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Ectopic expression of *Zmiz1* induces cutaneous squamous cell malignancies in a mouse model of cancer

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Abstract

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 non-melanoma 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. Additionally, 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 *ZMIZ1* may play a broader role in epithelial cancers.

Introduction

Non-melanoma skin cancer is the most common human cancer worldwide with ~1 million new cases reported in the United States annually (Alam and Ratner, 2001). While basal cell carcinoma (BCC) is more common than squamous cell carcinoma (SCC), SCC is a more

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Conflict of Interest

The authors state no conflict of interest.

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

aggressive disease and metastasizes at low frequency (Alam and Ratner, 2001)(Alam and Ratner, 2001)(Alam and Ratner, 2001)(Alam and Ratner, 2001). Current treatments rely upon surgical excision of the cancerous tissue. However, surgical removal can be disfiguring since tumors develop on sun-exposed skin. The major risk factor for developing non-melanoma skin cancer is UV exposure, and UV-induced *TP53* mutations are frequently observed in both BCC and SCC (Benjamin and Ananthaswamy, 2007; Brash *et al.*, 1991). Additionally, 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 over-expression of an N-terminally truncated protein (*i.e.* Zmiz1¹⁻¹⁸⁵). 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 ligase with homology to the PIAS (protein inhibitor of activated Stats) family. Prior work demonstrated that ZMIZ1 increases transcriptional activity of the androgen receptor (AR), p53, and Smad3/4 when overexpressed in cultured cells (Lee *et al.*, 2007; Li *et al.*, 2006; Sharma *et al.*, 2003). The increased AR activity was associated with sumoylation of AR, 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 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 Zmiz1¹⁻¹⁸⁵ 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, not only in SCC.

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 (ChIP) 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). 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, 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 SCC and, when possible, matched normal skin samples. Interestingly, we observed tumor-specific *ZMIZ1*^{short} up-regulation in 4 of 6 samples by qRT-PCR (Figure 1d). The full-length transcript (*i.e.* *ZMIZ1*) was up-regulated in only 2 of 6 samples.

Zmiz1¹⁻¹⁸⁵ expression in mouse skin induces squamous cell tumors

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

Each line was crossed to K14-Cre transgenic mice to induce expression of *Zmiz1*¹⁻¹⁸⁵ 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 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*¹⁻¹⁸⁵ was less efficient, as evidenced by lower EGFP protein levels in double transgenic skin (Supplementary Figure S2). Given that both *Zmiz1*¹⁻¹⁸⁵ transgenes are likely multi-copy concatemers, the discrepancy in EGFP expression is likely caused by Cre-mediated deletion of the transgene. Similar situations have previously been described (Grippio *et al.*, 2002). Nevertheless, both *Zmiz1*¹⁻¹⁸⁵ transgenic lines show Cre-inducible expression.

A cohort of *Zmiz1*¹⁻¹⁸⁵;K14-Cre double transgenic mice (n=54) was generated and aged alongside *Zmiz1*¹⁻¹⁸⁵ (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, so these will also be referred to as KAs for the remainder of this manuscript. *Zmiz1*¹⁻¹⁸⁵-induced masses compressed subjacent tissues and exhibited cutaneous muscle invasion in 23 of 147 tumors (15.6%). Double transgenic mice from line 163-*Zmiz1*¹⁻¹⁸⁵ also developed KAs, but exhibited lower penetrance with increased latency. This is likely due to the lower transgene expression levels observed (Supplementary Figure S2). 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 SCC.

Gene expression profiling of *Zmiz1*¹⁻¹⁸⁵ induced skin tumors

The *Zmiz1*¹⁻¹⁸⁵;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 5 independent tumors from 207-*Zmiz1*¹⁻¹⁸⁵;K14-Cre transgenic mice along with 6 normal control skin samples from 207-*Zmiz1*¹⁻¹⁸⁵;K14-Cre, 207-*Zmiz1*¹⁻¹⁸⁵, or K14-Cre transgenic mice.

Normalized gene expression profiles were compared to identify genes that showed 2-fold change in expression between tumor and normal skin with a false discovery rate less than 5%. This identified 701 and 603 genes that were up- or down-regulated, respectively (Supplementary Table S1). Differential expression of 6 of these genes was verified using qRT-PCR (Supplementary Figure S3). Analysis of these results using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang *et al.*, 2009) or gene set enrichment analysis (GSEA) (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 (IHC) staining of formalin-fixed paraffin-embedded skin sections. As predicted, tumors with transposon insertions in the *Zmiz1* locus displayed high levels *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*¹⁻¹⁸⁵;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 IHC 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, Zmiz1¹⁻¹⁸⁵ overexpression is a rare event that drives hyperplasia, and eventually tumorigenesis. If this is true, expression of the Zmiz1¹⁻¹⁸⁵ 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 (Figure S4). We found that some level of DsRed expression was retained even after Cre recombination, suggesting that recombination is inefficient. Furthermore, while transgenic Zmiz1¹⁻¹⁸⁵ 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. Likewise, a comparison of the normalized expression values for all probes corresponding to *Zmiz1* exons from our exon array datasets showed tumor-specific overexpression of exons encoded by the Zmiz1¹⁻¹⁸⁵ transgene. Expression of these exons in normal skin from Zmiz1¹⁻¹⁸⁵;K14-Cre double transgenic mice was similar to that of non-transgenic control skin samples (Supplementary Figure S4).

Our results indicate that *Zmiz1*¹⁻¹⁸⁵ is an oncogene involved in skin cancer in mice (Figure 2c). Additionally, *ZMIZ1*^{short} is overexpressed in human SCC, but not normal keratinocytes (Figure 1c and d). However, protein expression in human SCC has not been assessed. Therefore, we analyzed a panel of human SCCs by IHC 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). This data, combined with the fact that overexpression of Zmiz1¹⁻¹⁸⁵ in mouse skin is sufficient to produce skin lesions, indicates that ZMIZ1 is involved in the etiology of human SCC.

Finally, we obtained a tumor tissue array from the National Cancer Institute (NCI) to assess ZMIZ1 expression in breast, ovarian, colon and lung tumors. Twenty-eight percent of breast, 15% of ovarian, and 10% of colon cancers displayed strong nuclear ZMIZ1 expression when analyzed by IHC (Table 1 and Supplementary Figure S5). 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 truncated Zmiz1 expressed from recombinant retroviruses in multiple cell lines (Figure 4). Higher steady-state expression of Zmiz1¹⁻¹⁸⁵ 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*¹⁻¹⁸⁵ during tumorigenesis.

Discussion

We previously identified *Zmiz1* as a candidate cancer gene in cutaneous squamous cell malignancies using *Seeping Beauty* (*SB*) transposon insertional mutagenesis (Dupuy *et al.*, 2009). The frequency of *ZMIZ1* mutation in human cancer is unknown, and though *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 to normal skin. Furthermore, expression of the short-form in mouse skin leads to tumor formation with remarkably short latency, suggesting that *Zmiz1*¹⁻¹⁸⁵ provides a robust oncogenic stimulus. Taken together, we conclude that expression of the short-form not only correlates with tumor status in both human SCC and mouse models, but also that *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*¹⁻¹⁸⁵ expression was consistent with the diagnosis of keratoacanthoma (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 SCC, or if they remain a distinct class of squamous hyperplasia (Ko, 2010). Efforts to develop histological methods to distinguish KA from SCC have failed to provide reliable and specific markers (Karaa and Khachemoune, 2007; Mandrell and Santa Cruz, 2009). Regardless, skin-specific *Zmiz1*¹⁻¹⁸⁵ 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 non-melanoma skin cancer for a variety of reasons. For example, the majority of genetically engineered mouse models of non-melanoma skin cancer require promotion with a phorbol ester such as TPA (12-O-tetradecanoylphorbol-13-acetate) and produce mostly benign papillomas, only some of which progress to SCC (Jansen *et al.*, 2001; Liu *et al.*, 2006; Vassar *et al.*, 1992; Wang *et al.*, 1998). Most of these models have also been constructed on susceptible inbred strain backgrounds (*e.g.* FVB/n, BALB/c) (Diez *et al.*, 2009; Proweller *et al.*, 2006; van Hogerlinden *et al.*, 1999). Despite these features, many existing mouse models develop tumors with a long latency. In contrast, *Zmiz1*¹⁻¹⁸⁵; K14-Cre transgenic mice rapidly develop tumors on a resistant strain background without the need for promotion with TPA (Figure 2). Furthermore, we did not detect any spontaneous mutations in either *Hras* (n=14) or *Trp53* (n=13) in *Zmiz1*¹⁻¹⁸⁵-induced KAs (Supplementary Figure S6), 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 since 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 down-regulated genes in tumors are involved in muscle function (e.g. *Myh1*, *Tm*, *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). Some of these family members have previously been shown to be associated with KA and/or SCC (De Heller-Milev *et al.*, 2000; Kato and Torigoe, 1977). 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 (Bartek and Hodny, 2010; Potts, 2009). We report that *Zmiz1*¹⁻¹⁸⁵ 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*¹⁻¹⁸⁵ 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*¹⁻¹⁸⁵ could be due to its increased protein stability. Therefore, expression of *Zmiz1*¹⁻¹⁸⁵ 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 SCC, as well as in a subset of human breast, ovarian, and colon cancer, demonstrating a potential role in the development of a variety of human epithelial cancers. While *ZMIZ1*^{short} expression status in these cancers is unknown, the results of our experiments indicate that *ZMIZ1* is causally involved in tumorigenesis. Our data suggests that further study of the role *ZMIZ1* plays in cancer could have widespread impact on our current understanding of these diseases.

Materials & 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 cDNA 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, Carlsbad, CA) with the following primers: ZMIZ1altexF (5'-GCGACCGGTGCAACTTCTA-3'), ZMIZ1ex8R (5'-GACAGAGTGGGTTTCATGGAG-3'), ZMIZ1Ex6F (5'-AATCCTGCCAACTTCCACAA-3'), ZMIZ1ex7R (5'-AGCAGTCTGTAGCCGAGGTC-3'), TBPex2F (5'-GCTGAGAAGAGTGTGCTGGA-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 C_t computational method (Livak and Schmittgen, 2001). This study was conducted in adherence to the Helsinki guidelines. Patients provided written consent for studies using human tissues and all procedures were approved by the Institutional Review Board.

Generation of Zmiz1¹⁻¹⁸⁵ transgenic mice

Exons 9-24 of *Zmiz1* cDNA were PCR-amplified from the NIH Mammalian Gene Collection IRAV clone ID 6856060 (Invitrogen, Carlsbad, CA) using primers Zmiz1-F (5'-GCTAGCCCTATGGCCAATGCCAACAA-3') and Zmiz1-R (5'-AGATCTGTTGTTCTCAAAGAGAGACA-3'). This fragment was digested with NheI/BglII and cloned into the NheI/XhoI sites of pTraffic (Basheer *et al.*, in prep) 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 Zmiz1¹⁻¹⁸⁵ transgenic mice on a B6SJL F1 background. Animals used for aging were backcrossed to C57BL/6 for 3 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 5µm 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) at a 1:50 dilution overnight at 4°C. Sections were counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, CA) 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. Klingelutz (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*¹⁻¹⁸⁵. 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 (HeLa, MCF7) or 100 Wg/ml (N-HSK-1) G418. Selection was maintained for 12 days, whereupon resistant clones were pooled and expanded in selection media.

Total cDNA was generated as previously described. Ectopic expression of *Zmiz1* or *Zmiz1*¹⁻¹⁸⁵ mRNA in stably transduced cell lines was assessed by qPCR with primers against the neomycin resistance sequence (Neo forward: 5'-TGAATGAACTGCAGGACGAG-3' and Neo reverse: 5'-ATACTTTCTCGGCAGGAGCA-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 (1:5000, Covance, Princeton, NJ), anti-RAI17 (1:100, Abgent, San Diego, CA) and anti-β-Tubulin (1:1000, Sigma-Aldrich, St. Louis, MO).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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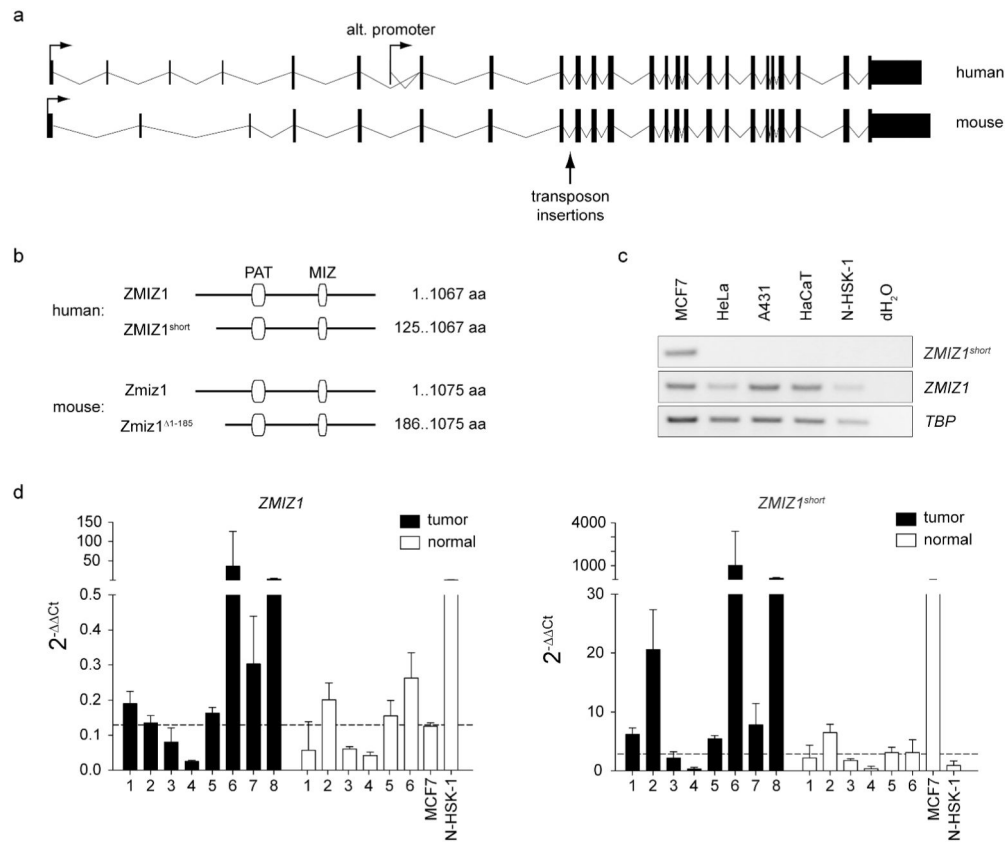


Figure 1. Tumor-specific expression of *ZMIZ1^{short}*

(a) Diagram of the *ZMIZ1* locus in mouse and human. The human alternative promoter is located in intron 6 and drives expression of the *ZMIZ1^{short}* transcript. This transcript is similar to the transcript produced by *SB* transposon insertion into mouse intron 8. (b) The amino acid sequence of human and mouse short-forms differs by approximately 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) 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 SCCs shows increased tumor-specific expression of *ZMIZ1^{short}*. Dotted lines indicate the average C_t values for normal skin.

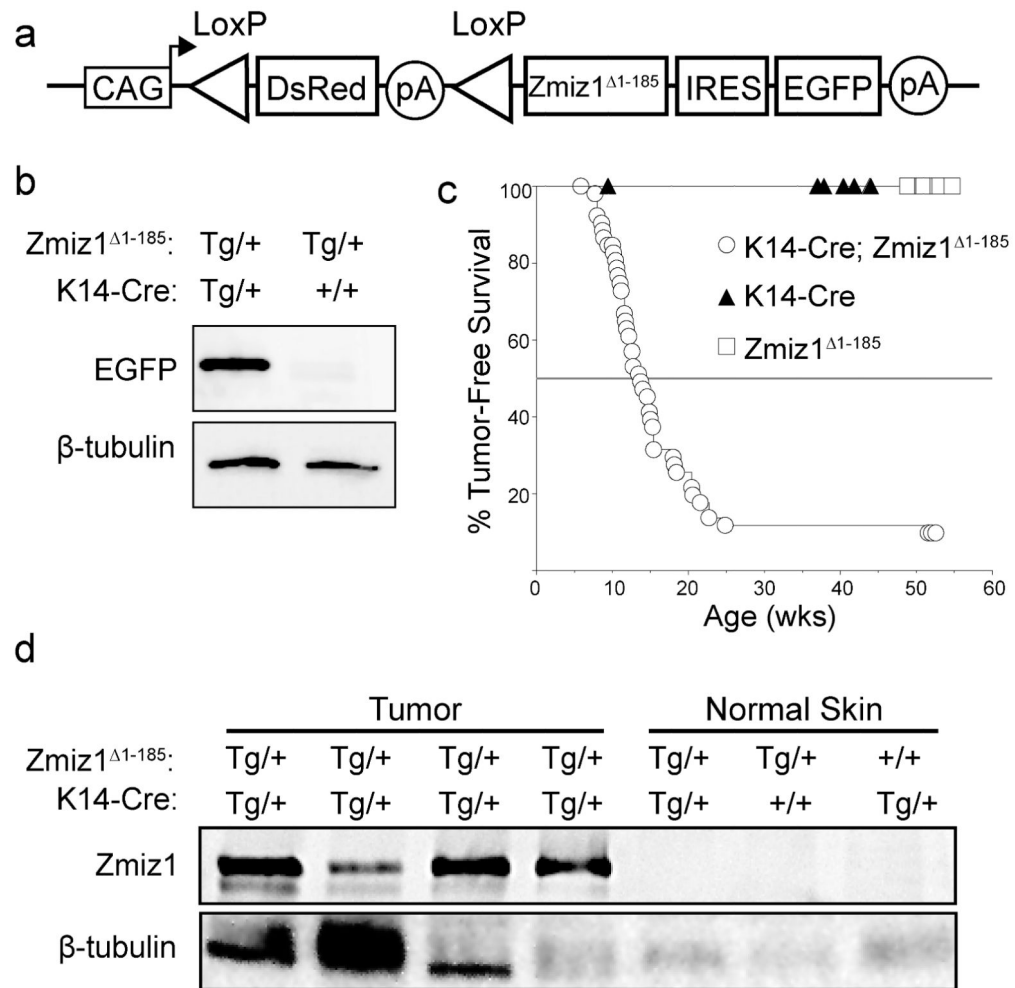


Figure 2. Zmiz1¹⁻¹⁸⁵ transgenic mice develop skin tumors

(a) Diagram of the Cre-inducible Zmiz1¹⁻¹⁸⁵ transgene. (b) Western blot showing skin from Zmiz1¹⁻¹⁸⁵ single transgenic mice has nearly undetectable expression of EGFP, whereas double transgenic mice display detectable EGFP expression. (c) Kaplan-Meier survival curve of mouse line 207 showing tumor-free survival of K14-Cre^{Tg/+}; Zmiz1¹⁻¹⁸⁵ Tg/+ double transgenic mice (circles, n=54), K14-Cre^{+/+}; Zmiz1¹⁻¹⁸⁵ Tg/+ single transgenic (squares, n=18), and K14-Cre^{Tg/+}; Zmiz1¹⁻¹⁸⁵ +/+ single transgenic mice (triangles, n=35). Double transgenic mice developed skin tumors with an average latency of 14 weeks after birth, while single transgenic controls did not develop skin tumors (log-rank, P<0.0001). (d) Western blot confirms overexpression of the Zmiz1¹⁻¹⁸⁵ transgenic protein in mouse tumors. Endogenous Zmiz1 expression is undetectable in normal skin.

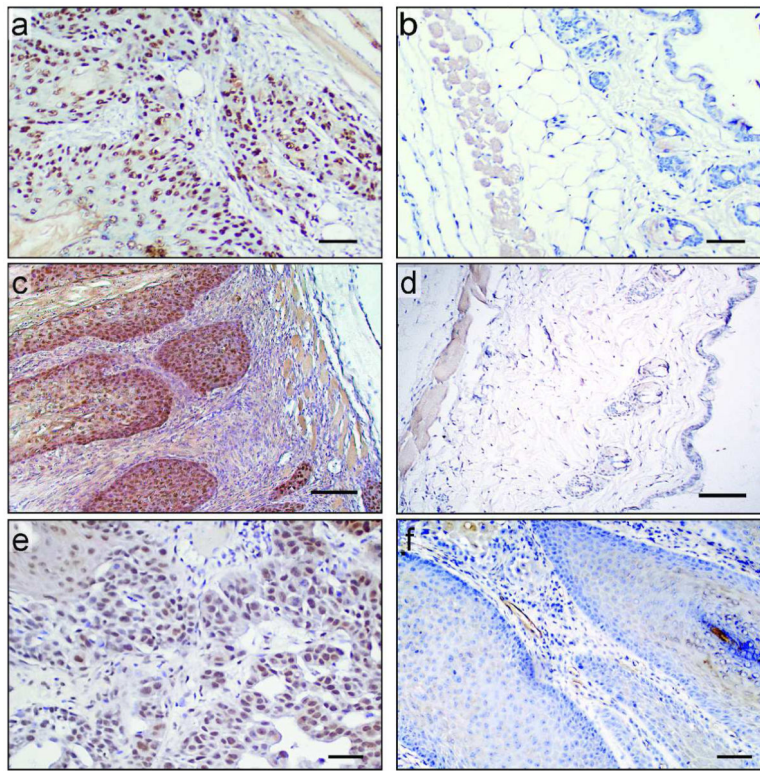


Figure 3. Aberrant nuclear accumulation of Zmiz1 in tumor tissue

(a) Immunohistochemistry (IHC) analysis reveals overexpression of Zmiz1 in *SB.* induced tumors with transposon insertions in intron 8. (b) Zmiz1 expression is undetectable by IHC in normal mouse skin. Scale bars, 50 μ m. Similarly, nuclear accumulation was observed in K14-Cre^{Tg/+}; Zmiz1^{1-185 Tg/+} double transgenic tumors (c), but not normal skin from double transgenic mice (d). Scale bars, 100 μ m. (e) Strong nuclear ZMIZ1 expression was observed in 14 of 19 human cutaneous SCCs when analyzed by IHC. (f) Example of a human SCC without ZMIZ1 overexpression. Scale bars, 100 μ m.

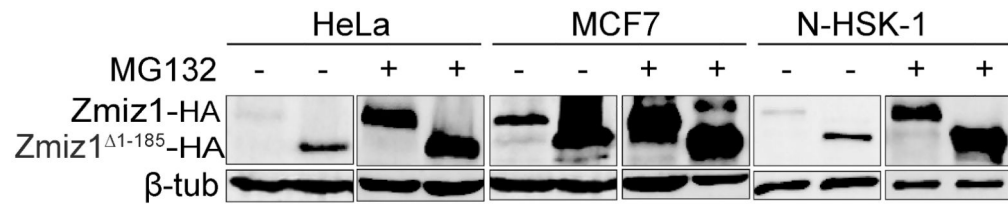


Figure 4. Zmiz1¹⁻¹⁸⁵ displays increased protein stability

Western blots show higher steady-state protein expression in Zmiz1¹⁻¹⁸⁵-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 qRT-PCR (data not shown). Where indicated, MG132 (20 μ m) treatment increased protein stability, suggesting Zmiz1 and Zmiz1¹⁻¹⁸⁵ are degraded by the proteasome and that stability is regulated at the protein level.

Table 1

Mixed tumor array ZMIZ1 expression data

Tumor type	% Nuclear Zmiz1	# of tumors examined
Breast	28%	32
Ovarian	15%	40
Colon	10%	28
Lung	0%	41

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