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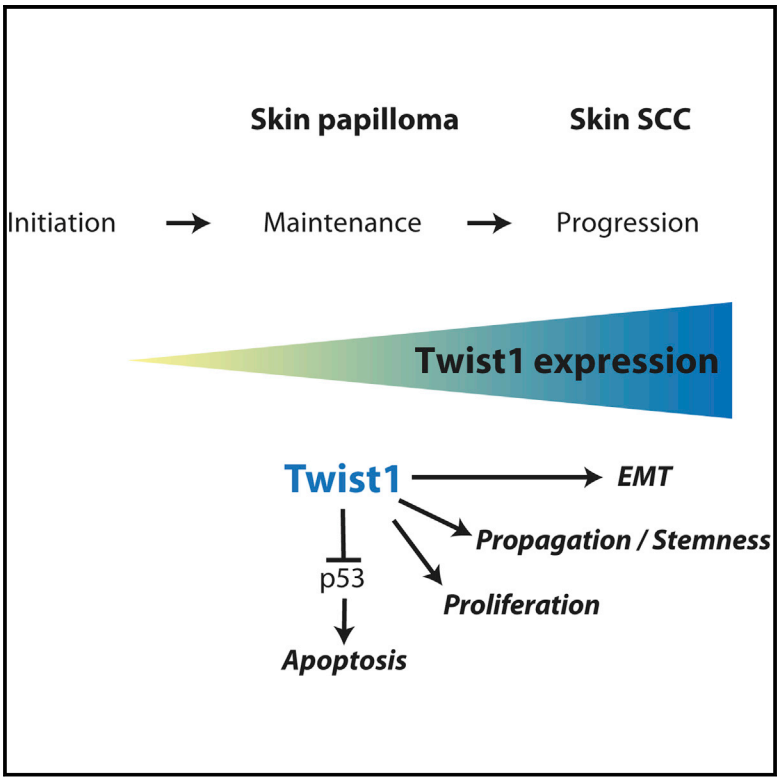
Cell Stem Cell

Different Levels of Twist1 Regulate Skin Tumor Initiation, Stemness, and Progression

Graphical Abstract



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In Brief

Twist1 promotes tumor stemness and EMT. It remains unclear whether Twist1 also controls tumor initiation and whether the different Twist1 functions are linked. Using Twist1 deletion at different stages of skin tumorigenesis, Beck et al. show that Twist1 is required for tumor initiation, stemness, and progression in a gene-dosage-dependent manner.

Highlights

- Twist1 is required for skin tumor initiation
- Twist1 controls tumor initiation and progression in a gene-dosage-dependent manner
- Twist1 controls apoptosis and propagation in a p53-dependent and -independent manner
- Twist1 controls tumor maintenance and stemness independently of its EMT function

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Article

Different Levels of Twist1 Regulate Skin Tumor Initiation, Stemness, and Progression

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SUMMARY

Twist1 promotes epithelial-to-mesenchymal transition (EMT), invasion, metastasis, and cancer stem cell (CSC) properties. However, it remains unclear whether Twist1 is also required for tumor initiation and whether Twist1-induced cancer stemness and EMT are functionally linked. Using a conditional deletion of Twist1 at different stages of skin carcinogenesis, we show that Twist1 is required for skin tumor initiation and progression in a gene-dosage-dependent manner. Moreover, conditional ablation of *Twist1* in benign tumors leads to increased apoptosis, reduced cell proliferation, and defective tumor maintenance and propagation independently of its EMT-inducing abilities. Concomitant deletion of Twist1 and p53 rescues the apoptotic response, but not the cell proliferation and propagation defects. These results reveal that Twist1 is required for tumor initiation and maintenance in a p53-dependent and -independent manner. Importantly, our findings also indicate that tumor stemness and EMT can be regulated by distinct mechanisms.

INTRODUCTION

The epithelial-to-mesenchymal transition (EMT) is a cellular process during which epithelial cells lose their adhesion properties and acquire mesenchymal features allowing their migration and invasion (Kalluri and Weinberg, 2009; Nieto, 2013; Thiery et al., 2009). EMT is essential during embryonic development for gastrulation, neurogenesis, and other developmental programs during morphogenesis (Nieto, 2013). The EMT program is activated in a wide variety of cancer cells as they leave the primary tumor and colonize distant organs and form metastases (Nieto, 2013; Puisieux et al., 2014). EMT has also been reported to suppress tumor apoptosis and oncogene-induced senescence (Ansieau et al., 2008; Burns et al., 2013; Emadi Baygi et al., 2010; Kwok et al., 2007; Lee and Bar-Sagi, 2010; Maestro et al., 1999; Sayan et al., 2009; Tran et al., 2012; Valsesia-Wittmann et al., 2004) and to produce cancer cells with stem cell-like properties (Mani et al., 2008; Masui et al., 2014; Morel et al., 2008; Wellner et al., 2009; Zhou et al., 2014). Transcription factors such as Twist, Snail, Zeb, and FoxC families are expressed in different cancers, and their overexpression induces EMT as well as cancer stem cell (CSC)-properties in a variety of cancer cell lines (Mani et al., 2008; Morel et al., 2008; Nieto, 2013; Puisieux et al., 2014).

Twist1, a basic-helix-loop-helix transcription factor, is one of the EMT-inducer prototypes (Ansieau et al., 2010). Twist1 deletion in mice leads to failure in neural tube closure associated with defects in the formation of the head mesenchyme and limb buds (Chen and Behringer, 1995). Twist1 is expressed in tumors of different origins such as sarcomas, gliomas, squamous cell carcinoma (SCCs), breast cancers, and melanomas (Ansieau et al., 2010). Mechanistically, Twist1 overexpression has been shown to rescue Myc-induced apoptosis through inhibition of p53 (Maestro et al., 1999; Valsesia-Wittmann et al., 2004). Twist1 knockdown decreases the metastatic potential of mammary tumor cell lines (Yang et al., 2004). In addition, Twist1 overexpression induces EMT and E-cadherin repression, suggesting that Twist1 promotes metastasis by inducing EMT (Morel et al., 2012; Tsai et al., 2012; Yang et al., 2004). In addition to these antiapoptotic and prometastatic roles, Twist1 overexpression in mammary epithelial and cancer cell lines has been shown to promote their ability to self-renew as mammospheres in vitro and to engraft into immunodeficient mice, suggesting that Twist1 promotes tumor stemness (Mani et al., 2008; Morel et al., 2008).

However, to what extent these different functions of Twist1 including its effect on EMT, stemness, proliferation, and apoptosis are functionally linked or whether these functions are independently regulated by Twist1 remains unknown. Also, while the role of Twist1 in cancer cell survival (Ansieau et al., 2008; Burns et al., 2013; Hasselblatt et al., 2009; Kwok et al., 2007; Lee and Bar-Sagi, 2010; Maestro et al., 1999; Tran et al., 2012; Valsesia-Wittmann et al., 2004) and progression by inducing invasion and extravasation through EMT is well-established both in vitro and in vivo (Elias et al., 2005; Hasselblatt et al., 2009; Lee et al., 2006; Mikheeva et al., 2010; Tsai et al., 2012; Yang et al., 2004; Yuen et al., 2007), the role of Twist1 in the early steps of tumor initiation remains elusive. Here, we used skin

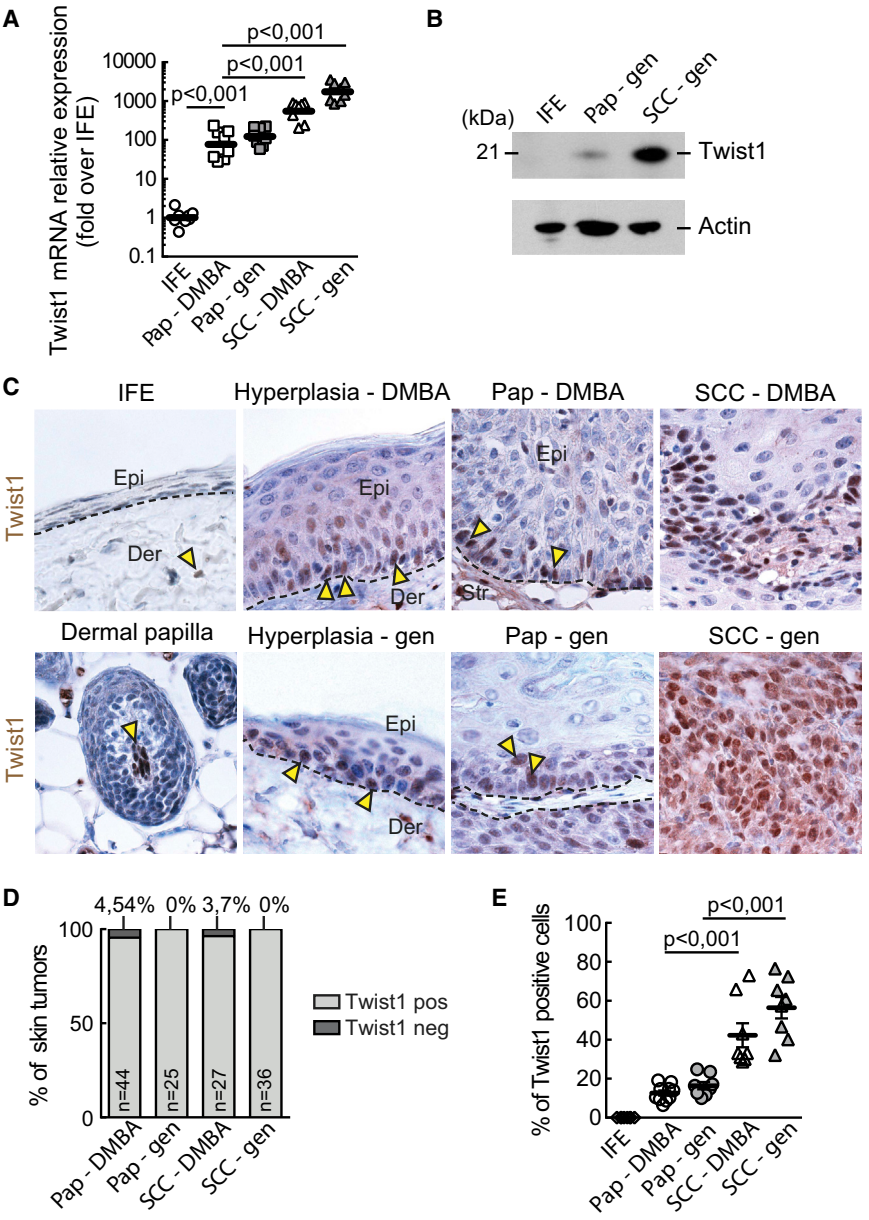


Figure 1. Twist1 Is Expressed at the Early Stages of Skin Tumorigenesis

(A) qRT-PCR analysis of Twist1 mRNA expression performed on FACS isolated normal interfolicular epidermis (IFE) and tumor cells. Data were normalized to normal IFE (n = 8/condition).

(B) Western blot for Twist1 was performed on FACS isolated normal IFE cells, genetically induced papilloma and SCC.

(C) Twist1 IHC performed on skin and tumor sections.

(D) Quantification of the proportion of Twist1 positive tumors.

(E) Quantification of the proportion of Twist1 positive epithelial cells by IHC, showing the increase of the proportion of Twist1 positive cells in invasive SCCs (n = 8/condition); dermis (Der); epithelium (epi); and stroma (str).

Scale bars, 50 μm (see also Figures S1 and S2). Error bars represent SEM.

tumorigenesis as a model to investigate these important and open questions. Mouse skin SCC is an ideal model to tackle these questions, as skin tumors arise from mutations in the *Ras* gene (Kemp, 2005), which have been previously shown to synergize with Twist1 during tumorigenesis (Morel et al., 2012). Skin tumorigenesis can be clearly separated into different stages including tumor initiation, malignant progression, and metastasis (Kemp, 2005). Finally, skin SCCs contain cells with CSC properties characterized by enhanced propagating capacity following their serial transplantation into immunodeficient mice (Boumahdi et al., 2014; Lapouge et al., 2012; Malanchi et al., 2008; Schober and Fuchs, 2011).

In this study, using conditional deletion of Twist1 at different stages of skin tumorigenesis, we investigated the different functions of Twist1 and their interdependence during tumor initiation, maintenance, propagation, and malignant progression. We found that Twist1 is expressed at the earliest step of tumorigenesis and is essential for the initiation and malignant progression of skin tumors in a gene-dosage-dependent manner. Conditional deletion of Twist1 in pre-existing tumors demonstrates the essential role of Twist1 in tumor maintenance. Twist1 inhibits oncogene-induced apoptosis in a p53-dependent manner, while the ability of Twist1 to promote tumor cell proliferation and propagation is regulated by a p53-independent mechanism. Strikingly, these oncogenic functions of Twist1 are not dependent upon its ability to induce EMT.

RESULTS

Twist1 Is Expressed at the Early Stages of Skin Tumorigenesis

Twist1 has been detected in tumor propagating cells (TPCs) of mouse skin SCCs (Lapouge et al., 2012; Schober and Fuchs, 2011), and Twist1 overexpression promotes malignant progression of skin tumors (Morel et al., 2012; Tsai et al., 2012). However, the temporal dynamic of Twist1 expression, particularly during the early stages of skin tumorigenesis, remains to be established. To address this question, we measured Twist1 mRNA levels by quantitative (q)RT-PCR in normal skin epidermis, in DMBA (9, 10-dimethyl-1, 2-benzanthracene)/TPA (12-*O*-tetradecanoyl phorbol-13-acetate), and genetically (K14CreER:KRas^{G12D} and K14CreER:KRas^{G12D}:p53$^{fl/fl}$) induced papilloma and SCCs isolated by fluorescence associated cell sorting (FACS) (Figure 1A and Figure S1A available online). Twist1 mRNA was upregulated by 100-fold in papilloma as compared to normal epidermis, and its expression further increased in SCCs (Figure 1A).

Twist1 protein was undetectable in normal epidermal cells by western blot (Figure 1B) and immunohistochemistry (Figures 1

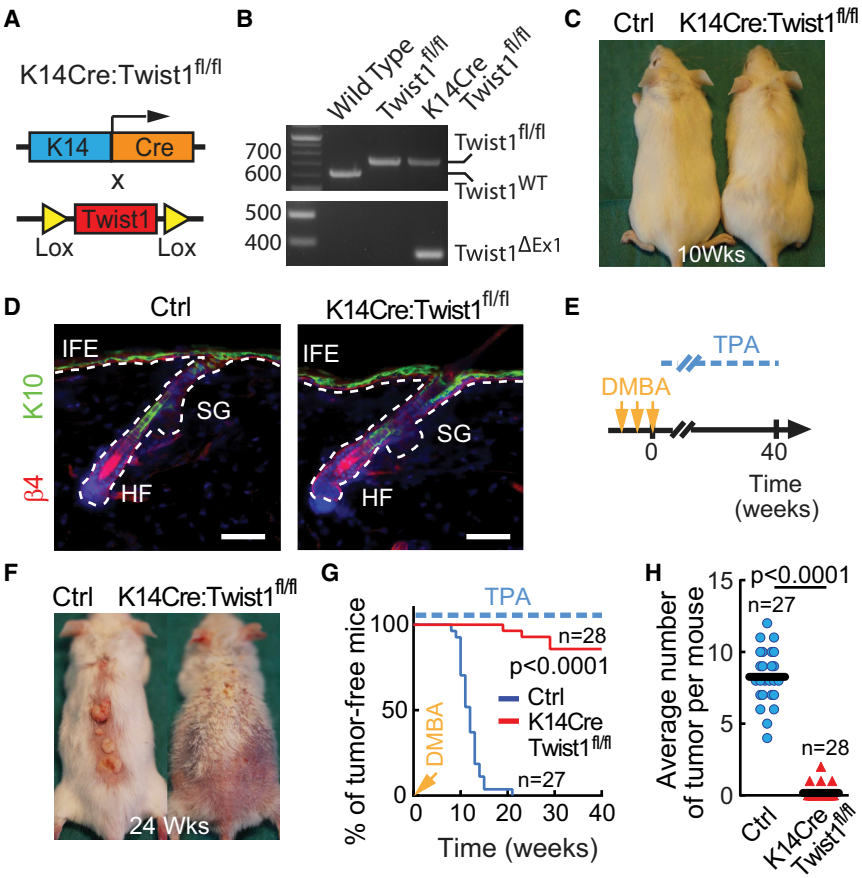


Figure 2. Twist1 Is Critical for Skin Tumor Initiation

(A) Genetic strategy used to study the role of Twist1 during skin tumor initiation.

(B) PCR for Twist1 flox, deleted ($Twist1^{\Delta Ex1}$) and wild-type alleles.

(C) Picture of representative 10-week-old $Twist1^{fl/fl}$ (Ctrl) and K14Cre:$Twist1^{fl/fl}$ mice, showing that Twist1cKO epidermis was macroscopically normal.

(D) Immunostaining for β4 integrin and K10 in control (Ctrl), showing that Twist1cKO epidermis is microscopically normal.

(E) Strategy used to determine the impact of Twist1 deletion in epidermal cells on skin carcinogenesis.

(F) Picture of representative Ctrl and K14Cre:$Twist1^{fl/fl}$ mice 24 weeks following the first DMBA application, showing the absence of skin tumors in K14Cre:$Twist1^{fl/fl}$ mice.

(G) Percentage of Ctrl and K14Cre:$Twist1^{fl/fl}$ mice with skin tumors over time. $p < 0.0001$ by log rank test.

(H) Average number of skin tumors on Ctrl and K14Cre:$Twist1^{fl/fl}$ mice 40 weeks following the first DMBA application. $p < 0.0001$ by unpaired t test.

and S1B–S1D); only fibroblasts from the dermis and the mesenchymal part of the hair follicle, called the dermal papillae, expressed Twist1 in the normal skin (Figure 1C). Twist1 was expressed in some epidermal cells following KRas-activation or following DMBA/TPA treatment, prior the appearance of benign tumors (Figure 1C), but not in hyperplasia induced by TPA or retinoic acid treatment in the absence of oncogenic mutations (Figures S1C and S1D). Twist1 was expressed in the vast majority of DMBA/TPA and genetically induced (KRasG12D/p53 conditional knockout [cKO]) skin tumors (Figure 1D), and the level of Twist1 further increased in SCCs (Figures 1A–1C). In addition to the increase of Twist1 expression, Twist1 was also expressed in more cells in SCC (50%) as compared to papilloma (15%) (Figure 1E). These results indicate that the induction of Twist1 expression is an early event during mouse skin tumorigenesis and precedes tumor development.

To investigate the relevance of our data to human SCCs, we analyzed Twist1 expression in a series of 32 SCCs and five normal skins. As found in the mouse skin, Twist1 was not expressed in the normal human epidermis and was expressed only in dermal cells (Figure S2A). However, more than 50% of human skin SCCs (Figure S2B) were positive for Twist1, showing that Twist1 is expressed in both mouse and human skin SCCs.

Twist1 Is Critical for Skin Tumor Initiation

While many studies have demonstrated a role for Twist1 in tumor cell survival (Ansieau et al., 2008; Kwok et al., 2007; Maestro et al., 1999; Tran et al., 2012; Valsesia-Wittmann et al., 2004) and propagation (Mani et al., 2008; Mikheeva et al., 2010; Morel et al., 2008), the role of Twist1 during tumor initiation has not been yet investigated. To determine whether Twist1 regulates the early steps of skin tumorigenesis, we conditionally deleted Twist1 in the developing skin epidermis using the K14-CRE allele (K14Cre:$Twist1^{fl/fl}$), which is active as early as E12 and results in the deletion of the Twist1 floxed alleles in the skin epidermis (Figures 2A and 2B). K14Cre:$Twist1^{fl:fl}$ mice were born at the expected Mendelian ratio; these mice were indistinguishable morphologically and histologically from control mice at 10 weeks of age (Figures 2C and 2D). These results indicate that Twist1 expression in the skin epidermis is not required for the morphogenesis, postnatal growth, and adult homeostasis of the skin epidermis. To assess the role of Twist1 during skin tumorigenesis, we treated K14Cre:$Twist1^{fl:fl}$ mice and their control littermates with DMBA/TPA. In the 40 weeks following the first DMBA administration (Figure 2E), only a small fraction (14%) of the K14Cre:$Twist1^{fl/fl}$ mice developed one or two tumors, while all of the control mice developed on average eight tumors per mouse (Figures 2F–2H). These results indicate that Twist1 controls skin tumor initiation. The rare tumors observed on Twist1 cKO mice were macroscopically and microscopically similar to the ones that developed on control animals and may arise from distinct genetic and/or epigenetic mechanisms that may render Twist1 dispensable to tumor initiation.

Twist1 Regulates Tumor Initiation and Progression in a Dose-Dependent Manner

Since Twist1 expression increases with tumor progression, we also assessed whether Twist1 is required for malignant progression by decreasing *Twist1* gene dosage in a genetic/inducible

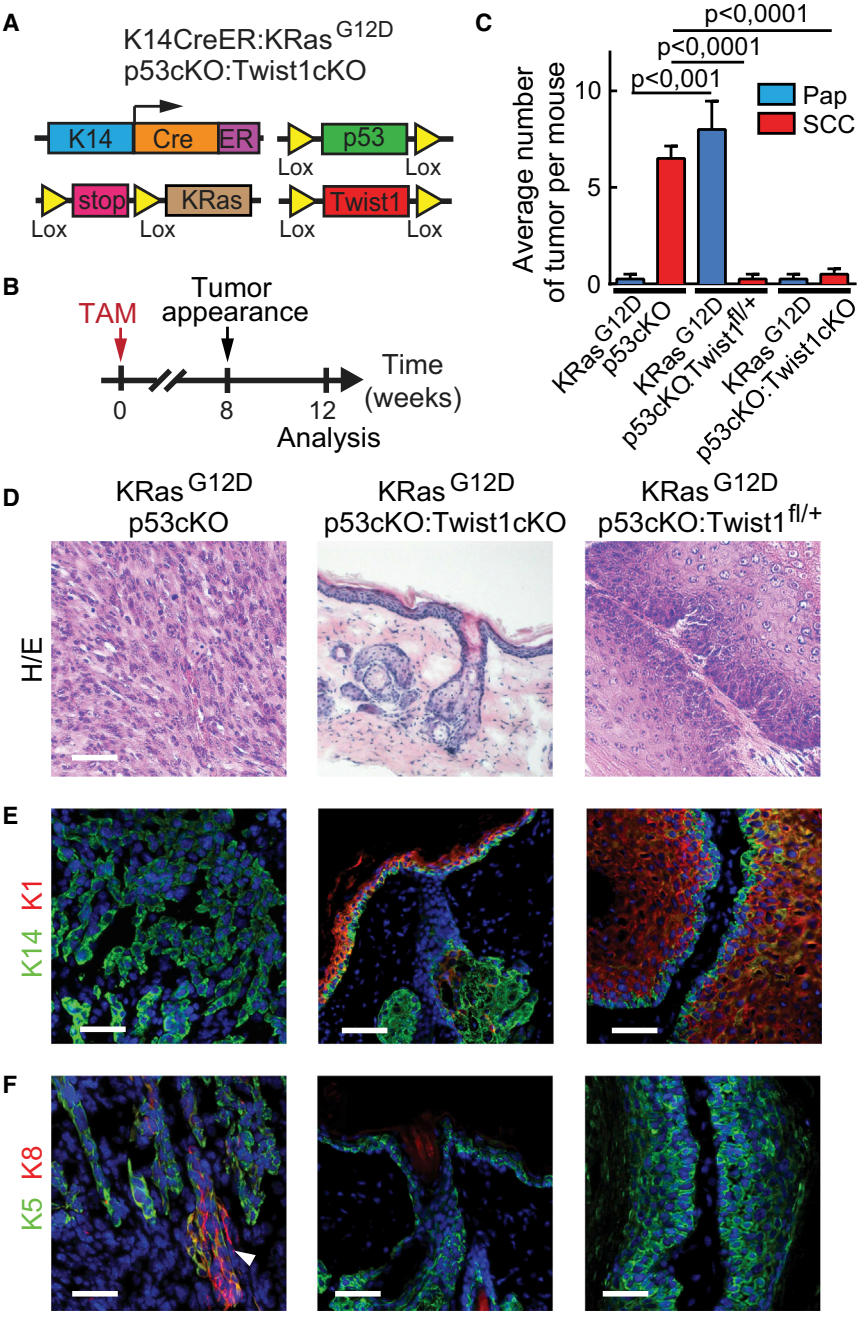


Figure 3. Twist1 Regulates Tumor Initiation and Progression in a Dose-Dependent Manner

(A and B) Genetic strategy (A) and experimental design (B) used to assess gene dosage requirement of Twist1 for tumor initiation and progression.

(C) Average number of papilloma and SCC on K14CreER:KRas^{G12D}:p53^{cKO}, K14CreER:KRas^{G12D}:p53^{cKO}:Twist1$^{fl/fl}$, and K14CreER:KRas^{G12D}:p53^{cKO}:Twist1$^{fl/+}$. $p < 0.0001$ by Kruskal-Wallis test followed by Dunn's multiple comparisons test (n = 7/genotype).

(D–F) Hematoxylin/Eosin (D) and Immunostaining for K14 and K1 (E) or for K5 and K8 (E), showing that K14CreER:KRas^{G12D}:p53$^{fl/fl}$:Twist1$^{fl/fl}$ mice did not develop any tumors, K14CreER:KRas^{G12D}:p53$^{fl/fl}$:Twist1$^{fl/+}$ mice developed papilloma, while K14CreER:KRas^{G12D}:p53$^{fl/fl}$ mice preferentially develop SCCs.

Scale bars, 50 μm. Error bars represent SEM.

skin cancer model (Figure 3A). Tamoxifen (TAM) administration to K14CreER:KRas^{G12D}:p53$^{fl/fl}$ mice led to the appearance of tumors within 12 weeks (Figure 3B) that rapidly progress into invasive SCC (Lapouge et al., 2011) (Figures 3C–3F). In sharp contrast, TAM administration to K14CreER:KRas^{G12D}:p53$^{fl/fl}$:Twist1$^{fl/fl}$ mice did not lead to the development of any tumor (Figures 3C–3F). This observation parallels the absence of tumor formation in DMBA/TPA-treated mice upon Twist1 inactivation and further confirms that Twist1 is required for skin tumor initiation. Interestingly, deletion of only one allele of *Twist1* (K14CreER:KRas^{G12D}:p53$^{fl/fl}$:Twist1$^{fl/+}$) did not change the number of tumors formed (Figure 3C), but was sufficient to block the conversion of papilloma to malignant SCCs, as illustrated by their benign histology (Figure 3D) and the persistence of K10 expression; a marker of differentiation in normal skin and papilloma (Figure 3E), and the absence of K8, a marker expressed in malignant skin SCC (Figure 3F). These results indicate that different levels of Twist1 are required for benign skin tumor initiation and malignant progression and show that low level of Twist1 that requires the expression of both Twist1 alleles is necessary for malignant progression.

Twist1 Controls Tumor Propagation of Primary Skin Tumors

The gold standard assay to study cancer stem cell properties is the transplantation of limiting dilution of tumor cells to assess their capacity to reform secondary tumors into immunodeficient mice. Overexpression as well as short hairpin RNA-induced knockdown of Twist1 in tumor cell lines affects E-cadherin expression, EMT, and the capacity of tumor cells to self-renew in vitro and to be propagated into immunodeficient mice in vivo (Mani et al., 2008; Morel et al., 2008). Hence, it was suggested that EMT induces tumor stemness. To determine whether tumor stemness and EMT are functionally linked, we first assessed in our experimental system whether Twist1 regulates tumor-propagating potential of primary skin tumors (Figure 4A). As previously reported (Lapouge et al., 2012), the transplantation of 10^6 total cells from papilloma, including tumor epithelial cells (TECs) and stromal cells, induced the formation of secondary tumors in about 80% of the recipient mice (Figure 4B). However, none of the Twist1 deleted TECs were able to reform secondary tumors in these experimental conditions (Figure 4B). Analysis of

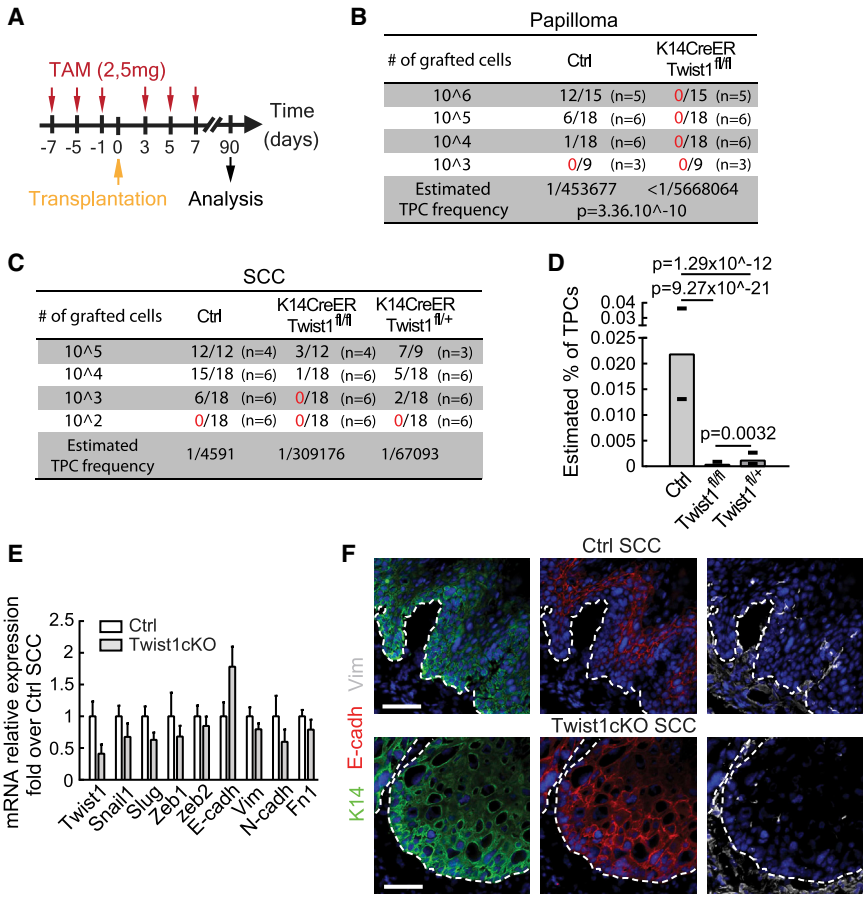


Figure 4. Twist1 Controls Tumor Propagation of Primary Skin Tumors

(A) Experimental design to assess the role of Twist1 in regulating tumor propagation into immunodeficient mice.

(B and C) Table summarizing number of tumors arising after the transplantation of different dilutions of Ctrl and Twist1 deficient papilloma TECs or (C) SCC cells. Exact Fisher test was performed.

(D) Estimated frequency of TPCs in Ctrl, K14CreER:Twist1$^{fl/fl}$, and K14CreER:Twist1$^{fl/+}$ SCC.

(E) qRT-PCR analysis of mRNA expression of EMT related genes performed on FACS isolated TECs from Ctrl and Twist1cKO mice 5 days after TAM administration (n = 3).

(F) Immunostaining for K14, E-cadherin, and vimentin in Ctrl and Twist1cKO SCC, showing that Twist1 deletion leads to an upregulation of E-cadherin in skin SCC.

Scale bars, 50 μm (see also Figure S3). Error bars represent SEM.

CD34 and Sox2 expression, two well-characterized skin cancer stem cell markers (Beck et al., 2011; Boumahdi et al., 2014; Lapouge et al., 2012; Schober and Fuchs, 2011; Siegle et al., 2014), showed no difference in the percentage of TEC expressing these two markers (Figures S3A–S3C), consistent with the essential role of Twist1 in regulating TPC function in benign tumors. To assess whether Twist1 also regulates the tumor-propagating potential in more advanced tumors, we transplanted FACS isolated TECs from SCC into immunodeficient mice following Twist1 deletion and found that Twist1 deletion decreased the proportion of TPCs by 100-fold (Figures 4C and 4D), indicating that Twist1 indeed modulates tumor stemness in both benign and malignant skin tumors. Interestingly, the loss of a single *Twist1* allele also greatly impaired tumor propagation, suggesting that the level of Twist1 promotes tumor propagation in a gene dosage manner (Figures 4C and 4D). Moreover, we observed that Twist1 deletion in SCC induced a small, but significant, upregulation of E-cadherin at mRNA and protein level (Figures 4E and 4F), suggesting that Twist1 regulates directly or indirectly E-cadherin expression during malignant progression.

Twist1 Is Essential for Tumor Maintenance In Vivo Independently of EMT

Our data indicate that *Twist1* conditional deletion before or concomitantly to oncogene expression prevents the initiation of benign skin tumors and conditional deletion in pre-existing tumors impairs their ability to be propagated into immunodeficient mice. However, it remains unclear whether Twist1 expression is required for the maintenance of benign papillomas in vivo in their native tumor environment. We therefore acutely deleted Twist1 in papillomas by administering TAM to K14CreER:Twist1$^{fl/fl}$ mice displaying DMBA/TPA induced skin tumors (Figures 5A and 5B). Remarkably, while control papillomas continued to grow during the 2 weeks following TAM administration, Twist1 deletion induced rapid tumor regression and after 14 days of treatment most of the skin papillomas had completely disappeared (Figure 5C). Twist1-deleted tumor cells were abnormally large with big nuclei (Figure 5D), exhibited a strong decrease in the tumor cellularity accompanied by a significant decrease in TEC proliferation, and a marked increase in apoptosis (Figures 5E–5J and S4). These results show that in skin papilloma, Twist1 expression is required for tumor maintenance in vivo by promoting proliferation and suppressing apoptosis.

Surprisingly, 7 days after TAM administration, immunostaining performed on skin sections showed that Twist1-deficient tumor cells express the same levels of E-cadherin as control tumors (Figure 5K). Quantitative (q)RT-PCR performed on FACS-isolated TECs confirmed that the levels of E-cadherin were not affected by Twist1 deletion (Figure 5L) and showed no change in the expression of mesenchymal markers (vimentin, fibronectin, and N-cadherin) or other EMT-transcription factors such as Snail, Slug, Zeb1, and Zeb2 (Figure 5L). These results indicate that in skin papilloma, Twist1 regulates tumor maintenance and tumor propagation independently of its ability to regulate expression of EMT-related genes.

Twist1 Inhibits p53 Stabilization in Primary Skin Tumors

Because Twist1-dependent tumor maintenance did not require induction of EMT, we searched for alternative mechanisms by

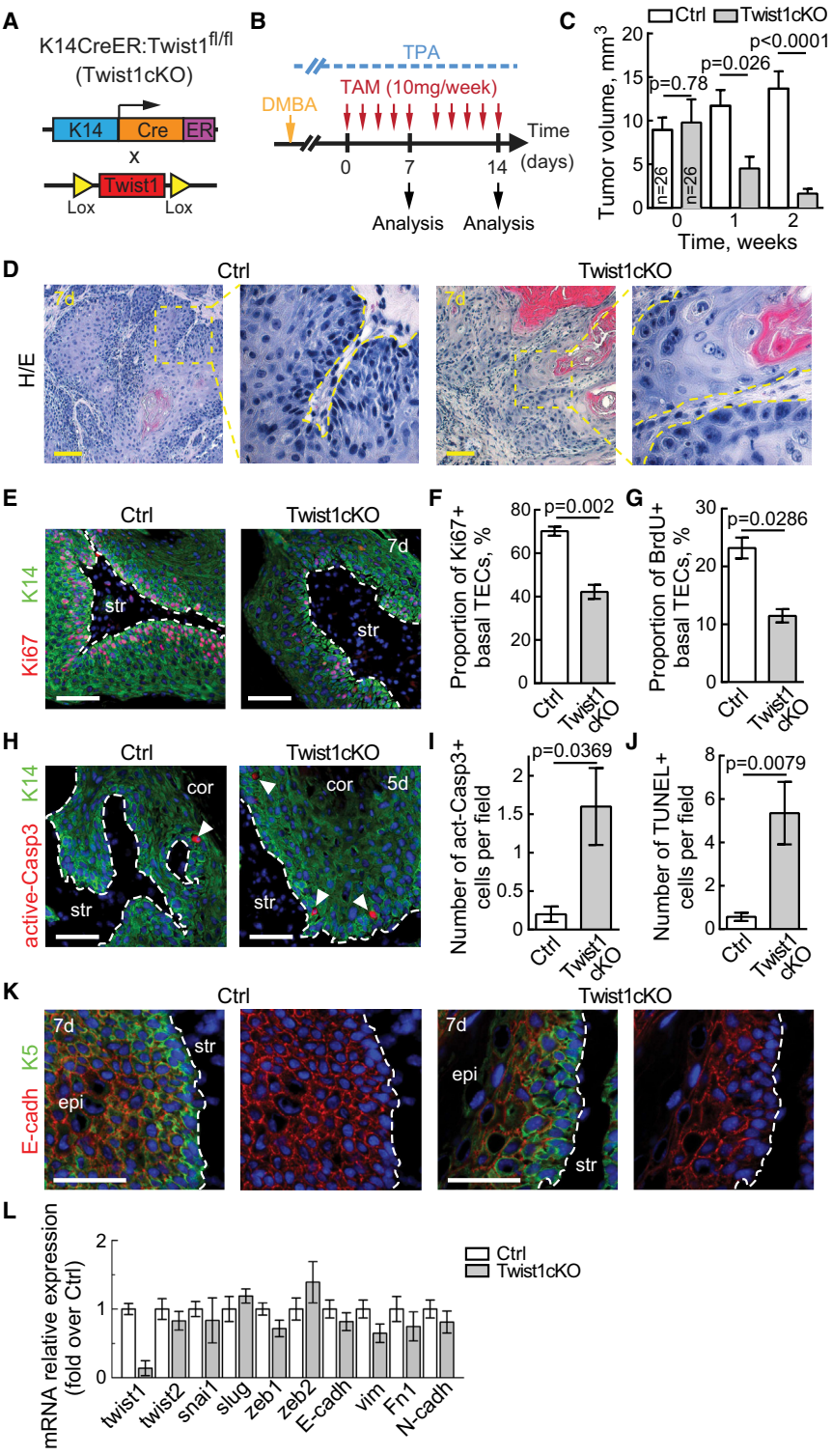


Figure 5. Twist1 Is Essential for Benign Tumor Maintenance In Vivo Independently of EMT

(A and B) Genetic strategy used to study the role of Twist1 in the maintenance of tumor cells in primary tumors vivo.

(C) Tumor size 0, 1, and 2 weeks after the beginning of TAM treatment in Ctrl and K14CreER:Twist1$^{fl/fl}$ mice (n = 26 from four animals). This data show rapid tumor regression upon Twist1 deletion.

(D) Hematoxylin/Eosin on tumors from Ctrl and K14CreER:Twist1$^{fl/fl}$ mouse treated with TAM for 7 days.

(E) Immunostaining for K14 and Ki67 on Ctrl and Twist1cKO tumors treated with TAM for 7 days.

(F) Quantification of the proportion of Ki67+ basal TECs (n = 6/condition).

(G) Quantification of the proportion of BrdU+ basal TECs (n = 4/condition).

(H) Immunostaining for active-caspase3 and K14 on tumors from a Ctrl and K14CreER:Twist1$^{fl/fl}$ mouse treated with TAM for 5 days.

(I) Quantification of the proportion of active-Caspase3+ TECs (n = 4/condition).

(J) Quantification of the proportion of TUNEL+ TECs (n = 5/condition), showing that cell death was increased upon Twist1 deletion.

(K) Immunostaining for E-cadherin and K5 on Ctrl and Twist1cKO tumors treated with TAM for 7 days, showing that Twist1 deletion does not lead to E-cadherin upregulation.

(L) qRT-PCR analysis of EMT regulators, E-cadherin, and mesenchymal markers in Ctrl and Twist1 deficient tumors treated with TAM for 7 days.

Scale bars, 50 μm (see also Figure S4). Error bars represent SEM.

which Twist1 controls this process. Twist1 had been previously reported to negatively regulate p53-signaling in response to HRas- and Myc-induced oncogenic stress and to prevent oncogene-induced senescence in different cell lines in vitro (Ansieau et al., 2008; Maestro et al., 1999; Valsesia-Wittmann et al., 2004). To determine whether endogenous Twist1 regulates the maintenance of skin tumors through similar mechanisms, we searched for evidence of p53 signaling upon Twist1 deletion in pre-existing skin papillomas. Immunostaining analysis 5 days after TAM administration revealed that p53 was rapidly stabilized in most TECs following Twist1 deletion, while p53 protein expression was not detected in control papilloma (Figures 6A and 6B). qRT-PCR analysis of the expression of well-established p53 target genes such as *Noxa, Bad, Bid, Bax, Mdm2*, and *p21* (Harris and Levine, 2005) showed that these genes were upregulated in TECs following Twist1 deletion, thus providing further evidence for an activation of p53 signaling pathway (Figure 6C). Immunostainings confirmed the upregulation of p21, which mediates p53-dependent cell cycle arrest in TECs following Twist1 deletion (Figures 6D and 6E). The prevention of oncogene-induced p53-dependent senescence controlled by Twist1 in vitro has been shown to involve the inhibition of p19ARF expression (Ansieau et al., 2008; Valsesia-Wittmann

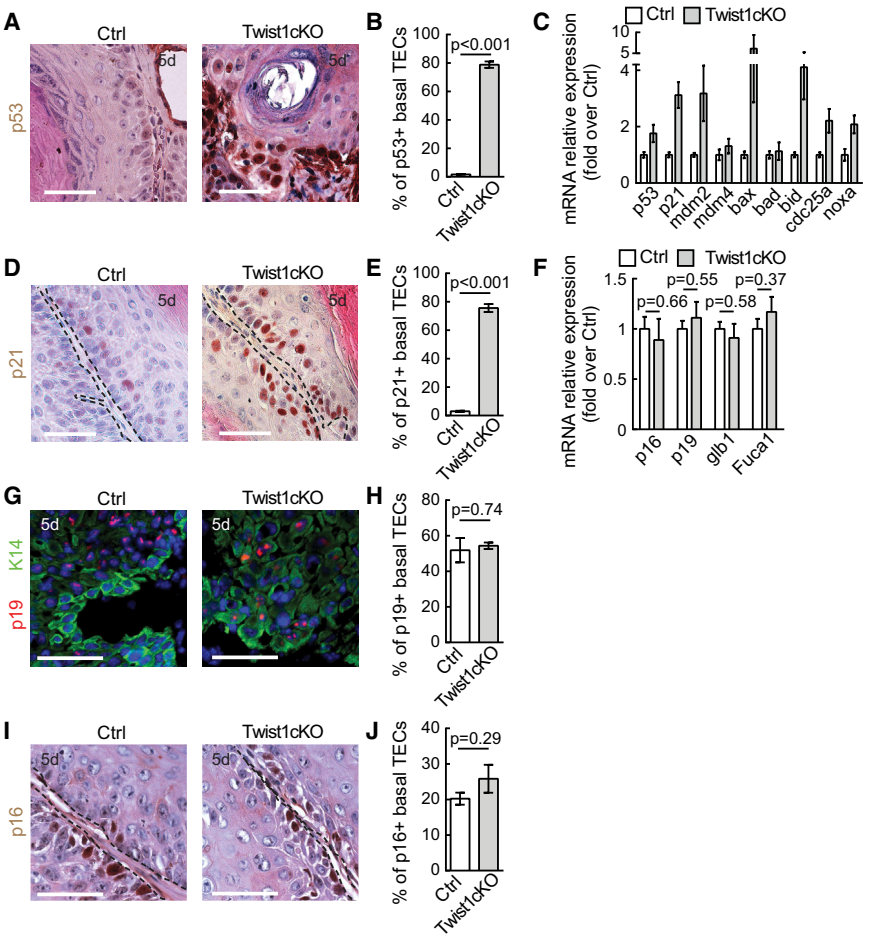


Figure 6. Twist1 Inhibits p53 Stabilization in Primary Skin Tumors

(A) IHC for p53 on tumors from a Ctrl and K14CreER:Twist1$^{fl/fl}$ mouse treated with TAM for 5 days.

(B) Quantification of the proportion of basal p53 positive TECs, showing a massive increase in p53 expression following Twist1 deletion (n = 3).

(C) qRT-PCR analysis of p53 target genes performed on FACS isolated TECs following TAM administration to K14CreER:Twist1$^{fl/fl}$ mice (n = 3).

(D) IHC for p21 on tumors from a Ctrl and K14CreER:Twist1$^{fl/fl}$ mice treated with TAM for 5 days.

(E) Quantification of the proportion of basal p21 positive TECs (n = 5).

(F) qRT-PCR analysis of the expression of senescence associated genes on FACS isolated TECs following TAM administration to K14CreER:Twist1$^{fl/fl}$ mice.

(G) Immunostaining for p19 and K14 on tumors from a Ctrl and K14CreER:Twist1$^{fl/fl}$ mouse treated with TAM for 7 days.

(H) Quantification of the proportion of basal p19 positive TECs (n = 5).

(I) IHC for p16 on a tumor from a Ctrl and K14CreER:Twist1$^{fl/fl}$ mouse treated with TAM for 5 days.

(J) Quantification of the proportion of basal p16 positive TECs (n = 3). IHC were counterstained with Hematoxylin/Eosin.

Scale bars, 50 μm. Error bars represent SEM.

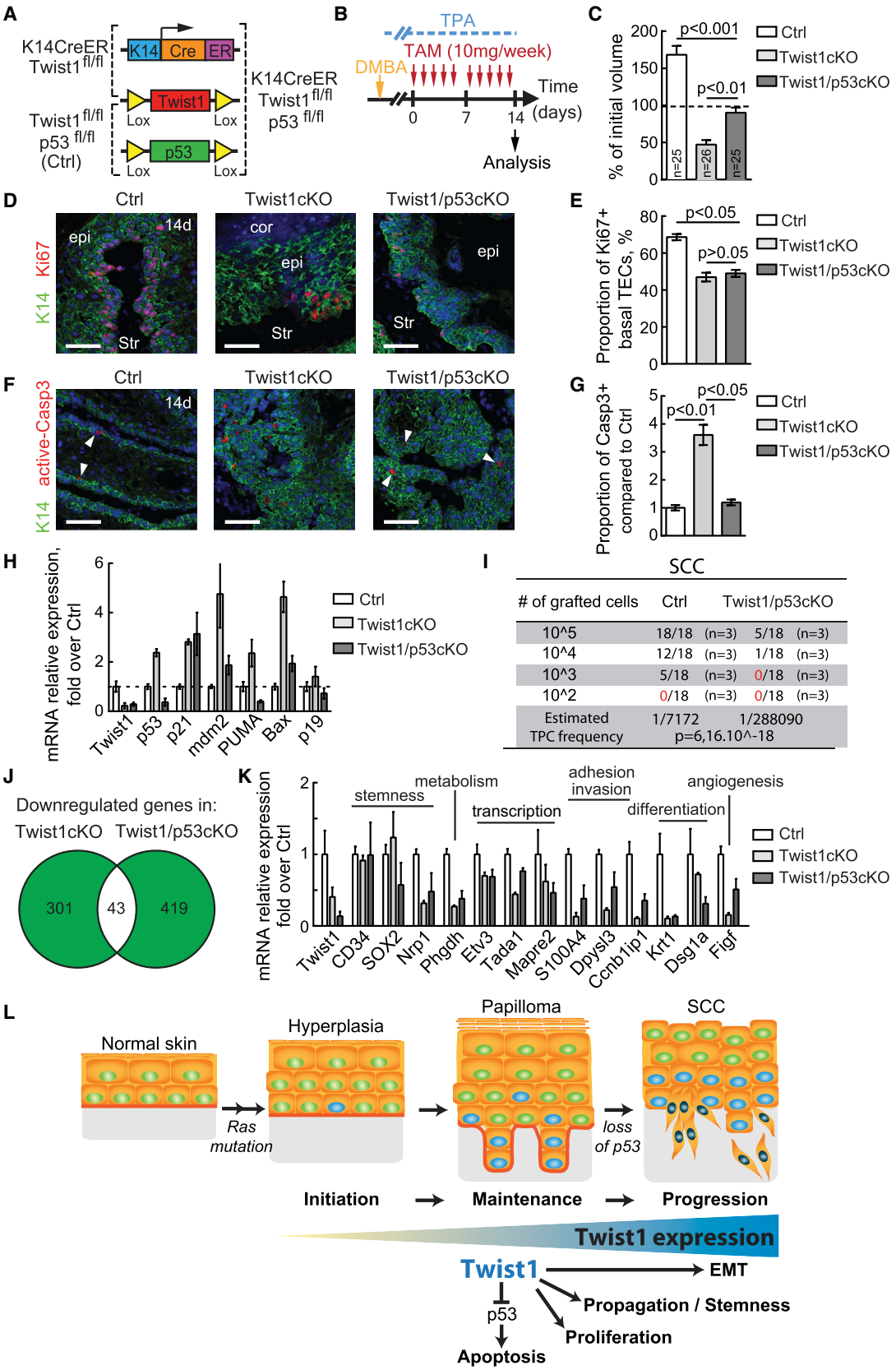
et al., 2004). To determine whether Twist1 sustains tumor proliferation and maintenance by inhibiting the p19ARF-p53 axis, we investigated the expression of p19 following inactivation of Twist1 in skin tumors. qRT-PCR and immunostaining analyses of p19, p16, as well as, glb1 (β-galactosidase) (Dimri et al., 1995) and Fuca1 (α-Fucosidase) (Hildebrand et al., 2013), two markers of oncogene induced senescence, revealed these genes were expressed in skin papilloma, but were not upregulated following Twist1 deletion (Figures 6F–6J). Altogether, these results indicate that while p53 is rapidly activated following Twist1 deletion in skin papilloma, which in turn induces the expression of cell cycle arrest and proapoptotic p53 target genes, p53 activation did not further induce the canonical senescence pathway in these cells.

p53-Dependent and -Independent Functions of Twist1 in Skin Tumors

To determine whether the impact of Twist1 deletion on tumor maintenance and propagation is strictly dependent on activation of the p53 pathway, we generated double cKO mice (K14CreER:Twist1$^{fl/fl}$:p53$^{fl/fl}$), allowing the specific ablation of Twist1 and p53 simultaneously in pre-existing DMBA/TPA-induced skin tumors (Figures 7A, 7B, and S5A–S5D). Control tumors continued to grow following TAM administration, while Twist1-inactivation alone led to tumor regression (Figure 7C). In contrast, deletion of both Twist1 and p53 prevented tumor growth, but did not lead to tumor regression (Figure 7C), indicating that p53 loss only partially rescues the impact of Twist1 deletion. Immunostaining analyses for apoptotic and proliferation markers showed that p53 deletion in Twist1-deficient cells rescued apoptosis, but did not rescue cell proliferation (Figures 7D–7G). qRT-PCR performed on FACS-isolated TECs following TAM administration confirmed the efficient deletion of p53 and Twist1 in the different conditional KO mice and revealed the efficient downregulation of the proapoptotic p53 target genes (Figure 7H). However, p21 was not downregulated either at the mRNA or the protein level in Twist1/p53 double KO tumor cells as compared to Twist1 single KO TECs (Figures S5C and S5D), indicating that Twist1 regulates cell proliferation in skin tumor independently of p53.

To determine whether the p53-independent functions of Twist1 regulate tumor stemness, we assessed, using the standard assay to assess tumor stemness (Magee et al., 2012), the ability of p53 deletion to rescue the decrease in tumor propagating potential induced by Twist1 deletion. Transplantation of serial dilution of TECs 7 days after TAM administration showed that p53 deletion did not rescue the defect in TPC potential induced by Twist1 deletion in primary skin SCCs (Figure 7I), supporting the notion that Twist1 can regulate tumor stemness independently of p53.

To further characterize the mechanisms by which Twist1 regulates tumor maintenance and propagation independently of p53 and of its ability to modulate EMT, we transcriptionally



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profiled using microarray FACS isolated TECs from control, Twist1cKO, and Twist1/p53cKO papilloma 5 days after TAM administration. We found 43 common genes downregulated and 78 common genes upregulated in both Twist1 single cKO and Twist1/p53 double cKO (Figures 7J, S6, and S7). Since no tumor regression was observed in Twist1/p53 double cKO, these genes are unlikely to reflect an indirect effect of Twist1 deletion on tumor regression. Importantly, we found that Twist1 deletion leads to the downregulation of Nrp1 (Figure 7K), a vascular endothelial growth factor (VEGF) coreceptor that we and others have previously reported as a major regulator of CSC in skin tumors (Beck et al., 2011; Siegle et al., 2014) and glioblastoma (Hamerlik et al., 2012). In addition, we found that Twist1 deletion led to decreased expression of phosphoglycerate dehydrogenase (Phgdh), a metabolic enzyme overexpressed in tumor cells, and whose knockdown induced decreased tumor cell proliferation (Locasale et al., 2011; Possemato et al., 2011). Twist1 deletion also directly or indirectly caused changes in the expression of other genes that promote proliferation (e.g., Etv3) and adhesion/invasion (e.g., S100A4, Dpysl3, ccnb1ip1, and Dsg1a) (Figure 7K).

DISCUSSION

While Twist1 is known for its ability to induce EMT, tumor progression, and dissemination (Chen and Behringer, 1995; Mani et al., 2008; Morel et al., 2008; Yang et al., 2004), its role in tumor initiation has not been investigated so far. Here, using conditional deletion of Twist1 in the skin epidermis at different stages of tumor initiation and progression, we demonstrate that Twist1 is expressed at early stages of tumorigenesis and is required for the initiation of skin tumors. Strikingly, while deletion of both alleles of Twist1 is necessary to prevent tumor initiation, deletion of a single allele is sufficient to prevent malignant progression. Whether Twist1 is monoallelically expressed before SCC conversion remains an open question. Although previous studies reported that transgenic overexpression of Twist1 stimulates the conversion of benign papilloma into malignant SCCs (Morel et al., 2012; Tsai et al., 2012), our results establish a previously unrecognized and essential role for Twist1 gene dosage in skin tumor initiation and malignant progression. Deletion of Twist1 in SCCs, but not in benign papilloma, upregulates E-cadherin expression, suggesting that higher levels of Twist are required for EMT as compared to lower levels required for its role in tumor initiation and maintenance.

Twist1 overexpression in breast cancer cell lines (Mani et al., 2008; Morel et al., 2008) and glioblastoma (Mikheeva et al., 2010) promotes EMT, in vitro proliferation, and tumor propagation into immunodeficient mice, raising the possibility that Twist1 controls tumor stemness through its EMT inductive properties. By deleting Twist1 in pre-existing skin tumors, we confirm its essential role for tumor maintenance in vivo and for tumor propagation into immunodeficient recipient mice. However, we also provide genetic evidence that in skin papilloma, Twist1 affects tumor maintenance and propagation independently of its ability to repress E-cadherin and to induce the EMT program, confirming that the mesenchymal phenotype and tumor initiating properties are not always linked (Celià-Terrassa et al., 2012; Ocaña et al., 2012; Sarrio et al., 2012). This finding demonstrates that Twist1 exerts different functions (i.e., EMT and tumor propagation) during skin tumorigenesis, and that these different activities are not necessarily functionally linked. A more general conclusion from this finding is that EMT is not a *sine qua non* condition for the establishment of tumor stemness in vivo.

Twist1 has been shown to prevent oncogene-induced apoptosis and/or senescence (e.g., Ras and Myc) by repressing the p16 and/or the p19ARF/p53 pathways (Ansieau et al., 2008; Maestro et al., 1999; Valsesia-Wittmann et al., 2004). Consistently, acute deletion of Twist1 in papilloma leads to a rapid stabilization of p53 as evidenced by a significant increase in the expression of its canonical target genes. However, the activation of the p53 pathway in this experimental setting is not the consequence of increased p19ARF expression and does not lead to senescence. These data indicate that Twist1 is capable of interfering with tumor suppression via distinct mechanisms depending on the cell types (breast, pancreas, or lung) (Lee and Bar-Sagi, 2010; Mani et al., 2008; Morel et al., 2008; Tran et al., 2012) and/or the nature and the extent of oncogene activation. Some of the discrepancies can also be explained by the fact that, in previous studies, oncogene activation was mimicked through overexpression of the oncogenes in cultured cells (Mani et al., 2008; Morel et al., 2008), whereas in our study, the oncogene is expressed in vivo and under the control of its native promoter/regulatory region. Different mechanisms have

Figure 7. p53-Dependent and -Independent Functions of Twist1 in Skin Tumors

(A and B) Genetic strategy used to dissect the p53-dependent and -independent functions of Twist1 in skin tumors.

(C) Tumor size 2 weeks after the beginning of TAM treatment in Ctrl, Twist1 cKO, and Twist1/p53 double KO mice, showing that concomitant deletion of Twist1 and p53 rescues tumor shrinkage that occurs following Twist1 deletion.

(D) Immunostaining for K14 and Ki67 on Ctrl, Twist1 cKO, and Twist1/p53 double cKO tumors treated with TAM for 10 days.

(E) Quantification of the proportion of Ki67+ basal TECs treated with TAM for 14 days (n = 6/condition). These data show that p53 deletion does not rescue the decrease in tumor proliferation mediated by Twist1 deletion.

(F) Immunostaining for active-caspase3 and K14 on Ctrl, Twist1 cKO, and Twist1/p53 double KO tumors treated with TAM for 14 days.

(G) Quantification of the proportion of active-Caspase3+ TECs treated with TAM for 14 days (n = 9/condition). These data show that p53 deletion rescues tumor apoptosis mediated by Twist1 deletion.

(H) qRT-PCR analysis of p53 related genes mRNA expression performed on FACS isolated TECs from Ctrl, Twist1 cKO, and Twist1/p53 double cKO tumors treated with TAM for 10 days.

(I) Table summarizing the number of tumors arising after the transplantation of different dilutions of Ctrl and Twist1/p53 double cKO TECs from invasive SCCs treated with TAM for 10 days. These data show that p53 deletion does not rescue the decrease in tumor propagating potential mediated by Twist1 deletion.

(J) Venn diagram, showing the genes downregulated in both Twist1cKo and Twist1/p53 double cKO.

(K) qRT-PCR analysis of genes downregulated in both Twist1cKo and Twist1/p53 double cKO (n = 3/condition).

(L) Scheme representing the dynamic expression and the different roles of Twist1 during skin tumor initiation, maintenance, and progression (see also Figures S5–S7).

Error bars represent SEM.

been proposed to explain how p53 gets stabilized and activated following Twist1 loss of function in tumor cells (Ansieau et al., 2008; Kwok et al., 2007; Lee and Bar-Sagi, 2010; Vichalkovski et al., 2010), including a direct interaction between p53 and Twist1 (Piccinin et al., 2012; Shiota et al., 2008). According to these studies, Twist1 directly antagonizes p53 functions by promoting Mdm2-mediated p53 degradation. Consistent with this possibility, whereas p53 protein is undetectable in skin papilloma expressing Twist1, p53 protein, but not mRNA, rapidly accumulates upon Twist1 deletion. However, whether p53 stability in skin papilloma is controlled through a direct interaction with Twist1 will require further studies.

Concomitant deletion of p53 and Twist1 in tumors allowed us to further dissect the p53-dependent and independent functions of Twist1 in the control of apoptosis and tumor stemness. Importantly, we show that Twist1 prevents apoptosis by suppressing p53-function and, at the same time, promotes tumor cell proliferation and propagation independently of p53. Using microarray analysis of TECs following the deletion of Twist1 or Twist1 and p53, we uncovered 121 genes positively and negatively regulated by Twist1 independently of p53 function. We found that Twist1 directly or indirectly promotes the expression of Nrp1, a VEGF coreceptor that promotes tumor stemness and proliferation in skin tumors (Beck et al., 2011; Siegle et al., 2014). Twist1 also regulates directly or indirectly the expression of Phgdh, an enzyme overexpressed in tumor cells essential for cancer cell proliferation (Locasale et al., 2011; Possemato et al., 2011). While it has been previously shown that overexpression of p53 dominant-negative mutant (p53-R175H) induces Twist1 expression in immortalized prostate cell lines (Kogan-Sakin et al., 2011), our data show that Twist1 is expressed in KRasG12D p53-deficient SCC, indicating that Twist1 expression in tumor cells is not strictly dependent on p53. In addition, our data show that Twist1 is essential for the initiation and maintenance of p53-deficient SCC. Together these results unambiguously establish both p53-dependent and -independent roles for Twist1 in tumor initiation, proliferation, apoptosis, and propagation.

In summary, our study uncovers a key role for Twist1 in the early steps of tumor initiation and a dose-dependent role in malignant progression (Figure 7L). Our results also demonstrate that the multiple functions of Twist1 including tumor initiation, progression, propagation, and EMT can be functionally uncoupled and are regulated by different mechanisms. Due to the importance of Twist1 functions in regulating different aspects of tumorigenesis, future studies will be required to better understand the dynamic of Twist1 expression during tumor initiation, progression, and metastasis, as metastatic colonization requires the downregulation of Twist1 expression (Tsai et al., 2012) and dissect in greater detail the molecular mechanisms underlying Twist1 functions at each of these important steps during tumorigenesis. Such studies are likely to lead to the development of therapeutic strategies aimed at interfering with the different oncogenic functions of Twist1 in cancer.

EXPERIMENTAL PROCEDURES

Mice

Nonobese diabetic (NOD)/severe combined immunodeficiency (SCID)/Il2Rγ null mice were obtained from Charles River. Twist1$^{fl/fl}$ (Chen et al., 2007), K14CRE (Vasioukhin et al., 1999), K14CREER (Vasioukhin et al., 2001), KRas$^{LSL-G12D}$ (Tuveson et al., 2004), and p53$^{fl/fl}$ (Jonkers et al., 2001) mice have been obtained from the Mouse Mutant Resource Regional Center at University of North Carolina, Chapel Hill, NC or obtained from Jackson Laboratories. Mouse colonies were maintained in a certified animal facility in accordance with the European guidelines.

Mice were treated with DMBA and TPA as previously described (Beck et al., 2011; Lapouge et al., 2012). Mice were treated three times at postnatal day 23, 25, and 27 with DMBA (50 μg) and then treated twice weekly with TPA (2.5 μg) until their sacrifice. K14CreER:KRas$^{LSL-G12D}$:p53$^{fl/fl}$ mice were treated with TAM resuspended in sunflower oil at postnatal day 23 and 25 (2.5 milligrams [mg]/injection) as previously described (Lapouge et al., 2011).

Measurement of Tumor Growth

Skin tumors were measured using a precision calliper. Tumor volumes were calculated using the formula $V = \pi \times [d2 \times D] / 6$, where d is the minor tumor axis and D is the major tumor axis.

Immunostaining

Optimal cutting temperature (OCT) (Tissue Tek) embedded samples were sectioned at 6 μm sections using CM3050S cryostat (Leica Microsystems) and fixed in 4% paraformaldehyde for 10 min at room temperature. Nonspecific antibody binding was prevented by incubating the slides with 5% horse serum (HS), 1% BSA, and 0.2% Triton X-100 for 1 hr at room temperature. Primary antibodies were incubated overnight at 4°C in blocking buffer. Sections were rinsed three times in PBS and incubated with secondary antibodies diluted at 1:400 for 1 hr at room temperature. Nuclei were stained in Hoechst solution (4 millimolar) and slides were mounted using Glycergel (Dako) supplemented with 2.5% 1,4-diazabicyclo(2.2.2)octane (Sigma-Aldrich).

For immunohistochemistry (IHC), 5-μm paraffin sections were deparaffinized and rehydrated. The antigen unmasking procedure was performed for 25 min at 98°C in citrate buffer (pH 6) + Tween 0.05% using the pretreatment module (labvision). Endogenous peroxydase was blocked using 3% H2O2 (Merck) in methanol (VWR) for 10 min at room temperature. Endogenous avidin and biotin were blocked using the Endogenous Blocking Kit (Invitrogen) for 20 min at room temperature. Mouse on mouse blocking kit was then used to block unspecific epitopes according to the manufacturer guideline (MOM, Vector Labs). For Twist1 staining, slides were incubated in a blocking solution with 5% HS, 1% BSA, and 0.2% Triton X-100 for 30 min at room temperature before incubation in the MOM blocking. Mouse anti-Twist1 antibody was incubated overnight at 4°C. Anti-mouse biotinylated (1/1,000 for 30 min at room temperature), Standard ABC Kit, and ImmPACT DAB (Vector Laboratories) were used for the detection of HRP activity. Slides were then dehydrated and mounted using Safemount (Labonord).

Antibodies

The primary antibodies used are listed in the Supplemental Experimental Procedures.

Microscopy

Microscope acquisition was performed on a Zeiss Axio Imager.M1 fluorescence microscope with a Zeiss Axiocam MR3 camera for immunofluorescence microscopy and a Zeiss AxioCam MRc 5 camera for bright-field microscopy using AxioVision release 4.6 software.

FACS Isolation of Tumor Cells

Tumors were digested in collagenase I (Sigma) (2.5 mg/ml) for 2 hr at 37°C on a rocking plate. TECs were sorted based on expression of Epcam and absence of lineage negative markers (CD45, CD31, and CD140a), living Epcam+/CD45-/CD31-/CD140a-. Fluorescence-activated cell sorting analysis was performed using FACSAria and FACSDiva software (BD Biosciences). Sorted cells were collected at 4°C either in culture medium for in vivo transplantation experiments and into lysis buffer for RNA extraction.

Tumor Transplantation into Immunodeficient Mice

For transplantation of papilloma cells, total living cells were sorted based on Hoechst dye exclusion, then resuspended in 50 μl of Matrigel (E1270, 970 mg/ml; Sigma) and injected subcutaneously to NOD/SCID/Il2Rg null

mice (Charles River). For transplantation of SCC cells, different dilutions of Lin-/Epcam+ TECs resuspended in 50 μl of Matrigel were injected subcutaneously to NOD/SCID/Il2Rg null mice. Technical triplicates injections per mouse were performed. In both cases, grafted mice were treated with TAM at day 3, 5, and 7 after transplantation (2.5 mg/injection). Secondary tumors were detected by palpation every week and their size monitored until tumor reached 1 cm^3 or when mice presented signs of distress, and the mice were sacrificed.

Estimation of the relative frequency of cancer propagating cells was performed using the extreme limiting dilution analysis (ELDA) as described (Hu and Smyth, 2009) and calculated using the ELDA online software (http://bioinf.wehi.edu.au/software/elda/). The statistical p value was obtained using a Chi-square test.

Conditional Deletion of Twist1 in Pre-existing Tumor Cells

K14CreER:Twist1$^{fl/fl}$ and K14CreER:Twist1$^{fl/fl}$:p53$^{fl/fl}$ and their littermate controls (K14CreER negative) were treated with DMBA/TPA as described above. After tumor appearance, mice were injected intraperitoneally with two cycles of 2 mg TAM per day during five consecutive days. Tumors were measured, as described above, on the day of injection, 7, and 14 days later to quantify tumor growth.

RNA Extraction, DNA Extraction, and qRT-PCR

RNA extraction was performed using the RNeasy Micro Kit (QIAGEN) according to the manufacturer's recommendations. Purified RNA was used to synthesize the first strand cDNA using Superscript II (Invitrogen) and random hexamers (Roche). qRT-PCR analyses were performed with 2 nanograms of cDNA as template, using a SYBR Green Mix (Applied Bioscience) and an Agilent Technologies Stratagene Mx3500P real-time PCR System.

Relative quantitative RNA was normalized using the housekeeping gene β-actin. Primers were designed using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Analysis of the results was performed using MxPro Software (Stratagene) and relative quantification was performed using the ΔΔCt method using β-actin as a reference. The entire procedure was repeated in at least three biologically independent samples.

All the primers used for genotyping and qPCR are listed in the Supplemental Experimental Procedures.

Microarray

RNA was extracted from FACS isolated TECs as described above. There were two control samples and two Twist1cKO samples that were sent to AROS Applied Biotechnology A/S (Aarhus) and analyzed on Affymetrix 430 2.0 chips. There were two control samples and two Twist1cKO:p53cKO samples that were sent to the IRB Functional Genomics Core and analyzed on Affymetrix 430 PM chips. Signals were normalized using frozen robust multiarray analysis to avoid batch effect (McCall et al., 2010). The list of genes upregulated and downregulated more than two times in Twist1cKO and in Twist1/p53cKO compared to their respective control was compared to find the common genes shown in Figures S6 and S7.

Twist1 Expression in Human Skin SCCs

Tissue samples were obtained from archival formalin-fixed and paraffin-embedded samples from five normal skins and 32 skin SCCs collected in the Department of Pathology of the Erasme Hospital, Brussels, Belgium. The 5-μm-thick sections were subjected to standard IHC as previously described (Boumahdi et al., 2014). The expression of TWIST was detected by immunostaining using a mouse monoclonal anti-TWIST antibody (clone Twist2c1a, 1:25). A case was considered as positive if some positive nuclei were noted in TECs.

ACCESSION NUMBERS

The Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/, accession number for the microarray data reported in this paper is GSE63334.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2014.12.002.

AUTHOR CONTRIBUTIONS

C.B., J.-C.M., and B.B. designed the experiments and performed data analysis. B.B., G.L., B.D., K.D., S.D., and K.W. performed all the experiments. S.R. and I.S. analyzed human samples. C.D. performed technical support. C.B. and B.B. wrote the manuscript.

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