RING protein Trim32 associated with skin carcinogenesis has anti-apoptotic and E3-ubiquitin ligase properties

Elizabeth J. Horn¹,², Amador Albor¹, Yuangang Liu¹, Sally El-Hizawi¹, Gretchen E. Vanderbeek¹, Melissa Babcock¹, G. Tim Bowden³, Henry Hennings⁴, Guillermina Lozano⁵, Wendy C. Weinberg⁶ and Molly Kulesz-Martín¹,²,⁷

¹Department of Dermatology and Department of Cell and Developmental Biology, Oregon Health and Science University, Portland, OR 97239, USA, ²Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, State University of New York at Buffalo, Buffalo, NY 14263, USA, ³Department of Radiation Oncology, University of Arizona, Tucson, AZ 85724, USA, ⁴Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute/National Institutes of Health, Bethesda, MD 20892, USA, ⁵Department of Molecular Genetics, The University of Texas M.D. Anderson Cancer Center Houston, TX 77030, USA and ⁶Laboratory of Immunobiology, US Food and Drug Administration, Rockville, MD 20857, USA.

To whom correspondence should be addressed
Email: kuleszma@ohsu.edu

Introduction

The TRIM protein family, originally described as the RBCC family, has been extended and re-named based on a characteristic tripartite motif that includes the RING, B1 and/or B2 tripartite domain, RING protein Trim32 associated with skin carcinogenesis has anti-apoptotic and E3-ubiquitin ligase properties.

Abbreviations: EGF, epidermal growth factor; GFP, green fluorescent protein; GST, glutathione S-transferase; HCEM, high calcium Eagle’s medium; HNSCC, head and neck SCC; LCEM, low calcium Eagle’s medium; LGMD2H, Limb-Girdle Muscular Dystrophy type 2H; M.D. Anderson Cancer Center Houston, TX 77030, USA and ¹Department of Immunobiology, US Food and Drug Administration, Rockville, MD 20857, USA.

In this study, we report evidence for Trim32 association with epidermal carcinogenesis and a fraction of human head and neck squamous cell carcinomas (HNSCC). Transduced Trim32 induced in vitro transformation of epidermal keratinocytes and epidermal thickening in vivo. These effects of wild-type Trim32 over-expression were coupled with inhibition of tumor necrosis factor α (TNFα)/ultraviolet B (UVB)-induced apoptosis in vitro and UVB-induced apoptosis.

In vitro

Increased colony number in an epidermal carcinogenesis model, in ultraviolet B (UVB)-induced squamous tumorigenic keratinocytes of a mouse epidermal carcinogenesis assay and epidermal thickening in vivo when skin grafted to athymic nude mice. These effects were not associated with proliferation and were not sufficient for tumorigenesis, even with 12-O-tetradecanoylphorbol-13-acetate treatment or defects in the tumor suppressor p53. However, transduced Trim32 inhibited the synergistic effect of tumor necrosis factor α (TNFα) on UVB-induced apoptosis of keratinocytes in vitro and the apoptotic response of keratinocyte grafts exposed to UVB-light in vivo. Consistent with its RING domain, Trim32 exhibited characteristics of E3-ubiquitin ligases, including being ubiquitylated itself and interacting with ubiquitylated proteins, with increases in these properties following treatment of cultured keratinocytes with TNFα/UVB. Interestingly, missense point mutation of human TRIM32 has been reported in Limb-Girdle Muscular Dystrophy type 2H, an autosomal recessive disease. We propose a model in which Trim32 activities as an E3-ubiquitin ligase favor initiated cell survival in carcinogenesis by blocking UVB-induced TNFα apoptotic signaling.

In vivo

However, transduced Trim32 inhibited the synergistic effect of tumor necrosis factor α (TNFα) on UVB-induced apoptosis of keratinocytes in vitro and the apoptotic response of keratinocyte grafts exposed to UVB-light in vivo. Consistent with its RING domain, Trim32 exhibited characteristics of E3-ubiquitin ligases, including being ubiquitylated itself and interacting with ubiquitylated proteins, with increases in these properties following treatment of cultured keratinocytes with TNFα/UVB. Interestingly, missense point mutation of human TRIM32 has been reported in Limb-Girdle Muscular Dystrophy type 2H, an autosomal recessive disease. We propose a model in which Trim32 activities as an E3-ubiquitin ligase favor initiated cell survival in carcinogenesis by blocking UVB-induced TNFα apoptotic signaling.

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in vivo. Furthermore, Trim32 expressed in keratinocytes had features of an E3-ubiquitin ligase that increased in response to TNFα/UVB treatment. Our results suggest that Trim32 contributes to cellular transformation and tumorigenesis by fostering the survival of cells that would otherwise undergo apoptosis.

Materials and methods

Cell culture

The clonal epidermal model of carcinogenesis (summarized in Figure 1) was derived and described previously (8). Non-transformed 291 keratinocytes exhibit characteristics of primary epidermal cultures, including regulation of proliferation and terminal differentiation by extracellular Ca\(^{2+}\), keratin patterns and comification envelope formation indistinguishable from that of primary epidermal cultures, and lack of tumorigenicity in syngeneic newborn mice. These cells were grown in ‘low calcium Eagle’s medium’ (LCEM), composed of Eagle’s minimal essential medium (EMEM) with Eagle’s salts without CaCl\(_2\) (Invitrogen, Carlsbad, CA), supplemented with 5% (v/v) fetal calf serum, and antibiotic-antimycotic (Invitrogen). EGFR transduced keratinocytes were selected and maintained with 10 μg/ml G418 (Invitrogen). The 09C, 05C and 03C initiated cells and the 09R tumorigenic cells were grown in high calcium Eagle’s medium’ (HCEM), composed of EMEM supplemented with 5% (v/v) fetal calf serum [pre-treated with Chelex-100 resin (Bio-Rad, Hercules, CA), to reduce calcium concentration], 10% (v/v) mouse dermal fibroblast conditioned media, 10 ng/ml EGF (UBI), 1% (v/v) antibiotic-antimycotic (Invitrogen) and 0.04 mM CaCl\(_2\). Transduced keratinocytes were selected and maintained with 100 μg/ml G418 (Invitrogen). The 09C, 05C and 03C initiated cells and the 09R tumorigenic cells were grown in ‘high calcium Eagle’s medium’ (HCEM), composed of EMEM supplemented with 5% (v/v) fetal calf serum, 10 ng/ml EGF, 1% (v/v) antibiotic-antimycotic and 1.4 mM CaCl\(_2\). Tumorigenic 05R and 03R cells were grown in HCEM medium without EGF supplementation. All cells were cultured under identical conditions in LCEM 24 h prior to RNA or protein harvest.

Cloning of mouse Trim32

mRNA differential display was performed as described (9). The complete Trim32 cDNA (GenBank AF347694, NM_035084) was obtained by screening a normal adult mouse testis cDNA library (Stratagene, Cedar Creek, TX) and performing ligation-anchored PCR using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) and BalbC adult mouse brain mRNA template.

Human tumor collection

Patients with HNSCC who gave informed consent were selected for the study. Tumor and uninvolved mucosa samples were removed during surgery. Tumors were macer dissected to remove non-cancerous tissue and samples were snap-frozen immediately for biochemical studies.

Northern blotting and qPCR

RNAs were extracted from cells at ~70% confluence using TRIZol reagent, and normal adult Balb/C mouse tissues and human tumor and tissue samples were homogenized in TRIZol Reagent using a Polytron (Kinematica, Littau-Lucerne). Ten micrograms of RNA was separated on a denaturing formaldehyde agarose gel, transferred to a nylon membrane, and incubated with [\(^{32}P\)]dCTP-labeled 1.5 kb Trim32 probe (3 × 10\(^5\) c.p.m./ml). After hybridization, radioactive signals were visualized by autoradiography and quantified by phosphorimaging (Amerham, Piscatway, NJ).

For quantitative real-time PCR (qPCR), total RNA was treated with DNase I (Invitrogen), and cDNA was generated using AMV reverse transcriptase (Roche, Indianapolis, IN) and random hexamers (Integrated DNA Technologies, Coralville, CA). Gene expression data were collected using the 7900HT thermocycler (Applied Biosystems, Foster City, CA) and gene-specific primers for human Trim32 [qTRIM1 (TGGTCCCTTTGACAGCACGATT) and qTRIM2 (GATCTTTAGCACTGTCAGATTGTCTGT)] and 18S [X81S1(CGCCTACCCACATCAGGAAA) and 18S2 (CCTGTTATTGTATTTTTGTCCTACTACCT)] in the presence of SYBR-Green I dye (Applied Biosystems). SYBR-Green I fluorescence upon binding to the minor groove of double-stranded DNA, allowing the quantification of the double-stranded amplicon in real time. Data were analyzed using the DACT method (ABI user bulletin 82, December 11, 1997).

Trim32-specific antibodies and immunoblotting

Trim32 cDNA was cloned into pGEX (Amerham), and an N-terminal GST–Trim32 fusion protein was produced in bacteria and purified as described (10). GST–Trim32 was injected into three female New Zealand white rabbits (RPCI Laboratory Animal Resources, Buffalo, NY). Antiserum specificity and titer for Trim32 antigen were tested by immunoblotting cell lysates and recombinant protein.

Cultured cells at 70% confluence were lysed at 4°C for 1 h in extraction buffer (20 mM HEPES, pH 7.5, 20% glycerol, 500 mM NaCl, 1.5 mM MgCl\(_2\), 0.2 mM EDTA, 0.1% Triton X-100, 1 mM NaVO\(_3\), 50 mM NaF, 1 mM DTT, 0.4 mM Pefabloc, 5 μl/mg FSC protector, 1 μM leupeptin, 1 μM pepstatin and 0.1 μM aprotinin), and lysates cleaned by centrifugation at 12,000 g for 15 min. Tumors and normal skin were isolated from SKH-1 hairless mice treated with UVB (9.0 kJ/m\(^2\) cumulative dose UVB) for 26 weeks or Senear mice treated with a sub-threshold dose of DMBA (5 μg DMBA/0.2 ml acetone) and treated with O-2-tetradecanolphorbol-13-acetate (TPA) (2 μg TPA/0.2 ml acetone once weekly) or mezerein (4 μg mezerein/0.2 ml acetone twice weekly) for 20 weeks. Tumor and normal skin samples were pulverized in liquid nitrogen, and protein was isolated from TRIzol lysates according to the manufacturer’s instructions. Protein was quantified using the Bradford colorimetric assay (Bio-Rad) according to manufacturer’s instructions.

Lysates (40 μg total protein) were resolved in SDS–PAGE, transferred onto nitrocellulose membranes (Schleicher & Schuller, Kenne, NH) and immunoblotted with monoclonal antibodies for green fluorescent protein (GFPS) (Santa Cruz Biotechnology, Santa Cruz, CA) or Hsp70 (Stressgen, San Diego, CA). Immuno-complexes were visualized by chemiluminescence and quantified using an Epson Perfecton 1650 Photo Scanner and OptiQuant (Packard) software. Fast-Green staining of total protein was used as a loading control.

Transformation assays

The in vitro transformation assay is based on altered response to extracellular calcium ion (Ca\(^{2+}\)) as described (11). Non-transformed 291 keratinocytes proliferate in culture media with 0.04 mM extracellular Ca\(^{2+}\) supplemented with EGF and fibroblast conditioned media. When the extracellular Ca\(^{2+}\) concentration is elevated (>1 mM), and EGF and conditioned media are removed, non-transformed keratinocytes accumulate differentiation-specific keratins, terminally differentiate and slough from the culture dish, while transformed keratinocytes continue to proliferate. In addition, 291 cells have a spontaneous transformation frequency of <0.001, indicating that the background of this assay is very low (11).

For transduction of 291 cells, full-length Trim32, GFP and activated (mutant) Ha-Ras (GenBank J00277) were cloned into the pLXS vector (Clontech) and transfected into ectotropic φNX packaging cells (provided by Dr Gary Nolan, Stanford University). Viral supernatant was titered in NIH-3T3 cells. Multiplicity of infection (MOI) was 0.6 p.f.u./cell for Trim32, anti(α)-sense Trim32, and GFP viruses and 0.006 p.f.u./cell for Ha-Ras. For each transformation assay, three independent 291 cultures were grown in LCEM and infected with retroviral supernatant in the presence of 5 mg/ml polybrene at 32°C. Twenty-four hours post-infection, cells were trypsinized and plated at clonal density (~100 surviving colonies/60-mm dish, 12 dishes per treatment group) in LCEM with 100 μg/ml G418 (Invitrogen) at 37°C. Due to a lower MOI, Ras-transduced cells were plated at 10 × greater density than Trim32-, α-sense Trim32- or GFP-transduced cells. After 10 days, three dishes were removed, cell colonies were fixed in methanol, stained with 10% Giemsa and counted to calculate plating efficiency (%PE = [number of colonies grown at 0.04 mM Ca\(^{2+}\)/number of viable cells plated]× 100). In the remaining dishes, HCEM media with 100 μg/ml G418 was added and exchanged twice weekly. Four weeks after the media switch to HCEM (day 38) colonies were fixed with formalin, stained with the keratin selective stain rhodamine (0.36% in water), and counted to calculate transformation frequency (%TF = [number of colonies grown at 1.4 mM Ca\(^{2+}\)/number of colonies grown at 0.04 mM Ca\(^{2+}\)]× 100). Where indicated, cells were treated with 10 ng/ml TPA beginning at day 5 and continued twice weekly to day 38.

Cell strain generation

Primary keratinocytes were isolated as described previously (8) from neonatal p53R172Hdg transgenic mice and their wild-type siblings (12) or neonatal p53+/- and p53+/- mice (13). Epidermal cells were infected with Trim32 or GFP retroviral supernatant as described above to generate 291-Tim32, 291-GFP, 291-Ha-Ras and respective p53R172Hdg, p53+/- and p53+/-...
Trim32 or GFP-expressing cell strains. Dishes expressing the same virus were pooled and maintained in LCEM with 100 μg/ml G418. RNA expression levels were tested by northern blotting (Trim32, GFP and activated Ha-Ras), genotype of p53-defective cells was tested by PCR, and Trim32, GFP and p53 protein expression levels were tested by immunoblotting.

Tumorigenesis studies

Cells were grown in LCEM with G418 (100 μg/ml), and to the skin biopsy sites of athymic nu/nu mice using an established skin-grafting technique (8). 5 × 10² cells were placed on each graft site. Two weeks after grafting, where indicated, TPA was applied topically once per week for 20 weeks (16 nmol or 2 μg TPA/0.2 ml acetone) to the backs of mice. Mice were killed when a tumor reached 1 cm in diameter. Samples of tumor and uninvolved skin were placed in formalin for histopathological analysis and snap-frozen for biochemical analysis (genotyping of p53 status and immunoblotting for Trim32 and GFP protein).

Apoptosis assays

291-Trim32 or 291-GFP cells (described above) were treated at 50% confluence with 5 ng/ml mouse TNFα (R&D Systems, Minneapolis, MN) or 230 J/m² UVB (using two Westinghouse FS20T12 sun lamps with maximum emission at 310 nm) alone or in combination. After 18 h cells were stained with the myc-epitope-specific 9E10 ascites fluid (8). 5 × 10⁴ body B-2 (Santa Cruz Biotechnology). Samples were incubated overnight at 4°C, and protein–antibody complexes were collected with Sepharose–protein A beads and eluted with 2 M M leupeptin, 1 M l, and a total of 400 μg lysate protein was sample buffer. Samples were resolved in sample buffer. Samples were resolved in 12% SDS±PAGE as described above. Myc-immunoprecipitated proteins were immunoblotted with 9E10.

Expression of Trim32 in an epidermal carcinogenesis model and in normal mouse tissues

The clonal epidermal model of carcinogenesis (Figure 1) consists of non-transformed progenitor cells and three independently initiated lineages with distinct tumor fates (8). Although tumors are morphologically identical to sporadic tumors induced by DMBA/TPA (7,12-dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol-13-acetate) treatment in vivo, they lack Ha-Ras gene over-expression or mutation (15), providing an opportunity to explore other cancer genes (9,16,17). The cell lineages cryopreserved at different stages of transformation and tumorigenesis also lend themselves to functional testing of candidate oncogene activities in growth, apoptosis and in vitro transformation. Trim32 elevation in the epidermal model, originally detected by differential display (data not shown), was confirmed by detection of a single 3 kb mRNA by northern blotting (Figure 2A). All initiated (09C, 05C and 03C) and tumorigenic (09R, 05R and 03R) cells exhibited 2–5-fold elevated expression compared with non-transformed 291 cells, suggesting that Trim32 mRNA is frequently elevated at initiation and persists with tumorigenic progression and malignancy. Trim32 mRNA was present in all normal mouse tissues examined by northern blotting, indicating ubiquitous expression (Figure 2B). The elevated expression of Trim32 protein in mouse brain was confirmed in human brain by immunoblotting with Trim32 antibody (data not shown). Our results are consistent with Frosh et al. (3) who

Fig. 2. Trim32 mRNA expression levels in the epidermal model and normal tissues. (A) Trim32 mRNA expression was elevated in transformed and tumorigenic derivatives of the epidermal model analyzed by northern blotting. Fold increases in Trim32 mRNA signals shown were normalized to G3PDH or 7 s. (B) Trim32 mRNA was expressed in normal adult mouse tissues analyzed by northern blotting as in (A).
found Trim32 mRNA elevated in human brain and Reymond \textit{et al.} (1) who reported ubiquitous expression of Trim32 mRNA in adult tissues and in developing mouse brain (http://www.tigem.it/TRIM/ish/ish/trim32ish.htm).

Mouse Trim32 cDNA was cloned and identified as the ortholog of human HT2A. HT2A protein was originally discovered by binding to TAT, the transcriptional activator of HIV (18) and renamed Trim32 based on functional motifs (1). The cloned mouse Trim32 cDNA sequence (GenBank AF347694, NM_053084) comprises a 1968 nt open reading frame encoding a 655 amino acid protein and is over 96% identical to human TRIM32 in deduced amino acid sequence (Figure 3). Mouse Trim32, like human TRIM32, contains a RING domain (differing from the human sequence by 1 amino acid), a B-box, and a coiled-coil domain, characteristic of the tripartite motif (TRIM) family (1), and the C-terminal NHL domain. The NHL domain in human TRIM32 is responsible for TAT protein interaction (18) and is mutated in LGMD2H (marked with a box) is found in LGMD2H.

Given this association of Trim32 expression with experimental carcinogenesis and human tumors, we next used an in vitro transformation assay (summarized in Figure 4A) was applied to 291 cells retrovirally transduced with GFP, Trim32, α-sense Trim32 or activated Ha-Ras, and selected with G418. As shown in Figure 5B, Trim32 increased transformation frequency 2–3-fold that of GFP or α-sense Trim32 negative controls ($P < 0.0001$, Wilcoxon Rank Sum test). A similar 2–3-fold increase in transformation frequency in Trim32 cells compared with GFP cells was observed in cells treated with TPA ($P < 0.0001$), and activated Ha-Ras as a skin taken from the back of these mice (lanes 5I and 6I, Figure 4B) showed elevation of Trim32 expression, suggesting that UVB-initiated skin may already have elevated Trim32 expression. A single treatment with 1500 J/m$^2$ UVB failed to increase Trim32 expression in mouse skin up to 8 days after irradiation (data not shown), ruling out the possibility that elevated Trim32 expression seen in tumors was an acute keratinocyte response to UVB irradiation. Twenty-four percent of tumors induced by DMBA–TPA or DMBA–mezelein had elevated Trim32 protein levels >2-fold (2/12 and 3/9, respectively) compared with uninvolved skin from age-matched control mice. Histopathology confirmed UVB-induced tumors (6/6) as SCCs and chemically induced tumors (21/21) as benign papillomas. These results suggest that elevation of Trim32 expression is common in UVB-induced carcinomas and present, although less frequently, in papillomas induced by two-stage chemical carcinogenesis protocols. They further support findings in the clonal model that elevation of Trim32 occurred in benign as well as in malignant stages of tumorigenesis.

To determine the relevance of TRIM32 elevation to human cancer development, qPCR was used to examine TRIM32 mRNA levels in HNSCC compared with uninvolved mucosa from the same patient. HNSCC samples from three of 14 patients (21%, see patient number with asterisk) had elevated TRIM32 mRNA expression levels compared with uninvolved mucosa, $P < 0.05$ (Figure 4C). Because HNSCC is associated with risk factors of alcohol and tobacco use, paired tumor and uninvolved mucosa samples from the same patient may have been exposed to the same carcinogenic factors. For this reason, TRIM32 expression levels were examined in normal mucosa from six sleep apnea patients. Relative TRIM32 expression levels were significantly higher in HNSCC patient uninvolved mucosa samples compared with normal mucosa, $P < 0.05$ Wilcoxon Rank Sum test (Figure 4C, inset). Thus, TRIM32 was elevated in a fraction of human HNSCC samples, similar to the fraction of chemically induced mouse epidermal cancers (21–24%). While verification in a larger cohort is necessary, the data from human samples support the findings in the mouse model that TRIM32 expression can be elevated early, prior to malignancy, and maintained or further increased in malignant tumors.

**Keratinocyte transformation in vitro and epidermal thickening in vivo by transduced Trim32**

Given this association of Trim32 expression with experimental carcinogenesis and human tumors, we next used an \textit{in vitro} transformation assay (11) to test whether Trim32 was sufficient for epidermal cell transformation. This assay, based on altered response to extracellular Ca$^{2+}$, measures an early step in epidermal cell transformation in response to a variety of chemical (19), physical or viral oncogenic factors applied \textit{in vitro} or \textit{in vivo} (20). The ability to maintain colonies under conditions that induce terminal differentiation \textit{in vitro} correlates with initiation, whether carcinogen is applied \textit{in vitro} or \textit{in vivo} (21). The \textit{in vitro} transformation assay (summarized in Figure 5A) was applied to 291 cells retrovirally transduced with GFP, Trim32, α-sense Trim32 or activated Ha-Ras, and selected with G418. As shown in Figure 5B, Trim32 increased transformation frequency 2–3-fold that of GFP or α-sense Trim32 negative controls ($P < 0.0001$, Wilcoxon Rank Sum test). A similar 2–3-fold increase in transformation frequency in Trim32 cells compared with GFP cells was observed in cells treated with TPA ($P < 0.0001$), and activated Ha-Ras as a
positive control efficiently induced transformation. Ha-Ras-transduced transformed colonies were larger with more darkly stained, tightly packed cells than Trim32 transformed colonies, suggestive of greater proliferative activity. Doubling times of the stably transduced cells (~2 days) and plating efficiencies in the transformation assays were equivalent between groups, although decreased in the presence of TPA (data not shown).

To assess Trim32 activity in cellular transformation in vivo, 291 cells were retrovirally transduced with Trim32, GFP or mutant Ha-Ras and selected with G418, to obtain the 291-Trim32, 291-GFP and 291-Ras cells, respectively. Expression levels of Trim32, GFP and mutant Ha-Ras were confirmed by immunoblotting (data not shown). Then, cells were engrafted to skin biopsy sites of athymic nu/nu mice (8) and exposed to...
mis with occasional dyskeratotic keratinocytes reminiscent of control. A biopsy of Trim32:TPA mice (Figure 6A, upper compared with GFP:TPA-treated mice or Trim32:solvent TPA. Trim32:TPA-treated mice (3/6) exhibited thickened skin in situ Bowen’s Disease, a SCC (22), shown for comparison (Figure 6A, lower left panel). Few cornified cell layers), loss of polarity, inflammation, breaking was not due to increased proliferation. Small mitotic figures were present, suggesting that epidermal thickening was not due to increased proliferation. Small erythematous nodules were seen on the backs of Ras:TPA-treated mice (3/5), and biopsy revealed parakeratosis (nuclei within the tuous nodules were seen on the backs of Ras:TPA-treated mice (3/5), and biopsy revealed parakeratosis (nuclei within the epidermal thickening seen in the Trim32:TPA mice persisted 12 months post-grafting (5 months after the last TPA treatment), but was seen in only a portion of the grafts and was not associated with enhanced proliferative features or papilloma formation, suggesting that additional carcinogenic events or cofactors are required.

Therefore, we combined elevated Trim32 expression with a malignant conversion-associated defect, loss of p53 function. The p53 gene is mutated in over 50% of human cancers (23) and p53 function is altered at malignant conversion in the clonal epidermal model ([16) and Knights and Kulesz-Martin, unpublished], p53-defective keratinocytes [p53 +/-, p53 +/- (12)] [p53 +/- R172H, p53 +/- (13)] were transduced with Trim32 or GFP retrovirus and engrafted to athymic nu/nu mice (8). Prior to implantation, p53 genotype and Trim32 and GFP protein levels were verified. Trim32-expressing TPA. Trim32:TPA-treated mice (3/6) exhibited thickened skin compared with GFP:TPA-treated mice or Trim32:solvent control. A biopsy of Trim32:TPA mice (Figure 6A, upper left panel) revealed spongiosis (edema) and thickened epidermis with occasional dyskeratotic keratinocytes reminiscent of Bowen’s Disease, a SCC in situ found on sun exposed skin (22), shown for comparison (Figure 6A, lower left panel). Few mitotic figures were present, suggesting that epidermal thickening was not due to increased proliferation. Small erythematous nodules were seen on the backs of Ras:TPA-treated mice (3/5), and biopsy revealed parakeratosis (nuclei within the cornified cell layers), loss of polarity, inflammation, breakdown of the epidermal/dermal junction and dysplasia consistent with early neoplasia (Figure 6A, lower right panel). Phenotypic abnormalities were absent in GFP:TPA (Figure 6A, upper right panel) and Trim32:solvent control mice (data not shown). The epidermal thickening seen in the Trim32:TPA mice persisted 12 months post-grafting (5 months after the last TPA treatment), but was seen in only a portion of the grafts and was not associated with enhanced proliferative features or papilloma formation, suggesting that additional carcinogenic events or cofactors are required.

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cells had 2.5–4-fold elevated Trim32 protein compared with their respective GFP-expressing control cell lines (data not shown). All cell strains were keratinocytes, as indicated by keratin 14 detection by immunoblotting (data not shown).

Tumors formed in p53−/− Trim32 grafts (two tumors per 24 graft sites) beginning at 12 weeks compared with p53−/− GFP grafts (0/24). When combined with mutant p53, tumors formed on 25–33% of 24 graft sites, with no statistically significant differences between p53−/−/R172H Trim32 and p53−/−/R172H GFP groups (Kaplan–Meier analysis, data not shown). Histopathological examination indicated high-grade, anaplastic, spindle cell tumors in all groups that formed tumors. Thus, Trim32 over-expression was insufficient or weakly favorable to tumorigenesis in p53-null keratinocytes and offered little advantage to genetically unstable malignant keratinocytes with the mutant p53 gene.

However, Trim32 significantly accelerated and increased the incidence of epidermal thickening (annular plaque formation) in p53−/− Trim32 compared with p53−/− GFP groups (Kaplan–Meier analysis, shown in days, Figure 6B). Annular plaques radiated out from the original graft site beginning at 9 weeks post-grafting, peaking at 16 weeks at 1–2 cm in diameter (data not shown), and then gradually subsiding until unapparent. Histopathology of the annular plaque revealed compacted collagen bundling (collagen similar to scar tissue or the graft site) compared with uninvolved skin (Figure 6C, upper left compared with upper right panel) and an increased number and length of hair follicles compared with uninvolved skin (Figure 6C, lower left compared with lower right panel). The annular plaque phenotype has not been observed previously in hundreds of grafts of cultured cells or skin. Genotyping of tumors confirmed expression of the p53 mutant or null alleles, while the null allele was absent in the annular plaque (determined by PCR specific for the null cassette, data not shown), indicating that tumors arose from the engrafted epidermal cells as expected, whereas annular plaques were comprised of host cells. Thus, elevated Trim32 expression was sufficient for an early stage of cellular transformation in vitro, but not sufficient for tumorigenesis in vivo in the presence of TPA or defects in the p53 gene.

Protection of Trim32-transduced keratinocytes from apoptosis induced by TNFα/UVB in vitro and UVB in vivo
The common phenotype of Trim32 cells in vivo was thickening of skin, due to epidermal hyperplasia or increased number of hair follicles. This was not associated with increases in mitotic figures in vivo or cellular proliferation rates in vitro, suggesting that Trim32 may function in enhancing cellular survival, and led us to evaluate keratinocytes in response to inducers of apoptosis. UVB was chosen because of its well-documented role in inducing apoptosis in normal epidermis, its significance in human skin cancer and the elevated expression of Trim32 in the UVB-induced mouse skin tumors observed in the current study. Apoptosis underlies the sunburn reaction, a mechanism that eliminates keratinocytes with irreparable UV-induced damage (24). In addition, TNFα is released by skin keratinocytes upon UVB-irradiation, enhancing its apoptotic effects, and is a key mediator of sunburn (25). Therefore, stable retrovirally transduced 291-Trim32 and 291-GFP cells, previously used for in vivo transformation assays (Figure 6A) were treated in vitro with TNFα/UVB and examined for apoptosis. Apoptotic and non-apoptotic cells were distinguished in vitro by phase contrast microscopy, DNA and mitochondrial fluorescence staining, or caspase-3 activation. 291-Trim32 cells were 77% less sensitive to TNFα/UVB treatment than 291-GFP cells (Figure 7A). Furthermore, 291-Trim32 cells exhibited 2–3-fold reduction in caspase-3 activity after TNFα/UVB treatment (Figure 7A, inset). Representative morphology of cells 24 h after treatment with TNFα/UVB is shown (Figure 7B). Non-apoptotic cells have faint blue Hoechst nuclear fluorescence and intense red cytoplasmic mitotracker fluorescence, while apoptotic cells have intense Hoechst fluorescence and faint cytoplasmic red mitotracker fluorescence. The apoptotic response of 291-GFP cells was equal to that of the parental 291 cells, indicating that cell line generation alone did not alter apoptotic potential (data not shown).

On the basis of this anti-apoptotic effect of Trim32, we next examined the response of Trim32-transduced cells to UVB-irradiation in vivo. Apoptotic cells in the epidermis called SBCs are distinguished by their condensed, pyknotic nuclei and shrunken, eosinophilic cytoplasm (26). They are evident in mouse skin within 24 h post-irradiation with 500–750 J/m² UVB-light (27), a dose also shown to induce human keratinocyte apoptosis (28). TNFα was not added in our in vivo experiments because it is present in epidermis (29) and increases after UVB-irradiation (30). Grafted non-initiated keratinocytes have a lifespan of ~21 days (31), and grafts were treated and harvested within 10 days.

Trim32 grafts were 2–2.6-fold less sensitive to apoptosis than GFP grafts irradiated with 600 J/m² UVB (P < 0.02, Wilcoxon rank sum test) based on fewer SBCs in the Trim32 grafts in two experiments. In the experiments shown in Figure 7C, SBCs in GFP grafts increased 10–20 times with increasing UVB doses of 600 and 1200 J/m², respectively, with Trim32 grafts 2-fold less sensitive to apoptosis (P < 0.02, Wilcoxon rank sum test) than GFP grafts (Figure 7C). Representative UVB-irradiated grafts are shown with SBCs indicated (Figure 7D). Apoptosis in UVB-irradiated grafts was confirmed by TUNEL assay (data not shown). The in vivo results indicate that cells expressing Trim32 were less sensitive to UVB-induced apoptosis than cells expressing GFP, suggesting Trim32 fosters cellular survival in the epidermis in response to UVB-irradiation. The in vitro results indicate an inhibition of the synergy between TNFα and UVB-irradiation in inducing apoptosis. We next sought to determine whether Trim32 could exhibit properties of an E3-ubiquitin ligase, as expected because of its RING domain, and whether these properties were responsive to TNFα/UVB treatment.

Trim32 a putative E3-ubiquitin ligase with increased activity after TNFα/UVB treatment
The RING domain of Trim32 suggests activity as an E3-ubiquitin ligase, as proven for TRIM families TRIM18 (7) and TRIM25 (2). E3-ligases link ubiquitin groups to substrate proteins and often self-ubiquitylate. To examine E3-ubiquitin ligase activity of Trim32 in keratinocytes, we determined Trim32’s ubiquitylation state and interaction with ubiquitylated proteins with or without TNFα/UVB treatment. First we ensured that the GFP–Trim32 protein localized predominantly in the cytoplasm, as expected from studies of GFP–TRIM32 (1) and endogenous TRIM32 localization in human fibroblasts (Dr Klaus Wrognemann, University of Manitoba, personal communication). GFP–Trim32 fluorescence was concentrated in bright dots over a diffuse cytoplasmic staining (Figure 8A, right lower panel). Treatment with TNFα/UVB had no effect on GFP or GFP–Trim32 protein levels compared
with untreated lysates (Figure 8B). Upon immunoprecipitation with a myc-specific antibody (that recognizes transfected, myc-tagged ubiquitin) and immunoblotting with a GFP-specific antibody, several bands corresponding to ubiquitylated GFP-Trim32 proteins were detected (Figure 8C, lane 3), and intensity of these bands increased after TNFα/UVB treatment (Figure 8C, lane 4 compared with lane 3). To determine if the GFP-Trim32 fusion protein was interacting with other ubiquitylated proteins, lysates were immunoprecipitated with a GFP-specific antibody and immunoblotted with a myc-ubiquitin-specific antibody. Myc-positive proteins were detected in the high molecular weight range (4150 kDa), corresponding to ubiquitylated cellular proteins interacting with GFP-Trim32 (Figure 8D, lane 3). Signal intensity increased after TNFα/UVB treatment (Figure 8D, lane 4 compared with lane 3). These results indicate that Trim32 has properties of an E3-ubiquitin ligase and that Trim32-associated ubiquitylation is stimulated by TNFα/UVB treatment.

Discussion

Trim32 was associated with carcinogenesis in benign and malignant tumorigenic keratinocytes and in early cellular transformation in vitro. The finding that Trim32 protein expression was uniformly elevated in sporadic cases of UVB-induced SCCs and in a fraction of chemically induced papillomas indicates that association of Trim32 expression with cancer was not limited to the clonal epidermal model and is likely to be of more general significance. Supporting relevance of the mouse model to human cancer, TRIM32 elevation in human HNSCC samples and adjacent mucosa suggests that TRIM32 is elevated early in HNSCC development and, as in the clonal keratinocyte model, maintained in malignant progression. In normal tissues, Trim32 expression was particularly high in brain and testis, two tissues that have very low rates of apoptosis and a blood-barrier that ensures tissue integrity. These observations support speculation that Trim32 has a role in cell survival of normal tissues.

Activated Ha-Ras is associated with proliferation of keratinocytes in the in vitro transformation assays and in vivo (32). Our results failed to provide evidence for proliferative stimulus by Trim32 in vitro or in vivo, and showed that Trim32 expression lowered the apoptotic response to UVB stimulation in vitro and in vivo. Thus, Trim32 may primarily confer a survival advantage, in contrast to activated Ha-Ras, which may confer a growth advantage. Tumor formation rates of Trim32-transduced epidermal cells with p53 null genotype or mutant p53 were

Fig. 7. Cellular survival in Trim32 cells. (A) 291-GFP and 291-Trim32 cells were treated with TNFα, UVB or in combination, and apoptosis was measured in two independent experiments (n = 4). Active caspase-3 was determined by colorimetry (inset) in two independent experiments (n = 3). Statistically significant differences (indicated) were tested by single tailed Wilcoxon Rank Sum test. (B) Panels show representative fields (300x) from untreated and TNFα/UVB-treated cells used to calculate percent apoptosis in (A). Arrowheads point to apoptotic cells, and the arrow points to a non-apoptotic cell. (C) 291-GFP and 291-Trim32 cells were engrafted to skin biopsy sites of athymic nu/nu mice and UVB-irradiated. SBCs were counted relative to total number of basal epidermal cells in eight serial sections, six grafts per condition (mean 746 ± 247 cells counted per graft), and statistical differences (indicated) were calculated using the Wilcoxon Rank Sum Test. (D) Micrographs represent hematoxylin and eosin stained grafts; arrow indicates SBC. Brightfield microscopy was performed at 640× magnification using a Leica DC50 upright Microscope and images captured with a Leica IM digital camera.
similar to that of their respective GFP control epidermal cells, while others have shown that p53⁻/⁻ or p53 +/+ cells transduced with activated Ha-Ras produced SCCs or papillomas (12). We speculate that the absence of an oncogenic proliferation stimulus may explain the insufficiency of Trim32 for tumorigenesis even in p53 null keratinocytes.

Trim32 did induce an in vivo phenotype of epidermal thickening and annular plaque formation, predominantly in mice engrafted with Trim32-transduced cells null for p53. A similar phenotype is seen in annular erythema centrifugum, in which an annular rash indicates paraneoplastic changes in human skin (33). Hair follicle density amplification within the annular plaque may be a precursor to tumorigenesis, as suggested by transgenic mouse models of beta-catenin (34) or ornithine decarboxylase (35). Hair cycling in mouse skin occurs in a wave pattern with interactive signaling between neighboring follicles with 10% of the follicles in anagen, the proliferative phase and 90% in telogen, the resting phase. By morphology, hair follicles of the annular plaque were in late anagen, contributing to the thickened appearance (36), and did not progress to telogen, as in the uninvolved skin, potentially due to anti-apoptotic signaling from the engrafted cells.

Evidence from in vitro and in vivo apoptosis studies suggests Trim32 is involved in the cellular survival response. In vitro Trim32 expression inhibited the synergistic induction of apoptosis by TNFα/UVB treatment but not by UVB alone, suggesting Trim32 may function in TNFα pathways. TNFα is secreted by keratinocytes in response to UVB-irradiation, and the TNFα pathway is required for efficient UVB-induced apoptosis of skin in vivo (25). Consequently, it is reasonable to hypothesize that Trim32 confers cellular survival by dampening the apoptotic cellular response to TNFα after UVB-induced damage, expanding the pool of target cells for further oncogenic events. TNFα type I receptor is essential for the keratinocyte apoptotic response (25). Binding of TNFα to its type I receptor induces activation of the caspase cascade, JNK/p38 kinases, and the NFκB transcription factor (37). While the JNK/p38 pathway has a pro-apoptotic effect, and inhibition of p38 prevents UVB-induced apoptosis (38), our results indicate that Trim32 inhibited apoptosis induced by combined TNFα/UVB treatment but not UVB alone. Therefore, it is unlikely that Trim32 functions by inhibiting the pro-apoptotic JNK/p38 activation in keratinocytes. NFκB is not well understood as a regulator of keratinocyte pathways, but is anti-apoptotic in

Fig. 8. E3-ubiquitin ligase activity of Trim32. (A) Localization of Trim32 was determined by GFP fluorescence, with the same field captured in phase contrast and fluorescence. (B) 293 cells were co-transfected with GFP or GFP-Trim32 expression plasmids and a myc-tagged ubiquitin expression plasmid. Cells were treated with or without TNFα/UVB, and GFP or GFP-Trim32 protein expression was verified by immunoblotting. (C) Lysates from (B) were immunoprecipitated with 9E10 (specific for myc-tagged ubiquitin) and immunoblotted with a GFP-specific rabbit polyclonal antibody to detect GFP or GFP-Trim32. The locations of the IgG heavy chain and ubiquitylated forms of GFP-Trim32 are indicated. (D) Lysates from (B) were immunoprecipitated with B-2 (specific for GFP) and immunoblotted with 9E10. The locations of ubiquitin conjugated proteins and the IgG heavy and light chains are indicated.
certain experimental conditions. Expression of inactivation-resistant IκB sensitizes keratinocytes to TNFα-induced apoptosis, and transgenic mice expressing a constitutively active IκB form show increased apoptosis in epithelial cells (39). However, inhibition of NFκB strongly promotes Ha-Ras tumorigenesis in human keratinocytes (40). Future experiments will be directed toward determining the effect of Trim32 expression on these TNFα signaling pathways and their contribution to transformation and inhibition of apoptosis.

Trim32 involvement in TNFα pathways is particularly intriguing due to TRIM32 gene mutation in LGMD2H (3). LGMD2H has an autosomal recessive inheritance, suggesting this mutation inactivates TRIM32. LGMD2A, another myopathy, is caused by null mutation of the calpain-3 gene resulting in inhibition of NFκB activation (41). It will be of interest to test whether TRIM32 positively modulates muscle survival in response to TNFα or other stress, and whether this effect is lost in the LGMD2H mutant.

The current evidence that Trim32 is ubiquitylated and co-immunoprecipitates with ubiquitylated proteins supports the prediction of Frosk et al. (3) that Trim32 is an E3-ubiquitin ligase. The relevance of these activities to the survival phenotype is supported by their increases after treatment with TNFα/UVB. An additional feature of TRIM proteins as E3-ligases remaining to be tested is RING domain-mediated interaction with E2-ubiquitin conjugating enzymes (42). TRIM19, a nuclear protein, has been shown to interact with UbcH9, an E2-SUMO conjugating enzyme (43), consistent with the observation that most sumoylation occurs in the nucleus and distinct from Trim32, which appears to be predominantly cytoplasmic.

Taken together, these results suggest that Trim32 imparts a survival phenotype to epidermal cells responding to TNFα/UVB-induced stress, whereby these epidermal cells persist and can accumulate additional UVB-induced DNA damage or other oncogenic events, leading to cancer development. Future studies will be directed towards elucidating the role of Trim32 in carcinogenesis, in cellular survival, and as an E3-ubiquitin ligase. We propose a model in which Trim32 activation promotes carcinogenesis by blocking certain stress-induced apoptotic signaling pathways, while inactivation of Trim32 signaling may exacerbate apoptotic signaling in muscle dystrophy. Understanding Trim32 function should provide insights into the control of cell growth and apoptosis in cancer development and muscular dystrophy.

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