Ectopic Expression of Zmiz1 Induces Cutaneous Squamous Cell Malignancies in a Mouse Model of Cancer

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Cutaneous squamous cell carcinoma (SCC) is the second most common form of cancer in the human population, yet the underlying genetic mechanisms contributing to the disease are not well understood. We recently identified *Zmiz1* as a candidate oncogene in nonmelanoma skin cancer through a transposon mutagenesis screen. Here we show that transposon-induced mutations in *Zmiz1* drive expression of a truncated transcript that is similar to an alternative endogenous *ZMIZ1* transcript found to be overexpressed in human SCCs relative to normal skin. We also describe an original mouse model of invasive keratoacanthoma driven by skin-specific expression of the truncated *Zmiz1* transcript. Unlike most mouse models, Zmiz1-induced skin tumors develop rapidly and in the absence of promoting agents such as phorbol esters. In addition, we found that the alternative Zmiz1 isoform has greater protein stability than its full-length counterpart. Finally, we provide evidence that ZMIZ1 is overexpressed in a significant percentage of human breast, ovarian, and colon cancers in addition to human SCCs, suggesting that ZMIZ1 may play a broader role in epithelial cancers.

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INTRODUCTION

Nonmelanoma skin cancer is the most common human cancer worldwide with ~1 million new cases reported in the United States annually (Alam and Ratner, 2001). Although basal cell carcinoma is more common than squamous cell carcinoma (SCC), SCC is a more aggressive disease and metastasizes at low frequency (Alam and Ratner, 2001). Current treatments rely upon surgical excision of the cancerous tissue. However, surgical removal can be disfiguring as tumors develop on sun-exposed skin. The major risk factor for developing nonmelanoma skin cancer is UV exposure, and UV-induced *TP53* mutations are frequently observed in both basal cell carcinoma and SCC (Brash *et al.*, 1991; Benjamin and Ananthaswamy, 2007). In addition, 46% of human SCCs have mutations in *HRAS*, many of which are also UV induced (Pierceall *et al.*, 1991). Despite knowledge of these prevalent mutations, targeted therapies are not in widespread use and greater understanding of the genetics contributing to this disease will aid in their design.

We recently performed a *Sleeping Beauty* (*SB*) transposon mutagenesis screen in mice in which a variety of epithelial tumor types were produced, the most common being cutaneous malignancies formerly designated as SCCs (Dupuy *et al.*, 2009). Nearly 90% of cutaneous tumors harbored transposon insertions within the *Zmiz1* locus, likely resulting in overexpression of an N-terminally truncated protein (i.e., Zmiz1$^{\Delta 1\text{-}185}$). Interestingly, Zmiz1 has previously been implicated in lymphoma through retroviral mutagenesis screens (Sauvageau *et al.*, 2008; Uren *et al.*, 2008).

The *ZMIZ1* locus encodes a protein with a PAT domain, an MSX-interacting zinc-finger (MIZ) domain, and a putative nuclear localization sequence. The molecular function of ZMIZ1 is not well characterized, but the MIZ domain is predicted to function as an E3 SUMO (Small Ubiquitin-like Modifier) ligase with homology to the PIAS (protein inhibitor of activated Stats) family. Prior work demonstrated that ZMIZ1 increases transcriptional activity of the androgen receptor, p53, and Smad3/4 when overexpressed in cultured cells (Sharma *et al.*, 2003; Li *et al.*, 2006; Lee *et al.*, 2007). The increased androgen receptor activity was associated with sumoylation of androgen receptor, apparently mediated by ZMIZ1 (Sharma *et al.*, 2003). A constitutive *Zmiz1* knockout mouse allele has been described, but homozygous deletion of *Zmiz1* causes early embryonic lethality, thus preventing the study of Zmiz1 function in adult tissues (Beliakoff *et al.*, 2008). Consequently, current knowledge regarding Zmiz1 function is

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Abbreviations: EGFP, enhanced green fluorescent protein; KA, keratoacanthoma; K14, keratin 14; qRT–PCR, quantitative real-time reverse-transcriptase–PCR; SB, Sleeping Beauty; SCC, squamous cell carcinoma; SUMO, Small Ubiquitin-like Modifier

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inadequate to explain how its overexpression might lead to skin cancer.

Here we report the results of experiments performed to validate Zmiz1 as an oncogene and to explore how Zmiz1 truncation by transposon insertion might confer selective advantage to tumor cells. We found that overexpression of Zmiz1$^{\Delta 1\text{-}185}$ in mouse skin was sufficient to induce tumors, thereby creating a transgenic model of cutaneous malignancy. Interestingly, we discovered that a similar endogenous isoform is expressed in a tumor-specific manner in human skin. We also observed nuclear accumulation of ZMIZ1 in human breast, ovarian, and colon cancers, supporting a widespread relevance of our mouse model to other epithelial cancers, and not only SCCs.

RESULTS

Tumor-specific expression of an alternative ZMIZ1 transcript

We recently identified *Zmiz1* as a cancer gene important for the induction of cutaneous SCC using *SB* transposon mutagenesis (Dupuy *et al.*, 2009). Significantly, all insertions were located within intron 8, and oriented in such a way as to promote expression of a truncated transcript including exons 9–24 (Figure 1a). Importantly, the *SB*-induced transcript strongly resembles an endogenous human *ZMIZ1* isoform (uc001kag.2), which includes an alternative promoter and exon that is spliced into exon 7 of the full-length transcript (Figure 1a).

Transcriptome and chromatin immunoprecipitation data generated by the ENCODE Project from a variety of cell lines are consistent with the presence of an alternative promoter just upstream of the alternative exon (Birney *et al.*, 2007) (Supplementary Figure S1 online). Expression of the short isoform (i.e., ZMIZ1^{short}) is likely regulated by the activity of this alternative promoter. All known and predicted functional domains are preserved in the predicted open reading frame of this short isoform (Figure 1b).

We detected endogenous expression of *ZMIZ1^{short}* in MCF7 breast cancer cells; however, the expression was not detected in the other human cell lines tested, including immortalized normal keratinocyte cell lines HaCaT and N-HSK-1 (Figure 1c). *ZMIZ1^{short}* transcript was also measured in human SCCs and, when possible, matched normal skin samples. Interestingly, we observed tumor-specific *ZMIZ1^{short}* upregulation in four of six samples by quantitative real-time

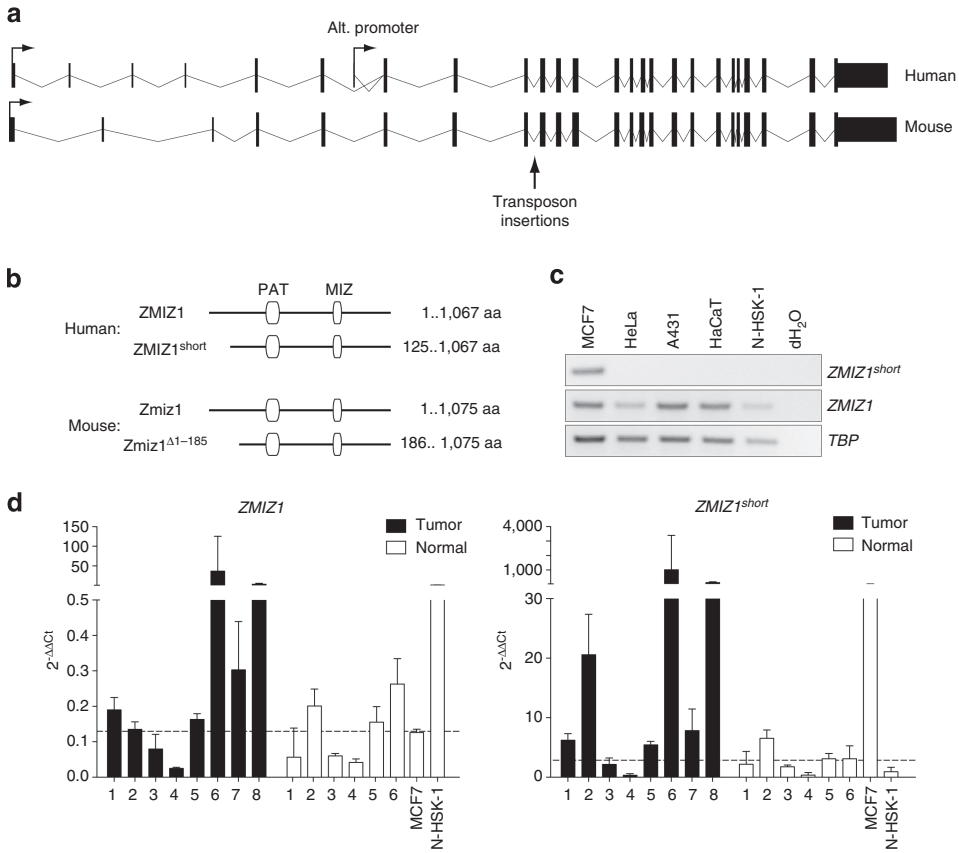

Figure 1. Tumor-specific expression of *ZMIZ1^{short}*. (**a**) Diagram of the *ZMIZ1* locus in mouse and human. The human alternative (Alt.) promoter is located in intron 6 and drives expression of the *ZMIZ1^{short}* transcript. This transcript is similar to the transcript produced by *Sleeping Beauty* (*SB*) transposon insertion into mouse intron 8. (**b**) The amino-acid sequence of human and mouse short forms differs by ~60 amino acids at the N-termini, but are otherwise nearly identical. Note that both full-length and ZMIZ1^{short} contain all currently annotated functional protein domains. (**c**) Reverse-transcriptase–PCR (RT–PCR) analysis confirmed that *ZMIZ1^{short}* is expressed in MCF7, but not in the other human cell lines tested. (**d**) Quantitative RT–PCR performed on human squamous cell carcinomas (SCCs) shows increased tumor-specific expression of *ZMIZ1^{short}*. Dotted lines indicate the average C_t values for normal skin.

reverse-transcriptase–PCR (qRT–PCR; Figure 1d). The full-length transcript (i.e., *ZMIZ1*) was upregulated in only two of six samples.

Zmiz1$^{\Delta 1\text{-}185}$ expression in mouse skin induces squamous cell tumors

As previously mentioned, we predicted that transposon-induced expression of the Zmiz1$^{\Delta 1\text{-}185}$ is oncogenic. To test this hypothesis, we engineered transgenic mice that conditionally express Zmiz1$^{\Delta 1\text{-}185}$ (Figure 2a). A lox-stop-lox strategy was employed to prevent ubiquitous expression of Zmiz1$^{\Delta 1\text{-}185}$. Cre-mediated recombination will delete the DsRed reporter embedded in the lox-stop-lox cassette and induce expression of the downstream Zmiz1$^{\Delta 1\text{-}185}$-IRES-EGFP cassette. Two Zmiz1$^{\Delta 1\text{-}185}$ founder animals showed detectable levels of the DsRed reporter in the skin and were used to establish transgenic lines 207-Zmiz1$^{\Delta 1\text{-}185}$ and 163-Zmiz1$^{\Delta 1\text{-}185}$.

Each line was crossed to keratin 14 (K14)-Cre transgenic mice to induce expression of Zmiz1$^{\Delta 1\text{-}185}$ in the proliferative basal layer of the cutaneous epidermis (Dassule *et al.*, 2000). Transgene expression in normal adult skin was assessed by western blot analysis of enhanced green fluorescent protein (EGFP) expression. As expected, EGFP expression was readily detectable in double-transgenic skin by western blot, but undetectable in skin from single-transgenic mice (Figure 2b). Cre-mediated expression of 163-Zmiz1$^{\Delta 1\text{-}185}$ was less efficient, as evidenced by lower EGFP protein levels in double-transgenic skin (Supplementary Figure S2 online). Given that both Zmiz1$^{\Delta 1\text{-}185}$ transgenes are likely multicopy concatemers, the discrepancy in EGFP expression is likely caused by Cre-mediated deletion of the transgene. Similar situations have previously been described (Grippo *et al.*, 2002). Nevertheless, both Zmiz1$^{\Delta 1\text{-}185}$ transgenic lines show Cre-inducible expression.

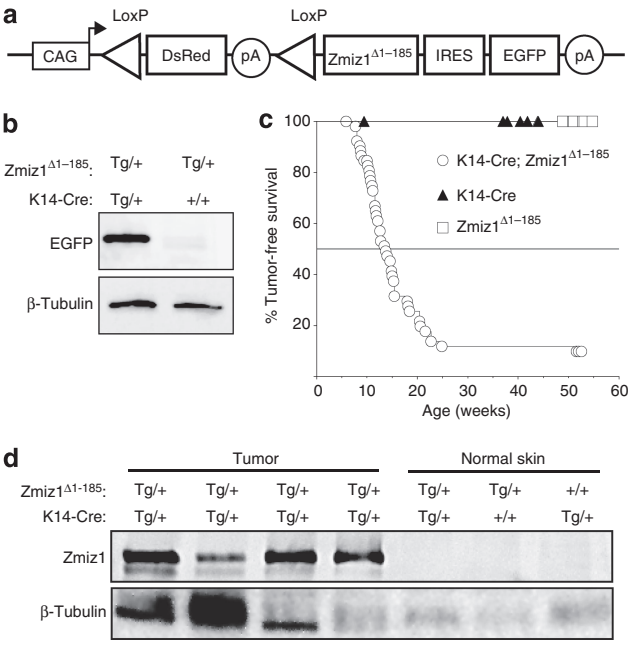

Figure 2. Zmiz1$^{\Delta 1\text{-}185}$ transgenic mice develop skin tumors. (**a**) Diagram of the Cre-inducible Zmiz1$^{\Delta 1\text{-}185}$ transgene. (**b**) Western blot showing that skin from Zmiz1$^{\Delta 1\text{-}185}$ single-transgenic mice has nearly undetectable expression of enhanced green fluorescent protein (EGFP), whereas double-transgenic mice display detectable EGFP expression. (**c**) Kaplan–Meier survival curve of mouse line 207 showing tumor-free survival of keratin 14 (K14)-Cre$^{Tg/+}$; Zmiz1$^{\Delta 1\text{-}185\ Tg/+}$ double-transgenic mice (circles, $n=54$), K14-Cre$^{+/+}$; Zmiz1$^{\Delta 1\text{-}185\ Tg/+}$ single-transgenic MICE (squares, $n=18$), and K14-Cre$^{Tg/+}$; Zmiz1$^{\Delta 1\text{-}185\ +/+}$ single-transgenic mice (triangles, $n=35$). Double-transgenic mice developed skin tumors with an average latency of 14 weeks after birth, whereas single-transgenic controls did not develop skin tumors (log-rank, $P<0.0001$). (**d**) Western blot confirms overexpression of the Zmiz1$^{\Delta 1\text{-}185}$ transgenic protein in mouse tumors. Endogenous Zmiz1 expression is undetectable in normal skin.

A cohort of Zmiz1$^{\Delta 1\text{-}185}$;K14-Cre double-transgenic mice ($n=54$) was generated and aged alongside Zmiz1$^{\Delta 1\text{-}185}$ ($n=18$) or K14-Cre ($n=35$) single-transgenic littermate controls (Figure 2c). Over 85% of double-transgenic mice spontaneously developed an average of 2.7 skin tumors per mouse with a mean latency of 14 weeks ($P<0.0001$). In all cases, the lesions were variably sized crateriform masses filled with compact keratin and lined by proliferative stratified squamous epithelium, a morphology consistent with keratoacanthoma (KA). Similarly, a more comprehensive study of the *SB*-induced tumors from Dupuy *et al.* (2009) showed identical histology, and hence these will also be referred to as KAs for the remainder of this article. Zmiz1$^{\Delta 1\text{-}185}$-induced masses compressed subjacent tissues and exhibited cutaneous muscle invasion in 23 of 147 tumors (15.6%). Double-transgenic mice from line 163-Zmiz1$^{\Delta 1\text{-}185}$ also developed KAs, but exhibited lower penetrance with increased latency. This is likely because of the lower transgene expression levels observed (Supplementary Figure S2 online). These results are consistent with the hypothesis that truncated Zmiz1 is an oncoprotein, and suggest that expression of *ZMIZ1^{short}* may also directly contribute to human SCCs.

Gene expression profiling of Zmiz1$^{\Delta 1\text{-}185}$-induced skin tumors

The Zmiz1$^{\Delta 1\text{-}185}$;K14-Cre double-transgenic mice represent the first published *in vivo* validation of *Zmiz1* as an oncogene, but the biological functions of Zmiz1 are not well studied. Thus, we sought to further characterize the Zmiz1-induced tumors to determine if any mechanistic insights could be gained. We generated gene expression profiles from five independent tumors from 207-Zmiz1$^{\Delta 1\text{-}185}$;K14-Cre transgenic mice along with six normal control skin samples from 207-Zmiz1$^{\Delta 1\text{-}185}$;K14-Cre, 207-Zmiz1$^{\Delta 1\text{-}185}$, or K14-Cre transgenic mice.

Normalized gene expression profiles were compared with identify genes that showed $\geqslant$2-fold change in expression between tumor and normal skin with a false discovery rate of $<5\%$. This identified 701 and 603 genes that were up- or down-regulated, respectively (Supplementary Table S1 online). Differential expression of six of these genes was verified using qRT–PCR (Supplementary Figure S3 online). Analysis of these results using DAVID (Database for Annotation, Visualization, and Integrated Discovery) (Huang da *et al.*, 2009) or gene set enrichment analysis (Subramanian *et al.*, 2005) did not reveal any significant conservation among the differentially regulated genes. Repeating these analyses using

only genes that showed a ⩾5-fold change in expression also failed to reveal any significant trends.

Zmiz1 accumulates in the nuclei of mouse KAs and human SCCs

Zmiz1 protein expression was evaluated in *SB*-induced KAs by immunohistochemical staining of formalin-fixed, paraffin-embedded skin sections. As predicted, tumors with transposon insertions in the *Zmiz1* locus displayed high levels of Zmiz1 expression (Figure 3a). Conversely, Zmiz1 protein was undetectable in normal mouse skin (Figure 3b), although the endogenous full-length transcript is expressed (Dupuy *et al.*, 2009). Based on these findings, we conclude that the majority of Zmiz1 protein detected in *SB*-induced tumors is derived from the truncated *Zmiz1* transcript.

Similarly, tumor tissue from Zmiz1$^{\Delta 1\text{-}185}$;K14-Cre double-transgenic mice displayed the same strong Zmiz1 nuclear expression pattern that was observed in spontaneous *SB*-induced KAs (Figure 3c). Interestingly, normal adult double-transgenic skin did not express detectable levels of Zmiz1 protein by immunohistochemistry or western blot (Figures 2d and 3d). This led us to hypothesize that Cre-mediated recombination results in complex transgene rearrangements with high frequency and results in a lack of transgene expression in most cells. Thus, Zmiz1$^{\Delta 1\text{-}185}$ overexpression is a rare event that drives hyperplasia, and eventually tumorigenesis. If this is true, expression of the Zmiz1$^{\Delta 1\text{-}185}$ transgene should be tumor specific.

DsRed and EGFP RNA expression was measured by qRT–PCR in normal skin from double- and single-transgenic mice, as well as in tumors (Supplementary Figure S4 online). We found that some level of DsRed expression was retained even after Cre recombination, suggesting that recombination is inefficient. Furthermore, although transgenic Zmiz1$^{\Delta 1\text{-}185}$ expression in normal skin is not detectable at the protein level, we are able to detect expression of EGFP at the RNA level. Consistent with our hypothesis, EGFP RNA expression is greatly increased in tumors. Similarly, a comparison of the normalized expression values for all probes corresponding to *Zmiz1* exons from our exon array data sets showed tumor-specific overexpression of exons encoded by the Zmiz1$^{\Delta 1\text{-}185}$ transgene. Expression of these exons in normal skin from Zmiz1$^{\Delta 1\text{-}185}$;K14-Cre double-transgenic mice was similar to that of nontransgenic control skin samples (Supplementary Figure S4 online).

Our results indicate that *Zmiz1*$^{\Delta 1\text{-}185}$ is an oncogene involved in skin cancer in mice (Figure 2c). In addition, *ZMIZ1*^{short} is overexpressed in human SCCs but not normal keratinocytes (Figure 1c and d). However, protein expression in human SCCs has not been assessed. Therefore, we analyzed a panel of human SCCs by immunohistochemistry to determine the frequency of ZMIZ1 nuclear accumulation. Consistent with our observations in mouse KAs, 14 of 19 human SCCs showed nuclear accumulation of ZMIZ1 (Figure 3e and f). These data, combined with the fact that overexpression of Zmiz1$^{\Delta 1\text{-}185}$ in mouse skin is sufficient to produce skin lesions, indicate that ZMIZ1 is involved in the etiology of human SCCs.

Finally, we obtained a tumor tissue array from the National Cancer Institute to assess ZMIZ1 expression in breast, ovarian, colon, and lung tumors. In all, 28% of breast, 15% of ovarian, and 10% of colon cancers displayed strong nuclear ZMIZ1 expression when analyzed by immunohistochemistry (Table 1 and Supplementary Figure S5 online). This result suggests a conserved role for ZMIZ1 in cancer development across diverse epithelial tissue types.

Biological impact of Zmiz1 protein truncation

As previously mentioned, transposon insertions in our *SB* screen clustered in intron 8 of *Zmiz1*, demonstrating a strong preference for a transcript mimicking *ZMIZ1*^{short} rather than full-length *ZMIZ1*. In an attempt to explain why the short-form might give tumor cells a selective advantage, we examined steady-state protein expression levels of full-length and

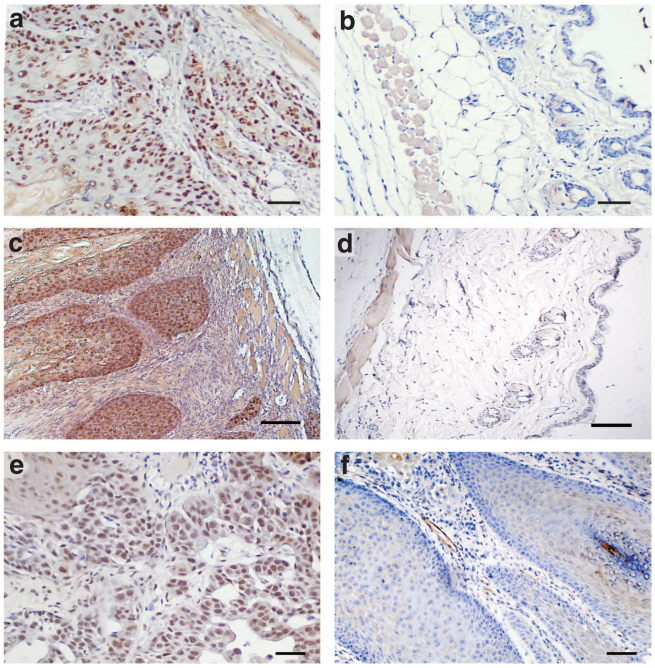

Figure 3. Aberrant nuclear accumulation of Zmiz1 in tumor tissue. (**a**) Immunohistochemical analysis (IHC) reveals overexpression of Zmiz1 in *Sleeping Beauty* (*SB*)-induced tumors with transposon insertions in intron 8. (**b**) Zmiz1 expression is undetectable by IHC in normal mouse skin. Bars = 50 μm. Similarly, nuclear accumulation was observed in keratin 14 (K14)-Cre$^{Tg/+}$; Zmiz1$^{\Delta 1\text{-}185\ Tg/+}$ double-transgenic tumors (**c**), but not normal skin from double-transgenic mice (**d**). Bars = 100 μm. (**e**) Strong nuclear ZMIZ1 expression was observed in 14 of 19 human cutaneous squamous cell carcinomas (SCCs) when analyzed by IHC. (**f**) Example of a human SCC without ZMIZ1 overexpression. Bars = 100 μm.

Table 1. Mixed tumor array ZMIZ1 expression data

Tumor type	% Nuclear Zmiz1	No. of tumors examined
Breast	28%	32
Ovarian	15%	40
Colon	10%	28
Lung	0%	41

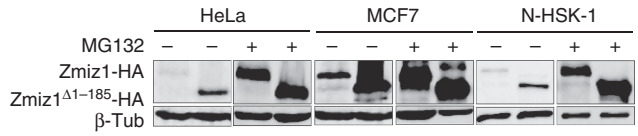

Figure 4. Zmiz1$^{\Delta 1\text{-}185}$ displays increased protein stability. Western blots show higher steady-state protein expression in Zmiz1$^{\Delta 1\text{-}185}$-HA-expressing HeLa, MCF7, and N-HSK-1 cells than those stably expressing full-length Zmiz1-HA, despite lacking significant differential transcript expression as analyzed by quantitative real-time reverse-transcriptase–PCR (qRT–PCR; data not shown). Where indicated, MG132 (20 μM) treatment increased protein stability, suggesting that Zmiz1 and Zmiz1$^{\Delta 1\text{-}185}$ are degraded by the proteasome and that stability is regulated at the protein level.

truncated Zmiz1 expressed from recombinant retroviruses in multiple cell lines (Figure 4). Higher steady-state expression of Zmiz1$^{\Delta 1\text{-}185}$ was consistently observed across independent cell lines, despite no significant differences in transcript levels as assessed by qRT–PCR (data not shown). This suggests that Zmiz1 protein stability is regulated. Consistent with this hypothesis, Zmiz1 protein stability was increased upon treatment with the proteasomal inhibitor MG132 (Figure 4). This result indicates that increased protein stability might confer a selective advantage to tumor cells expressing *Zmiz1*$^{\Delta 1\text{-}185}$ during tumorigenesis.

DISCUSSION

We previously identified *Zmiz1* as a candidate cancer gene in cutaneous squamous cell malignancies using *SB* transposon insertional mutagenesis (Dupuy *et al.*, 2009). The frequency of *ZMIZ1* mutation in human cancer is unknown, and although *ZMIZ1* transcripts have been sequenced in a variety of tumor types, very few independent tumors have been tested for any single tumor type (Forbes *et al.*, 2011). However, an alternative *ZMIZ1* promoter has recently been annotated and is located just upstream of an alternative exon that splices into exon 7 of the full-length transcript. This endogenous transcript is predicted to produce an N-terminally truncated protein (ZMIZ1^{short}) that is strikingly similar to the Zmiz1 truncation selected by the *SB* system.

Endogenous *ZMIZ1*^{short} is expressed in MCF7 breast cancer cells, but not in two immortalized human keratinocyte cell lines (HaCat and N-HSK-1). When we assessed *ZMIZ1*^{short} expression status in a panel of human SCC samples, we observed increased tumor-specific expression when compared with normal skin. Furthermore, expression of the short form in mouse skin leads to tumor formation with remarkably short latency, suggesting that *Zmiz1*$^{\Delta 1\text{-}185}$ provides a robust oncogenic stimulus. Taken together, we conclude that expression of the short form not only correlates with tumor status in both human SCCs and mouse models, but also *ZMIZ1*^{short} expression may play a causal role in tumor development.

It should be noted that the histology of skin tumors induced by skin-specific Zmiz1$^{\Delta 1\text{-}185}$ expression was consistent with the diagnosis of KA rather than SCC. KAs can be defined by their tendency to spontaneously regress. However, it is debated whether these are early-stage tumors that may progress into invasive SCCs, or if they remain a distinct class of squamous hyperplasia (Ko, 2010). Efforts to develop histological methods to distinguish KAs from SCCs have failed to provide reliable and specific markers (Karaa and Khachemoune, 2007; Mandrell and Santa Cruz, 2009). Regardless, skin-specific Zmiz1$^{\Delta 1\text{-}185}$ expression is clearly capable of producing invasive squamous cell malignancies that did not spontaneously regress. Whether these would become even more aggressive upon the acquisition of additional mutations is unknown.

Until now, the role of *Zmiz1* in cutaneous malignancy has been unappreciated, and our transgenic skin tumor model may lend unique insight into the biology of nonmelanoma skin cancer for a variety of reasons. For example, the majority of genetically engineered mouse models of nonmelanoma skin cancer require promotion with a phorbol ester such as 12-*O*-tetradecanoylphorbol-13-acetate and produce mostly benign papillomas, only some of which progress to SCC (Vassar *et al.*, 1992; Wang *et al.*, 1998; Jansen *et al.*, 2001; Liu *et al.*, 2006). Most of these models have also been constructed on susceptible inbred strain backgrounds (e.g., FVB/n, BALB/c) (van Hogerlinden *et al.*, 1999; Proweller *et al.*, 2006; Diez *et al.*, 2009). Despite these features, many existing mouse models develop tumors with a long latency. In contrast, Zmiz1$^{\Delta 1\text{-}185}$; K14-Cre transgenic mice rapidly develop tumors on a resistant strain background without the need for promotion with 12-*O*-tetradecanoylphorbol-13-acetate (Figure 2). Furthermore, we did not detect any spontaneous mutations in either *Hras* ($n=14$) or *Trp53* ($n=13$) in Zmiz1$^{\Delta 1\text{-}185}$-induced KAs (Supplementary Figure S6 online), demonstrating the strong oncogenic potential of Zmiz1. The status of Zmiz1^{short} expression in mouse models of SCC is currently unknown, and it may be useful to investigate whether Zmiz1 also plays a role in these models as KAs are rarely seen in two-stage chemical carcinogenesis experiments.

In an effort to further characterize our model, gene expression profiling was performed on transgenic tumors. Many of the downregulated genes in tumors are involved in muscle function (e.g., *Myh1*, *Ttn*, *Acta1*). It should be noted that, unlike normal skin, tumors are largely devoid of hair follicles and their associated arrector pili muscles. As a consequence, the decreased expression of muscle genes observed in tumors could be a result of variation in cellular composition and may not directly contribute to Zmiz1-induced transformation.

Among the genes showing the greatest increase in expression in tumor relative to normal skin samples are members of the small proline-rich protein (Sprr) and serine peptidase inhibitor (Serpin) families (Supplementary Figure S3 online). Some of these family members have previously been shown to be associated with KA and/or SCC (Kato and Torigoe, 1977; De Heller-Milev *et al.*, 2000). In particular, overexpression of Serpinb3a has been shown to mediate susceptibility to skin tumors in mice (Gariboldi *et al.*, 2003). Therefore, these genes represent likely candidates for elucidating a mechanism by which Zmiz1 induces tumor formation.

One of the few proposed biological functions of ZMIZ1 is an E3 SUMO ligase activity (Sharma *et al.*, 2003). A variety of E3 SUMO ligases have been reported to have roles in promoting genomic stability and DNA repair (Potts, 2009;

Bartek and Hodny, 2010). We report that Zmiz1$^{\Delta 1\text{-}185}$ has oncogenic function in the skin, but whether its SUMO ligase activity is required for oncogenesis is currently unknown. Presumably, both full-length Zmiz1 and Zmiz1$^{\Delta 1\text{-}185}$ could function as E3 SUMO ligases, yet there is clearly selection for the short form in *SB*-induced tumors. Our data suggest that expression of Zmiz1$^{\Delta 1\text{-}185}$ could be because of its increased protein stability. Therefore, expression of Zmiz1$^{\Delta 1\text{-}185}$ or ZMIZ1^{short} would lead to prolonged sumoylation activity, which may ultimately contribute to the oncogenic potential of Zmiz1.

Our experimental results include data directly supporting *Zmiz1* as an oncogene, the development and initial characterization of an original mouse model of skin cancer, and the discovery that an endogenous human alternative *ZMIZ1* transcript is expressed in a tumor-specific manner. We also report that frequent aberrant ZMIZ1 expression occurs in human SCCs, as well as in a subset of human breast, ovarian, and colon cancers, demonstrating a potential role in the development of a variety of human epithelial cancers. Although *ZMIZ1*^{short} expression status in these cancers is unknown, the results of our experiments indicate that *ZMIZ1* is causally involved in tumorigenesis. Our data suggest that further study of the role that ZMIZ1 plays in cancer could have widespread affect on our current understanding of these diseases.

MATERIALS AND METHODS

ZMIZ1 and ZMIZ1^{short} qRT–PCR

Total RNA was extracted from the stably transduced cell lines or human tissues using the PerfectPure RNA Tissue Kit (5-Prime, Gaithersburg, MD). Total complementary DNA was generated with the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) using the oligo dT primer. qPCR was performed using the Platinum SYBR Green qPCR SuperMix (Invitrogen) with the following primers: ZMIZ1altexF (5′-GCGACCGGTGCAACTTCT A-3′), ZMIZ1ex8R (5′-GACAGAGTGGGTTTCATGGAG-3′), ZMIZ1Ex6F (5′-AATCCTGCCAACTTCCACAA-3′), ZMIZ1ex7R (5′-AGCA GTCTGTAGCCGAGGTC-3′), TBPex2F (5′-GCTGAGAAGAGTGTGC TGGA-3′), and TBPex3R (5′-GCCATAAGGCATCATTGGAC-3′). *ZMIZ1* or *ZMIZ1*^{short} C_t values were first normalized to *TBP* C_t values, and then to N-HSK-1 using the $\Delta\Delta C_t$ computational method (Livak and Schmittgen, 2001). This study was conducted in adherence to the Declaration of Helsinki Principles. Patients provided written consent for studies using human tissues and all procedures were approved by the Institutional Review Board.

Generation of Zmiz1$^{\Delta 1\text{-}185}$ transgenic mice

Exons 9–24 of *Zmiz1* complementary DNA were PCR amplified from the National Institutes of Health (NIH) Mammalian Gene Collection IRAV clone ID 6856060 (Invitrogen) using primers Zmiz1-F (5′-GCTAGCCCTATGGCCAATGCCAACAA-3′) and Zmiz1-R (5′-AG ATCTGTTGTTCTCAAAGAGAGACA-3′). This fragment was digested with *Nhe*I/*Bgl*II and cloned into the *Nhe*I/*Xho*I sites of pTraffic (Basheer W *et al.*, in preparation) along with a linker to add a stop codon (annealed Linker+ (5′-GATCCTAAAGATCTC-3′) and Linker− (5′-TCGAGAGATCTTTAG-3′) oligos). This construct was sent to the University of Iowa Transgenic Animal core facility to generate Zmiz1$^{\Delta 1\text{-}185}$ transgenic mice on a B6SJL F1 background. Animals used for aging were backcrossed to C57BL/6 for three generations before breeding to K14-Cre mice (004782, Jackson Laboratory, Bar Harbor, ME). All aging experiments were performed following guidelines approved by the University of Iowa Institutional Animal Care and Use Committee.

Immunohistochemistry

Formalin-fixed, paraffin-embedded skin tumors were cut into 5 μm sections and mounted on glass slides, The ImmPRESS Antibody Peroxidase Kit was used for immunolabeling (Vector Laboratories, Burlingame, CA) using anti-RAI17 (AbGent, San Diego, CA) in a 1:50 dilution overnight at 4 °C. Sections were counterstained with Hematoxylin QS (Vector Laboratories) and mounted in Permount (Fisher Scientific, Pittsburgh, PA) for light microscopy. Primary antibody incubation was omitted in negative controls.

Generation of stable cell lines and MG132 treatment

MCF7 and HeLa cells were obtained from ATCC (Manassas, VA). N-HSK-1 keratinocytes were a gift from Aloysius J Klingelhutz (Gourronc *et al.*, 2010). Retroviral Zmiz1 constructs were produced by co-transfection of GP2-293 packaging cells with the pVSV-G packaging plasmid and pQCXIN, or pQCXIN containing Zmiz1 or Zmiz1$^{\Delta 1\text{-}185}$. Retroviral supernatants were collected 24 hours later and applied to proliferating cells. Stably transduced cells were selected 24 hours later with media containing 400 μg ml^{-1} (HeLa, MCF7) or 100 μg ml^{-1} (N-HSK-1) G418. Selection was maintained for 12 days, whereupon resistant clones were pooled and expanded in selection media.

Total complementary DNA was generated as previously described. Ectopic expression of *Zmiz1* or *Zmiz1*$^{\Delta 1\text{-}185}$ mRNA in stably transduced cell lines was assessed by qPCR with primers against the neomycin resistance sequence (Neo forward: 5′-TGAATGAACTGC AGGACGAG-3′ and Neo reverse: 5′-ATACTTTCTCGGCAGGAGC A-3′). Proteasomal degradation was inhibited by culturing cells in 20 μM MG132 (Sigma, St Louis, MO) for 24 hours.

Western blotting

Total protein was collected by lysing with RIPA. Samples were boiled in a reducing buffer and SDS-PAGE was performed. Protein was transferred to nitrocellulose membranes for western blotting. Antibodies used were anti-HA (1:5,000, Covance, Princeton, NJ), anti-RAI17 (1:100, Abgent), and anti-β-Tubulin (1:1,000, Sigma-Aldrich, St Louis, MO).

See Supplementary Methods online for microarray analysis, transgenic mouse qRT-PCR, *Hras1 and Trp53* sequencing, and *Zmiz1* expression vector generation.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Alam M, Ratner D (2001) Cutaneous squamous-cell carcinoma. *N Engl J Med* 344:975–83

Bartek J, Hodny Z (2010) SUMO boosts the DNA damage response barrier against cancer. *Cancer Cell* 17:9–11

Beliakoff J, Lee J, Ueno H *et al.* (2008) The PIAS-like protein Zimp10 is essential for embryonic viability and proper vascular development. *Mol Cell Biol* 28:282–92

Benjamin CL, Ananthaswamy HN (2007) p53 and the pathogenesis of skin cancer. *Toxicol Appl Pharmacol* 224:241–8

Birney E, Stamatoyannopoulos JA, Dutta A *et al.* (2007) Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* 447:799–816

Brash DE, Rudolph JA, Simon JA *et al.* (1991) A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc Natl Acad Sci USA* 88:10124–8

Dassule HR, Lewis P, Bei M *et al.* (2000) Sonic hedgehog regulates growth and morphogenesis of the tooth. *Development* 127:4775–85

De Heller-Milev M, Huber M, Panizzon R *et al.* (2000) Expression of small proline rich proteins in neoplastic and inflammatory skin diseases. *Br J Dermatol* 143:733–40

Diez FR, Garrido AA, Sharma A *et al.* (2009) RasGRP1 transgenic mice develop cutaneous squamous cell carcinomas in response to skin wounding: potential role of granulocyte colony-stimulating factor. *Am J Pathol* 175:392–9

Dupuy AJ, Rogers LM, Kim J *et al.* (2009) A modified sleeping beauty transposon system that can be used to model a wide variety of human cancers in mice. *Cancer Res* 69:8150–6

Forbes SA, Bindal N, Bamford S *et al.* (2011) COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. *Nucleic Acid Res* 39:D945–50

Gariboldi M, Peissel B, Fabbri A *et al.* (2003) SCCA2-like serpins mediate genetic predisposition to skin tumors. *Cancer Res* 63:1871–5

Gourronc FA, Robertson M, Herrig AK *et al.* (2010) Proliferative defects in dyskeratosis congenita skin keratinocytes are corrected by expression of the telomerase reverse transcriptase, TERT, or by activation of endogenous telomerase through expression of papilloma-virus E6/E7 or the telomerase RNA component, TERC. *Exp Dermatol* 19:279–88

Grippo PJ, Nowlin PS, Cassaday RD *et al.* (2002) Cell-specific transgene expression from a widely transcribed promoter using Cre/lox in mice. *Genesis* 32:277–86

Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4:44–57

Jansen AP, Verwiebe EG, Dreckschmidt NE *et al.* (2001) Protein kinase C-epsilon transgenic mice: a unique model for metastatic squamous cell carcinoma. *Cancer Res* 61:808–12

Karaa A, Khachemoune A (2007) Keratoacanthoma: a tumor in search of a classification. *Int J Dermatol* 46:671–8

Kato H, Torigoe T (1977) Radioimmunoassay for tumor antigen of human cervical squamous cell carcinoma. *Cancer* 40:1621–8

Ko CJ (2010) Keratoacanthoma: facts and controversies. *Clin Dermatol* 28:254–61

Lee J, Beliakoff J, Sun Z (2007) The novel PIAS-like protein hZimp10 is a transcriptional co-activator of the p53 tumor suppressor. *Nucleic Acids Res* 35:4523–34

Li X, Thyssen G, Beliakoff J *et al.* (2006) The novel PIAS-like protein hZimp10 enhances Smad transcriptional activity. *J Biol Chem* 281:23748–56

Liu B, Park E, Zhu F *et al.* (2006) A critical role for I kappaB kinase alpha in the development of human and mouse squamous cell carcinomas. *Proc Natl Acad Sci USA* 103:17202–7

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402–8

Mandrell JC, Santa Cruz D (2009) Keratoacanthoma: hyperplasia, benign neoplasm, or a type of squamous cell carcinoma? *Semin Diagn Pathol* 26:150–63

Pierceall WE, Goldberg LH, Tainsky MA *et al.* (1991) Ras gene mutation and amplification in human nonmelanoma skin cancers. *Mol Carcinog* 4:196–202

Potts PR (2009) The Yin and Yang of the MMS21-SMC5/6 SUMO ligase complex in homologous recombination. *DNA Repair (Amst)* 8:499–506

Proweller A, Tu L, Lepore JJ *et al.* (2006) Impaired notch signaling promotes de novo squamous cell carcinoma formation. *Cancer Res* 66:7438–44

Sauvageau M, Miller M, Lemieux S *et al.* (2008) Quantitative expression profiling guided by common retroviral insertion sites reveals novel and cell type specific cancer genes in leukemia. *Blood* 111:790–9

Sharma M, Li X, Wang Y *et al.* (2003) hZimp10 is an androgen receptor co-activator and forms a complex with SUMO-1 at replication foci. *EMBO J* 22:6101–14

Subramanian A, Tamayo P, Mootha VK *et al.* (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102:15545–50

Uren AG, Kool J, Matentzoglu K *et al.* (2008) Large-scale mutagenesis in p19(ARF)- and p53-deficient mice identifies cancer genes and their collaborative networks. *Cell* 133:727–41

van Hogerlinden M, Rozell BL, Ahrlund-Richter L *et al.* (1999) Squamous cell carcinomas and increased apoptosis in skin with inhibited Rel/nuclear factor-kappaB signaling. *Cancer Res* 59:3299–303

Vassar R, Hutton ME, Fuchs E (1992) Transgenic overexpression of transforming growth factor alpha bypasses the need for c-Ha-ras mutations in mouse skin tumorigenesis. *Mol Cell Biol* 12:4643–53

Wang XJ, Greenhalgh DA, Jiang A *et al.* (1998) Expression of a p53 mutant in the epidermis of transgenic mice accelerates chemical carcinogenesis. *Oncogene* 17:35–45