TIG3: An Important Regulator of Keratinocyte Proliferation and Survival

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Tazarotene-induced gene 3 (TIG3) is a tumor suppressor protein. In normal human epidermis, TIG3 is present in the differentiated, suprabasal layers, and it regulates terminal differentiation. TIG3 level is reduced in hyperproliferative diseases, including psoriasis and skin cancer, suggesting that loss of TIG3 is associated with enhanced cell proliferation. Moreover, transient expression of TIG3 leads to terminal differentiation in normal keratinocytes and apoptosis in skin cancer cells. In both cell types, TIG3 distributes to the cell membrane and to the centrosome. At the cell membrane, TIG3 interacts with and activates type I transglutaminase to enhance keratinocyte terminal differentiation. TIG3 at the centrosome acts to inhibit centrosome separation during mitosis and to alter microtubule function. These findings argue that TIG3 is involved in the control of keratinocyte differentiation and that loss of TIG3 in transformed cells contributes to the malignant phenotype.

Journal of Investigative Dermatology (2014) **134,** 1811–1816; doi:10.1038/jid.2014.79; published online 6 March 2014

THE EPIDERMIS AND TERMINAL DIFFERENTIATION

The epidermis is a multilayered tissue designed to provide protection from the environment. The major epidermal cell type is the keratinocyte, which differentiates to form basal, spinous, granular, and cornified layers (Eckert *et al.*, 1997). Formation of these layers involves a process called differentiation. The innermost epidermal layer, the basal layer, contains undifferentiated/proliferative cells that undergo regulated cell division (Pincelli and Marconi, 2010). A fraction of basal cells undergo differentiation to form the spinous, granular, and cornified layers. The layer immediately adjacent to the basal layer is the spinous layer that is distinguished by a spinous appearance. The next layer, the granular layer, consists of cells having intracellular membrane-bound granules, which contain proteins and lipids required for the formation of the epidermal barrier. Basal layer cells have proliferative potential, but cells in the spinous and granular layers, although viable, do not proliferate. This change in differentiation status is associated with stage-specific changes in protein expression. For example, basal cells express keratins 5 and 14, whereas suprabasal cells express differentiation markers including type I transglutaminase (TG1), involucrin, loricrin, filaggrin, keratin 1, and keratin 10 (Eckert *et al.*, 1997). Cells in the outermost ''cornified layer'' undergo terminal differentiation, which involves loss of the nuclei and assembly of the cornified envelope. These fully differentiated cells are called corneocytes, which consist of a network of stabilized keratin surrounded by a covalently cross-linked envelope of protein. Many envelope precursor proteins have been identified, including involucrin, cystatin-α, loricrin, elafin, small proline-rich proteins, filaggrin, and keratin (Eckert *et al.*, 1997). These precursor proteins are covalently cross-linked to form the cornified envelope by TG1, which is anchored to the inner surface of the plasma membrane and catalyzes the formation of ε-(γ-glutamyl)lysine protein–protein cross-links (Chakravarty and Rice, 1989). The ultimate function of the epidermis is to provide a protective barrier. This process is tightly controlled, and a current area of interest is identifying proteins that control this process. Tazarotene-induced gene 3 (TIG3) is an important regulator of cell survival that has an important role in this process (Eckert *et al.*, 2009).

TIG3

TIG3, also known as retinoid-induced gene 1 and retinoic acid receptor responder 3, is a tumor suppressor protein composed of 164 amino acids (Huang *et al.*, 2000; Jiang *et al.*, 2005; Tsai *et al.*, 2007; Ou *et al.*, 2008). TIG3 was discovered in keratinocytes treated with the synthetic retinoid tazarotene (DiSepio *et al.*, 1998). TIG3 shares sequence similarity with members of the H-rev107 family of tumor suppressors, and is over 50% identical to human and rat H-rev107 (DiSepio *et al.*, 1998). H-rev107 family members also show homology with the lecithin retinol acyltransferase family and NlpC/P60 super family (Hajnal *et al.*, 1994; Husmann *et al.*, 1998; Anantharaman and Aravind, 2003; Jahng *et al.*, 2003). The H-rev107 family of proteins shares a common structure that includes N-terminal hydrophilic and C-terminal hydrophobic domains. The TIG3 N-terminal domain comprises 134 amino

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Abbreviations: TG1, type I transglutaminase; TIG3, tazarotene-induced gene 3

Received 9 November 2013; revised 11 December 2013; accepted 16 January 2014; published online 6 March 2014

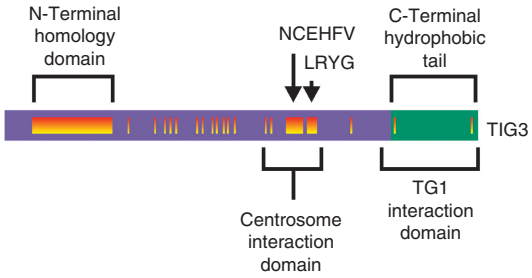

Figure 1. Structure of tazarotene-induced gene 3 (TIG3) and interaction with transglutaminase. TIG3 is a 164-amino-acid protein that is divided into N-terminal (purple, amino acids 1–134) and C-terminal (green, amino acids 135–164) domains. The N-terminal hydrophilic domain includes regions (orange vertical bars) that are highly conserved among members of the H-rev107 family of tumor suppressor proteins (Deucher *et al.*, 2000). The type I transglutaminase (TG1) interaction domain and the centrosome interaction domain are indicated, as are the N-terminal homology domain and the NCEHFV and LRYG motifs.

acids and the C-terminal region comprises 30 amino acids (Figure 1). The N terminus includes several conserved elements, including the N-terminal homology domain, and the NCEHFV and LRYG motifs. The C terminus contains fewer conserved elements, and mutagenesis studies suggest that it serves as a membrane-anchoring domain (Figure 1) (Deucher *et al.*, 2000).

TIG3 REGULATES KERATINOCYTE TERMINAL DIFFERENTIATION

TIG3 is selectively expressed in differentiating cells and during programmed cell death. As such, TIG3 is expressed at vanishingly low levels in proliferating keratinocytes in monolayer culture, but it is expressed at high levels in differentiated human epidermis and keratinocytes grown as epidermal equivalents (Duvic *et al.*, 1997, 2000; Sturniolo *et al.*, 2003; Jans *et al.*, 2008). This pattern of expression suggests that TIG3 is involved in differentiation and survival processes. Transient expression of TIG3 in keratinocytes, at levels within the physiological range observed in differentiated keratinocytes (Jans *et al.*, 2008), is associated with a marked morphological change. TIG3-positive cells display a morphology that resembles that of a terminal corneocyte (Sturniolo *et al.*, 2003). This morphological change is associated with reduced cell survival (Deucher *et al.*, 2000; Sturniolo *et al.*, 2003, 2005; Jans *et al.*, 2008; Eckert *et al.*, 2009). In fact, the expression of TIG3 in cultures of normal keratinocytes results in nearly quantitative cornification (Sturniolo *et al.*, 2003, 2005), suggesting that TIG3 is an inducer of keratinocyte differentiation. Consistent with a potential role in enhancing keratinocyte differentiation, TIG3 levels are reduced in epidermal hyperproliferative disease and cancer (Eckert *et al.*, 1997; DiSepio *et al.*, 1998; Deucher *et al.*, 2000; Duvic *et al.*, 2000, 2003; Jans *et al.*, 2008).

Several studies have examined the role of TIG3 in controlling intracellular processes and how this correlates with intracellular localization. These studies show that TIG3 localizes at the keratinocyte plasma membrane via a mechanism that requires the TIG3 C-terminal hydrophilic membrane-anchoring domain (Sturniolo *et al.*, 2003, 2005; Eckert *et al.*, 2009). A key finding is that membrane-associated TIG3 interacts with TG1 at this location (Sturniolo *et al.*, 2003, 2005; Eckert *et al.*, 2009). TG1 is anchored to the inner surface of the plasma membrane by a lipid anchor (Chakravarty and Rice, 1989; Phillips *et al.*, 1993) and is responsible for catalyzing the formation of the protein–protein cross-links required for cornified envelope assembly. It has been proposed that newly synthesized TIG3 inserts in the plasma membrane, translocates in the membrane to locate TG1, and that a TIG3/TG1 interaction drives the activation of TG1. Indeed, confocal studies reveal that TIG3 colocalizes with TG1 in keratinocytes (Sturniolo *et al.*, 2003, 2005; Jans *et al.*, 2008), and that TG1 activity is observed at these sites. Moreover, biochemical pull-down experiments reveal that TIG3 co-precipitates with TG1 (Sturniolo *et al.*, 2003, 2005; Jans *et al.*, 2008). This requires the full-length TIG3 protein, as removal of the C-terminal membrane-anchoring domain from TIG3 results in a loss of interaction (Sturniolo *et al.*, 2003, 2005). These studies suggest that the inability of the TIG3 C-terminal truncation mutant to interact with TG1 is because the mutant does not localize at the plasma membrane (Deucher *et al.*, 2000; Sturniolo *et al.*, 2003; Jans *et al.*, 2008). Additional studies identify a TIG3 region at the junction between the N-terminal domain and the C-terminal membrane-anchoring domain as being required for interaction with TG1 (Jans *et al.*, 2008) (Figure 1). The interaction of TIG3 with TG1 leads to increased TG1 activity (Sturniolo *et al.*, 2003, 2005), and the increase in TG1 activity is associated with reduced cell survival. The reduction in cell survival can be partially inhibited by monodansylcadaverine, a TG1 substrate competitive inhibitor (Sturniolo *et al.*, 2005). TIG3 is one of the only few proteins that interact with TG1 to increase activity (Jans *et al.*, 2008; Eckert *et al.*, 2009). Similar to most proteins that interact with TG1, TIG3 also functions as a substrate (Sturniolo *et al.*, 2005). As expected, the TIG3 mutant lacking the membrane-localization domain does not interact with TG1 and is not a substrate (Sturniolo *et al.*, 2003, 2005). These findings support the hypothesis that TIG3 regulates key events during terminal keratinocyte differentiation and that interaction at the cell membrane is a requirement for TIG3 function. We propose that TIG3 is produced, moves to the membrane where it is anchored by the C-terminal hydrophobic domain, and that it then moves in the membrane until it contacts and activates TG1. This ultimately leads to the cross-linking of cornified envelope precursors and envelope assembly (Figure 2). We further propose that removing the C-terminal region (green) results in an inability of TIG3 to drive activation, owing to its inability to localize with TG1.

TIG3 AT THE CENTROSOME

In addition to localizing at the plasma membrane, TIG3 is also observed at a perinuclear location. Keratinocytes were stained with anti-TIG3 and antibodies that detect organelle-specific proteins including pericentrin (centrosome), γ-tubulin

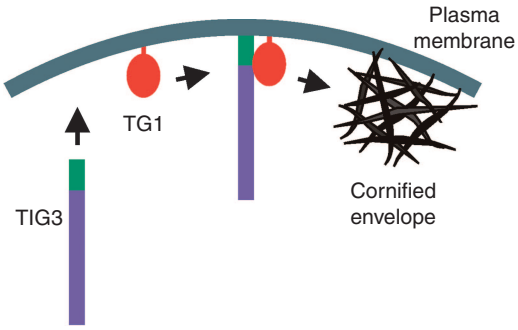

Figure 2. Tazarotene-induced gene 3 (TIG3) interaction with type I transglutaminase (TG1). TIG3 is produced in differentiated keratinocytes and is anchored to the plasma membrane via the C-terminal membrane-anchoring domain (green). TG1 is tethered to the plasma membrane via a lipid anchor (Chakravarty and Rice, 1989). TIG3 interaction with TG1 produces a complex that activates TG1 enzymatic activity, which leads to cornified envelope assembly. It is very likely that the TIG3/TG1 complex also includes other proteins.

(centrosome), GM130 (cis-Golgi apparatus), mannose-6-phosphate receptor (M6PR, trans-Golgi and late endosome), rab11 (recycling endosome), EEA1 (early endosome), clathrin heavy chain (intracellular transport vesicle), lamp1 (lysosome), calnexin (endoplasmic reticulum), and Mitotracker Red (mitochondria). TIG3 localizes with pericentrin and γ-tubulin in 90% of cells (Scharadin *et al.*, 2011). In addition, some colocalization is observed with the Golgi (GM130 and M6PR) markers, which is consistent with the observation that TIG3 localizes at the Golgi in cervical cancer cells (Tsai *et al.*, 2007; Scharadin *et al.*, 2012). TIG3 localization in the vicinity of the centrosome suggests that TIG3 may affect centrosome-related functions.

TIG3 impact on microtubule distribution and elongation

The centrosome (microtubule organizing center) is a 1–2-μm-diameter organelle that functions as a site of microtubule assembly (Doxsey *et al.*, 2005; Tsai *et al.*, 2007; Lim *et al.*, 2009). Microtubules form from α- and β-tubulin heterodimers that nucleate at the centrosome (Doxsey *et al.*, 2005). This microtubule network has two main functions in the cell. In interphase cells, the microtubules serve as a highway to guide and regulate intracellular cargo movement. The cargo includes proteins, vesicles, and organelles. Cargo is bound to the microtubules by molecular motors, called kinesins and dineins, which control anterograde (away from centrosome) and retrograde (toward centrosome) movement. The second role of the centrosome is to assemble the mitotic spindles that guide chromosome movement during mitosis. Both of these functions are absolutely essential for cell division and survival (Doxsey *et al.*, 2005).

The presence of TIG3 in the vicinity of the centrosome suggests that TIG3 may alter centrosome function. Recent reports support this hypothesis. Keratinocytes that transiently overexpress TIG3 display marked changes in cell division and status of the microtubule network. In control cells, the microtubule network radiates from the centrosome throughout the cell. In contrast, in TIG3-expressing cells, the microtubules redistribute to the cell periphery where they form a thick band adjacent to the cell membrane that is linked to the centrosome by thin microtubule ''spokes'' (Scharadin *et al.*, 2012). This rearrangement is accompanied by microtubule stability-inducing post-translational modification of α-tubulin (Scharadin *et al.*, 2011), including increased acetylation of lysine 40 and detyrosination of the C-terminal tyrosine of α-tubulin (Hammond *et al.*, 2008; Ikegami and Setou, 2010; Quinones *et al.*, 2011). The latter modification results in the formation of Δ2-α-tubulin, a highly stable form of α-tubulin. These modifications markedly increase microtubule stability (Gundersen *et al.*, 1987). However, in spite of the presence of these stabilizing modifications in TG3-positive cells, the peripheral microtubule band remains susceptible to disruption by nocodazole (Scharadin *et al.*, 2012) and reforms upon nocodazole removal (Scharadin *et al.*, 2012). Thus, the impact of TIG3 on microtubule dynamics requires further study. The changes in microtubule organization are consistent with an impact of TIG3 on centrosome function. This is supported by studies of microtubule elongation. Microtubule anterograde growth can be measured in real time in live cells using the plus-end binding protein EB1 (Piehl *et al.*, 2004; Jaworski *et al.*, 2008; Dixit *et al.*, 2009). Fluorescently labeled EB1 studies in control cells reveal abundant anterograde microtubule growth and show that this growth is markedly attenuated in TIG3-expressing cells. In contrast, although the initiation of microtubule formation is maintained in TIG3-positive cells, subsequent elongation of these microtubules is severely attenuated (Scharadin *et al.*, 2012). This suggests that TIG3 may suppress centrosome-dependent intracellular cargo distribution.

TIG3 IMPACT ON CENTROSOME SEPARATION

TIG3 also affects the centrosome during mitosis. During normal cell division, the centrosome is duplicated along with the chromosomes during the S-phase of the cell cycle. Subsequently, in early prophase, the daughter centrosomes separate and initiate the formation of radial arrays of microtubules, known as asters. The asters then move to opposite poles of the cell during metaphase and serve to form the mitotic spindle, which interacts with the chromosomes throughout mitosis. A critical and necessary step is the separation of the daughter centrosomes and their migration to the spindle poles. The impact of TIG3 on centrosome function during mitosis was studied by monitoring centrosome separation (Doxsey *et al.*, 2005). In control keratinocytes, 16% of cells display separated daughter centrosomes, which corresponds to the percentage of dividing cells. In comparison, only one percent of TIG3-positive cells display centrosome separation (Scharadin *et al.*, 2012). This difference is not due to TIG3-associated suppression of new centrosome formation, as centrosome synthesis is unchanged in control and TIG3-positive cells (Scharadin *et al.*, 2012). This inhibition of centrosome separation affects cell division. Keratinocytes that express TIG3 display a significant reduction in the uptake of the S-phase cell cycle marker bromodeoxyuridine. Approximately 14% of TIG3-negative keratinocytes, versus 1% of TIG3-expressing keratinocytes, are in S-phase

(Scharadin *et al.*, 2012). These findings support the hypothesis that TIG3 distribution to the centrosome inhibits centrosome separation and secondarily inhibits cell division.

TIG3 INTRACELLULAR LOCALIZATION AND CONTROL OF CELL FUNCTION

A key question is how the cell regulates TIG3 distribution between the plasma membrane and the centrosome. The mechanism is not well understood. Recent studies used a series of TIG3 mutants fused to enhanced green fluorescent protein. These studies identified amino acids 102–125 as sufficient to mobilize enhanced green fluorescent protein to the centrosome (Scharadin *et al.*, 2013). The presence of a centrosome localization sequence at amino acids 102–125 is particularly interesting, as this region encodes amino acids that are highly conserved when TIG3 is compared with other members of the H-rev107 tumor suppressor family (Deucher *et al.*, 2000), suggesting that centrosome-associated action may be a property of other family members. Moreover, TIG3 peptides, derived from within this region (amino acids 111-123), are able to reduce cell survival in some cell types (Simmons *et al.*, 2006).

The microtubule rearrangement displayed in cultured TIG3-expressing keratinocytes may have an important *in vivo* correlate. In the basal cells of the mouse epidermis, the microtubule network emanates from the centrosome. In suprabasal differentiated cells, in contrast, the microtubules preferentially distribute to cell–cell junctions and form a microtubule band at the cell periphery (Lechler and Fuchs, 2007; Scharadin *et al.*, 2011, 2012). It has been proposed that this microtubule redistribution is driven by ninein, a microtubule-anchoring protein. Ninein moves to the cell periphery in differentiated cells (Mogensen *et al.*, 2000; Dammermann and Merdes, 2002; Delgehyr *et al.*, 2005; Lechler and Fuchs, 2007). Preliminary studies indicate that TIG3 expression in human keratinocytes does not alter ninein distribution in human keratinocytes (TM Scharadin and RL Eckert, unpublished). Although further studies are necessary to elucidate the mechanism whereby TIG3 alters microtubule distribution, it is intriguing that TIG3 causes microtubules to distribute to the cell periphery in a pattern similar to that observed in suprabasal keratinocytes in the epidermis (Scharadin *et al.*, 2013). Thus, elevated TIG3 in the suprabasal epidermis may contribute to the microtubule redistribution observed in differentiated cells *in vivo*.

TIG3 IN HYPERPROLIFERATIVE DISEASE

As mentioned above, TIG3 is observed at reduced levels in skin cancer and epidermal hyperproliferative disease (DiSepio *et al.*, 1998). This suggests that the absence of TIG3 may be a permissive event in skin carcinogenesis. On the basis of this information, restoration of TIG3 expression in hyperproliferative diseases would be predicted to reduce cell proliferation and survival. Endogenous TIG3 levels can be increased by treating cells with tazarotene or other retinoids (DiSepio *et al.*, 1998; Huang *et al.*, 2000; Higuchi *et al.*, 2003; Jiang *et al.*, 2005). Retinoid-dependent increased TIG3 expression is also observed in retinoid-treated psoriatic lesions (Duvic *et al.*, 2000, 2003; ; Talpur *et al.*, 2009). This is consistent with observations in cultured keratinocytes. Transient expression of TIG3 in normal keratinocytes leads to a reduction in cell proliferation (Deucher *et al.*, 2000; Sturniolo *et al.*, 2003, 2005; Jans *et al.*, 2008; Eckert *et al.*, 2009). Further, transient expression of TIG3 suppresses growth in cell lines created from several types of cancers, including lung, head and neck, gastric, cervical, and prostate cancer (DiSepio *et al.*, 1998; Deucher *et al.*, 2000; Huang *et al.*, 2000, 2002; Higuchi *et al.*, 2003; Kawakami *et al.*, 2006). Consistent with a role in suppressing proliferation/increasing cell death, enhanced TIG3 expression is observed in tau neuropathies where it is associated with increased neuronal cell death (Wilhelmus *et al.*, 2011). TIG3 suppression of cell survival was also studied using epidermis-derived squamous cell carcinoma cells. These tumor cells proliferate rapidly, and restoring TIG3 expression slows cell proliferation (Scharadin *et al.*, 2011). Expression of TIG3 in cancer cells activates caspases 3 and 9 and PARP cleavage, leading to apoptosis (Scharadin *et al.*, 2011). In addition, TIG3 reduces the level of G1 and G1/S-phase cell cycle regulatory proteins including cyclin D, cyclin E, cdk4, and cdk6. This leads to a cell cycle block at the G1/S-phase transition (Scharadin *et al.*, 2011). The G1/S block is associated with increased expression of $p21^{Cip1}$ mRNA and protein (Scharadin *et al.*, 2011). Elevated TIG3 expression in other keratinocyte cell lines, including the immortalized HaCaT keratinocyte cell line and the transformed A431 squamous cell carcinoma cell line, yields similar results (TM Scharadin and RL Eckert, unpublished). The observation that TIG3 causes apoptosis in cancer cells is in contrast to the findings in normal human keratinocytes where it causes differentiation (Sturniolo *et al.*, 2003, 2005; Jans *et al.*, 2008; Eckert *et al.*, 2009; Scharadin *et al.*, 2011). As mentioned above, TIG3 enhances normal keratinocyte terminal differentiation, in part through binding to, and activation of, TG1, but it does not activate apoptosis (Sturniolo *et al.*, 2003; Jans *et al.*, 2008). Interestingly, TIG3 is distributed to the cell membrane and the centrosome in normal keratinocytes and skin cancer cell lines (Scharadin *et al.*, 2011). Thus, although TIG3 displays a similar pattern of localization in cancer and normal cells, the net outcome (differentiation or apoptosis) is different in the two cell types. Understanding the mechanistic basis for this difference will require additional study.

SUMMARY

TIG3 is a regulator of keratinocyte proliferation and terminal differentiation that is expressed in the suprabasal differentiated epidermis. We propose that onset of TIG3 expression in the suprabasal epidermis is an integral event in the process that reduces cell proliferation and enhances terminal differentiation. In this role, recent studies suggest that TIG3 operates via interaction at two major subcellular locations. The first location is the plasma membrane where it interacts with TG1 to increase activity leading to increased cornified envelope assembly (Duvic *et al.*, 2000; Sturniolo *et al.*, 2003, 2005; Jans *et al.*, 2008) (Figure 3c). The second location is the centrosome, where TIG3 acts to alter microtubule function and

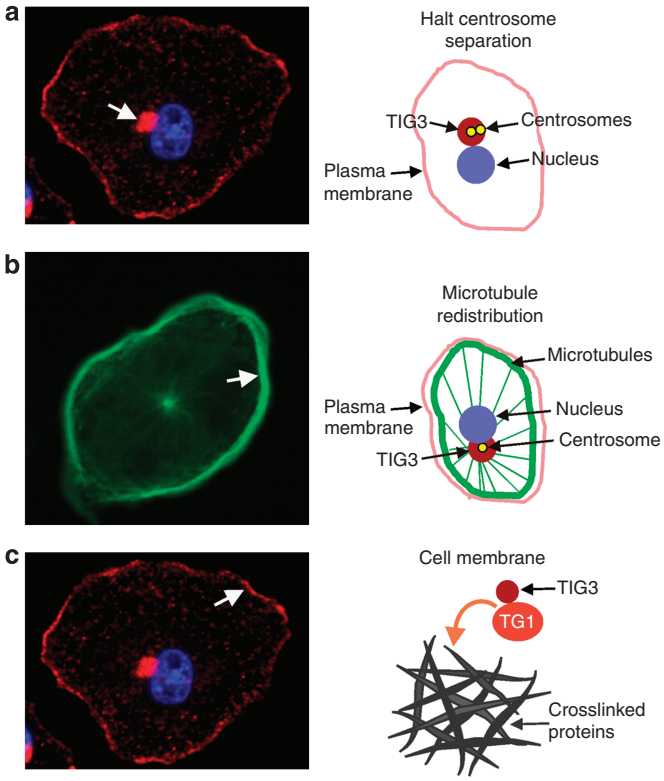

Figure 3. Role of tazarotene-induced gene 3 (TIG3) in keratinocytes. (**a**) TIG3 halts centrosome separation. Cultured normal foreskin keratinocytes were infected with TIG3-encoding adenovirus and after 24 hours stained with anti-TIG3. The arrow indicates TIG3 distribution at the centrosome. The schematic indicates the distribution of the centrosomes, the nucleus, the plasma membrane, and TIG3. The daughter centrosomes are produced during G2 of interphase of the cell cycle, but in the presence of TIG3 they never separate in prophase and move to the poles. (**b**) Impact of TIG3 on microtubule distribution. TIG3 causes a redistribution of the microtubules from a uniform array that extends throughout the cell to the distribution shown in this image stained with anti-β-tubulin (arrow indicates marginal microtubule distribution). Note the presence of the thin microtubule spokes emanating from the centrosome. The schematic representation shows the location of various cellular structures and the localization of TIG3. (**c**) TIG3 activates transglutaminase type I (TG1). The image shows that some of TIG3 distributes to the plasma membrane (arrow). The schematic representation shows that it interacts with the plasma membrane, where it interacts with and activates TG1, which leads to cross-linking of envelope precursor proteins to form the cornified envelope.

inhibit centrosome separation. TIG3 presence promotes a redistribution of the microtubules to form a peripheral band near the plasma membrane (Figure 3b). The α-tubulin subunit in these microtubules is altered by covalent modifications that reduce microtubule turnover and increase stability (Scharadin *et al.*, 2011, 2012). TIG3 also suppresses daughter centrosome separation, which is associated with reduced cell proliferation (Scharadin *et al.*, 2011, 2012) (Figure 3a). Finally, consistent with enhanced cell survival, TIG3 levels are reduced in hyperproliferative skin disorders, including psoriasis and skin cancer (Duvic *et al.*, 1997, 2000, 2003; Talpur *et al.*, 2009). We propose that TIG3 has a dual role to drive growth cessation and differentiation in maturing epidermis. It acts at the centrosome to inhibit centrosome separation and promote microtubule rearrangement to impede cell division, and it also acts at the plasma membrane to activate transglutaminase to stimulate cornified envelope formation.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants (CA184027, AR049713) awarded to Richard L Eckert.

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