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

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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 Boxes, and the coiled-coil domain (1). Several TRIM family members are involved in human developmental disorders or cancer. TRIM20 (PYRIN/MARENOSTRIN), TRIM18 (Midline one/MID1) and TRIM37 (Multiple/MUL) are mutated in familial Mediterranean fever, X-linked Opitz/GBBB syndrome and mulibrey nanism (dwarfism), respectively. TRIM19 (promyeloctytic leukemia/PML), TRIM27 (Ret finger protein/RFP) and TRIM24 (transcriptional intermediary factor 1/TIF1) form oncogenic fusion proteins with RARα (retinoic acid receptor alpha), RET (Ret proto-oncogene) and B-RAF (v-raf murine sarcoma viral oncogene homolog B1), respectively (1), while TRIM25 (estrogen responsive finger protein/ERFP) enhances breast tumor growth (2). TRIM25 and Trim32, the focus of this study, are unique among TRIM proteins so far in being linked to cancer without being oncogenic fusion proteins. Further, the TRIM32 gene is mutated in Limb-Girdle Muscular Dystrophy type 2H (LGMD2H), a mild autosomal recessive myopathy (3).

While the biochemical activity of TRIM32 is currently unknown, the conserved TRIM domains give clues to its function. The RING and B-box domains are zinc fingers with conserved cysteine residues. RING domains are present in E3-ubiquitin ligases and mediate interaction with E2-ubiquitin conjugating enzymes (4), while the coiled-coil domain mediates homo- and heterodimerization (5). TRIM25 also contains a C-terminal NHL domain (6). The RING domain suggests that TRIM32 is an E3-ubiquitin ligase, as proposed by Frosk et al. (3). The RING domain is characteristic of proteins with E3-ubiquitin ligase activity, including proteins involved in the control of apoptosis (cIAP1, cIAP2, XIAP and all TRAF proteins except TRAF1), transcription (Sina, Rbx1 and Mdm2), cell cycle (APC11), tyrosine kinase growth factor receptor signaling (CBL family members) and the tumor suppressor protein BRCA1 (4). Furthermore, other TRIM proteins are E3-ubiquitin ligases, including TRIM18, which targets the degradation of phosphatase 2A, PP2Ac (7) and TRIM25, which targets the degradation of 14-3-3-σ (2).

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 vivo.
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 described and derived previously (8). Non-transformed 291 keratinocytes exhibit characteristics of primary epidermal cultures, including regulation of proliferation and terminal differentiation by extracellular Ca²⁺. 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 and composed of Eagle’s minimal essential medium (EMEM) with Eagle’s salts without CaCl₂ (Invitrogen, Carlsbad, CA), 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 CaCl₂. 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 CaCl₂. Tumorigenic normal 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_055084) was obtained by screening a normal adult mouse testis cdna library (Stratagene, Cedar Creek, TX) and performing ligation-anchored PCR with the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) and Bal3C 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 macrodissected 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 [32P]dCTP-labeled 1.5 kb Trim32 probe (3′[-TTTTTCGGTCACTACCT-3′]) in the presence of SYBR-Green I dye (Applied Biosystems). SYBR-Green I fluoresces upon binding to the minor groove of DNA and excited at 515 nm and emits a green fluorescence colored at 516 nm. Fluorescence was monitored using the ABI PRISM 7700 sequence detection system (ABI). SYBR-Green I fluorescence was used to calculate the amount of RNA in the sample. qPCR was performed with the ABI 7900HT realtime PCR system (Applied Biosystems). SYBR-Green I fluorescent signal was used as an internal control to correct for differences in the amount of RNA present in each sample. Gene expression data were collected using the 7900HT Gene Expression Array (ABI) according to 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 & Schuell, Keene, NH) and immunoblotted with Trim32-specific antibodies and immunoblotting with GST±Trim32 was injected into three female New Zealand White rabbits (RPCL Laboratory Animal Resources, Buffalo, NY). Antibody specificity and titer for Trim32 antigen were tested by immunoblotting cell lysates and recombinant protein.

Transformation assays

The in vitro transformation assay is based on altered response to extracellular calcium ion (Ca²⁺) as described (11). Non-transformed 291 keratinocytes proliferate in culture media with 0.04 mM extracellular Ca²⁺ supplemented with EGF and fibroblast conditioned media. When the extracellular Ca²⁺ concentration is elevated (>1 mM), and EGF and conditioned media are removed, non-transformed keratinocytes accumulate differentiation-specific keratins, terminal 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 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 and mouse Trim32 were designed (3′[-TTTTTCGGTCACTACCT-3′] in the presence of SYBR-Green I dye (Applied Biosystems). SYBR-Green I fluoresces 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 ΔΔCT method (ABI user bulletin 82, December 11, 1997).

Primary keratinocytes were isolated as described previously (8) from neonatal p53R172Hdg transgenic mice and their wild-type siblings (12) or neonatal p53+/- mice and p53+/- mice (13). Epidermal cells were infected with Trim32 or GFP retroviral supernatant as described above to generate 291-Trim32, 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 engrafted to the skin biopsy sites of athymic nu/nu mice using an established skin-grafting technique (8). 5 × 10^5 cells were placed on each graft site. Two weeks after grafting, where indicated, TPA was applied topically once per week for 20 weeks (16 nmoles 2 μg TPA/O.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) and 200 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 5 mg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) and 10 nM mitotracker (Molecular Probes). Hoechst dye intercalates into DNA, and mitotracker accumulates in intact mitochondria in response to mitochondrial oxidation. Cells were observed under phase contrast and fluorescence microscopy using an X170 Olympus Inverted Microscope and images captured with a Magnafire digital camera. Apoptotic and non-apoptotic cells (~300 cells/condition) were counted visually on the captured digital images.

To measure caspase-3 proteolytic activity, cytoplasmic lysates were prepared by freeze thawing of the cell pellets in hypotonic buffer (14) 4.5 h after treatment with TNFα/UVB. Reactions were performed with 30 μg cytosolic protein extract in 230 μl buffer containing 100 nM HEPEPS, pH 7.5, 20% glycerol, 0.1% CHAPS, 10 mM DTT, 0.1 mg/ml BSA and 200 μM Ac-DEVD-NA (a colorimetric substrate for active caspase-3). Absorbance was measured at 405 nm every 30 min for up to 4 h inside a microplate spectrophotometer, and results were plotted as 405 nm absorbance versus incubation time and fitted to a straight line (typical correlation coefficients of 0.989 or better).

The slope of the line is proportional to caspase-3 proteolytic activity.

291-Trim32 and 291-GFP cells were engrafted to skin biopsy sites of athymic nu/nu mice (as described above). Nine days post-grafting, mice were anaesthetized and irradiated with 600, 1200 or 1800 J/m² UVB (as described above) and killed 24 h later. Grafts were harvested and placed in formalin for histopathological analysis. Sunburn cells (SBC) were counted per number of 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 Frosk et al. (3) who 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).

**Ubiquitination studies**

291 cells were transfected with myc-tagged ubiquitin (provided by Dr David Ransom, Oregon Health & Science University) and GFP or GFP-Trim32 expression plasmids. Transfected cells were treated with TNFα/UVB (as described above), and protein extracts were prepared 4.5 h after treatment. The proteasome inhibitor MG132 (20 μM) was added to the culture medium 2.5 h prior to protein extraction. Cells were lysed in a buffer containing 50 mM HEPES, pH 7.5, 0.1% Triton X-100, 150 mM NaCl and 20% glycerol with protein concentration of 1 mg/ml. Samples were incubated overnight at 4°C, and protein-antibody complexes were collected with Sepharose–protein A beads and eluted with 2× sample buffer. Samples were resolved in SDS–PAGE as described above. Myc-immunoprecipitated proteins were immunoblotted with a GFP-specific rabbit polyclonal antibody (Santa Cruz Biotechnology), while GFP-immunoprecipitated proteins were immunoblotted with SE10.

**Results**

**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). 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found TRIM32 mRNA elevated in human brain and Reymond 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 from aspartic acid to asparagine at amino acid 487 (3). Trim32 sequencing at the genomic level indicated that both the 291 non-transformed and 03R squamous cell carcinoma (SCC) cells had wild-type Trim32 (data not shown), verifying association of transformation-related changes with over-expression of wild-type protein.

**Trim32 protein and TRIM32 mRNA elevated expression in independently derived tumors**

Consistent with findings for Trim32 mRNA, Trim32 protein levels (2–6-fold) were elevated in all initiated (09C, 05C and 03C) and tumorigenic (09R, 05R and 03R) cells of the clonal epidermal cell model, compared with non-transformed 291 cells (Figure 4A). To determine the broader relevance of elevated Trim32 expression to epidermal cancers, protein levels were measured in mouse tumors derived by UBV-irradiation or two-stage carcinogenesis protocols (Figure 4B). Trim32 was present in all samples examined, and all tumors (6/6) induced by UBV had elevated Trim32 protein levels, ranging from 2 to 6 times non-irradiated skin from the same mouse. Interestingly, two samples of non-tumorous UBV-irradiated skin taken from the back of these mice (lanes 5I and 6I, Figure 4B) showed elevation of Trim32 expression, suggesting that UBV-initiated skin may already have elevated Trim32 expression. A single treatment with 1500 J/m² UBV 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 UBV irradiation. Twenty-four percent of tumors induced by DMBA–TPA or DMBA–mezerein had elevated Trim32 protein levels >2-fold (2/12 and 3/9, respectively) compared with uninvolved skin from age-matched control mice. Histopathology confirmed UBV-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 UBV-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 in vitro transformation assay (11) to test whether Trim32 was sufficient for epidermal cell transformation. This assay, based on altered response to extracellular Ca²⁺, measures an early step in epidermal cell transformation in response to a variety of chemical (19), physical or viral oncogenic factors applied in vitro or in vivo (20). The ability to maintain colonies under conditions that induce terminal differentiation in vitro correlates with initiation, whether carcinogen is applied in vitro or in vivo (21). The 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 UVB or DMBA/TPA/mezerein treatments (two panels at right). N1 and N2 are representative skin samples from control untreated mice, while T1–T5 are representative independent tumors (nine from a total of 21 tumors) from six treated mice. Trim32 protein expression was analyzed by immunoblotting as in (A). Fold increase in Trim32 expression of tumors was calculated relative to matched control untreated skin of the same mouse (UVB) or average value of untreated control mice (DMBA–TPA/mezerein) normalized to fast green staining of total protein. (C) Trim32 mRNA relative expression levels were analyzed by qPCR, and data are shown relative to normal human liver. Trim32 relative expression of normal mucosa samples is shown in the inset. Each sample was run in triplicate per plate (three plates total), and error bars depict standard deviation across the mean value of three plates. Patients indicated with an asterisk had statistically significant Trim32 expression levels between tumor and uninvolved mucosa tissue (P < 0.05) using a two-tailed Student's t-test.

Fig. 4. Elevated expression of Trim32 in independently derived tumors. (A) Elevated expression of Trim32 protein (78 kDa) in the epidermal model was detected by immunoblotting using Trim32 rabbit antiserum. Fold increases in Trim32 signal values shown were normalized to loading control Hsp70. (B) Trim32 expression was determined in mouse skin tumors. For UVB samples (two panels at left), 1N-6N represent control unirradiated normal skin from the abdomen matched with tumor samples 1T-6T, respectively, from the back of each of six mice. Mouse samples 5 and 6 indicate irradiated back skin (5I and 6I) without obvious tumor. For DMBA–TPA/mezerein treatments (two panels at right), N1 and N2 are representative skin samples from control untreated mice, while T1–T5 are representative independent tumors (nine from a total of 21 tumors) from six treated mice. Trim32 protein expression was analyzed by immunoblotting as in (A). Fold increase in Trim32 expression of tumors was calculated relative to matched control untreated skin of the same mouse (UVB) or average value of untreated control mice (DMBA–TPA/mezerein) normalized to fast green staining of total protein. (C) Trim32 mRNA relative expression levels were analyzed by qPCR, and data are shown relative to normal human liver. Trim32 relative expression of normal mucosa samples is shown in the inset. Each sample was run in triplicate per plate (three plates total), and error bars depict standard deviation across the mean value of three plates. Patients indicated with an asterisk had statistically significant Trim32 expression levels between tumor and uninvolved mucosa tissue (P < 0.05) using a two-tailed Student's t-test.
mis with occasional dyskeratotic keratinocytes reminiscent of Bowen’s Disease, a SCC (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.

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. Genotype 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 vitro was thickening of skin, due to epidermal hyperplasia or increased number of hair follicles. This was not associated with increases in mitotic figures in vitro 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 vitro 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 ISOL, a refinement of the 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 family members 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 Wrogemann, 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 (>150 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 (300×) 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 epidermal 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 Trim32 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 toward 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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