Office license.

#### Immunofluorescence, Immunohistochemistry, and Microscopy

Primary and secondary antibodies and labeling procedures are described in the [Extended Experimental Procedures](#) and [Table S1](#). Immunofluorescence (IF) slides were counterstained with the nuclear dye DAPI. Immunohistochemistry slides were counterstained with hematoxylin.

#### qRT-PCR

RNA isolation, cDNA preparation, and qRT-PCR were performed using the Trizol method as described previously ([Driskell et al., 2011a](#)) and described in detail in the [Extended Experimental Procedures](#).

#### Human Tissue

Samples were collected, diagnosed, and provided by H.G. and S.R.Q. or S.A. and K.N. All samples were obtained with informed consent and processed for research in accordance with the recommendations of the relevant local ethics committees: CRUK Cambridge Research Institute (number 08/H0306/30), German Medical Council, and/or the Japanese Ministry of Health, Labor, and Welfare.

#### Quantitation and Statistics

Quantitation of Ki67, p53+ve cells, and the average cross-sectional area of the SG differentiation compartment were determined from at least ten 10x images of H&E-stained tissue sections per mouse. Only vertical sections of back skin were quantitated. The SG differentiation compartment was identified in H&E sections on the basis of differentiating sebocytes exhibiting pale and enlarged cytoplasm from lipid accumulation. Examples of how measurements were made are shown in [Figure S2B](#). Statistical analysis was performed using the unpaired Student's *t* test.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Results, Extended Experimental Procedures, five figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2013.01.013>.

#### LICENSING INFORMATION

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#### ACKNOWLEDGMENTS

We thank K. Jensen, R. Driskell, and M. Mastrogriannaki for advice; E. Nascimento and M. Frye for reagents; A. Lamb for mammalian Myc expression constructs; and CRI and SCI core staff for expert technical assistance, in particular P. Humphreys, M. McLeish, H. Skelton, A. Mowbray, M. Webb, and J. Fox. F.M.W. gratefully acknowledges the financial support of the MRC, Wellcome Trust, CRUK, and the EU FP7 programme. We also acknowledge the support of the University of Cambridge and Hutchison Whampoa Ltd.

K.K. is the recipient of a MRC PhD studentship. P.J.S. is the recipient of a CRUK PhD studentship.

Received: March 27, 2012

Revised: October 28, 2012

Accepted: January 14, 2013

Published: February 7, 2013

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