(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 September 2011 (09.09.2011)

PCT

(10) International Publication Number
WO 2011/106839 A1

(51) International Patent Classification:
A61K 38/17 (2006.01) *A61P 35/00* (2006.01)
A61K 31/07 (2006.01) *C07K 14/435* (2006.01)

(21) International Application Number:
PCT/AU2011/000236

(22) International Filing Date:
3 March 2011 (03.03.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2010900897 3 March 2010 (03.03.2010) AU
2010904813 29 October 2010 (29.10.2010) AU

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

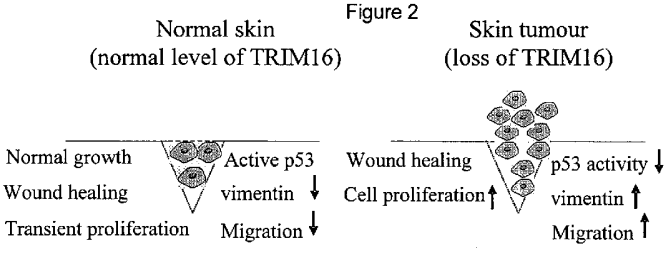
Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: SKIN CANCER PREVENTION AND TREATMENT

Figure 2



(57) **Abstract**: The present invention relates to compositions and methods for minimizing the risk of skin cancer developing in an individual, as well as compositions and methods for treating existing skin cancers to manage the symptoms and/or minimize the risk of the skin cancer metastasising. To this effect, the inventors have identified a protein, TRIM16, whose pattern of expression is linked to a skin cancer or pre-cancer phenotype

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Skin cancer prevention and treatment

Field of the invention

The present invention relates to detection and diagnosis of skin cancers and/or skin pre-cancers, as well as methods of reducing the risk of an individual developing skin cancer and treating existing skin cancers and/or skin pre-cancers to minimise the symptoms and progression of the cancer.

Background of the invention

Skin cancer represents the most common type of cancer in the Caucasian population worldwide. Malignant melanoma is the most aggressive form of skin cancer, and there is an urgent need to develop methods of preventing the development of malignant melanoma, as well as methods and therapeutics to treat it. Other skin cancers, such as primary cutaneous squamous cell carcinomas (SCC) and basal cell carcinoma (BCC) are usually treatable, but have the potential to recur locally and even metastasize and are therefore also important considerations in the development of preventative and therapeutic measures.

The present invention seeks to address these needs.

Reference to any prior art in the specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in Australia or any other jurisdiction or that this prior art could reasonably be expected to be ascertained, understood and regarded as relevant by a person skilled in the art.

Summary of the invention

The present invention seeks to provide compositions and methods for minimizing the risk of skin cancer developing in an individual, as well as compositions and methods for treating existing skin cancers to manage the symptoms and/or minimize the risk of the skin

cancer metastasising. To this effect, the inventors have identified a protein, TRIM16, whose pattern of expression is linked to a skin cancer or pre-cancer phenotype.

Accordingly, in one aspect of the invention, there is provided a pharmaceutical composition for minimising the risk of an individual developing skin cancer, or for treating an individual having a skin cancer or pre-cancer, the composition including TRIM16 (tripartite motif-containing protein 16) and a pharmaceutically acceptable diluent, carrier or excipient. Preferably, the composition includes a tumor suppressor domain of TRIM16.

In one embodiment of this aspect of the invention, the composition further includes a retinoid capable of binding to a retinoic acid receptor, such as one of more of retinol, retinal, retinoic acid, isotretinoin, alitretinoin, etretinate, acitretin, tazorotene, bexarotene and adapalene.

The composition of the invention may also be used in methods to prevent an individual developing skin cancer or at least reduce the risk of an individual developing skin cancer. There is therefore provided, in another aspect of the invention, a method of minimising the risk of an individual developing skin cancer comprising the step of applying an effective amount of TRIM16 to skin cells on an area of the individual's skin, wherein the amount of TRIM16 applied is effective to minimise the risk of skin cancer developing in the skin cells.

Preferably, it is the tumor suppressor domain of TRIM16 that is administered, or a protein containing the tumor suppressor domain of TRIM16, and optionally further includes administration of a retinoid or small molecule compound capable of binding to a retinoic acid receptor or activating retinoid acid receptor expression and transactivation.

In one embodiment, the method of minimising the risk of an individual developing skin cancer may further include the step of:

a) determining the level of expression of TRIM16 in a cell; or

b) determining the level of expression of a compound, the expression of which is regulated by TRIM16

thereby determining the susceptibility of the individual to developing skin cancer

The invention also seeks to provide a kit for determining an individuals susceptibility to skin cancer, including a TRIM16 binding agent. The kit may also include instructions for their use in carrying out the methods of the invention.

The composition of the invention may also be useful as a therapeutic for individuals who already have skin cancer or pre-cancer lesions. Accordingly, in yet another aspect of the invention, there is provided a method of treating a skin cancer including the step of applying TRIM16 to the skin cancer. Preferably, it is the tumor suppressor domain of TRIM16 that is administered, or a protein containing the tumor suppressor domain of TRIM16, and optionally further includes administration of a retinoid or small molecule compound capable of binding to a retinoic acid receptor or activating retinoid acid receptor expression and transactivation.

The skin cancer or pre-cancer may be a melanoma, squamous cell carcinoma or basal cell carcinoma, and may be retinoid treatment resistant.

Brief description of the drawings / figures

Figure 1: (a) nucleotide sequence of TRIM16 and (b) corresponding protein sequence. The protein sequence of the tumor suppressor domain is further provided in (c).

Figure 2: Summary illustration of the role of TRIM16 in normal verses cancerous skin cells.

Figure 3: Tabulation of the grade of TRIM16 staining from histopathological sections from normal, old scars (OC), new scars (NC), actinic keratosis (AK), small cell carcinoma (SCC) and basal cell carcinoma (BCC) skin samples.

Figure 4: Sequence alignment of the tumour suppressor domain of TRIM16 with TRIM16-002 and TRIM16-202.

Figure 5: 5A illustrates the effect of increasing concentrations of retinoic acid (13-cis-RA) on proliferation of human, non-cancerous keratinocytes. Figure 5B is a western blot analysis of TRIM16 levels in the cytoplasm and nucleus of the cells in the presence of retinoic acid. Figure C illustrates the change in cell viability of cancerous skin cells (MET-1) receiving TRIM16 at increasing concentrations of retinoic acid. Figure 5D is a western blot analysis of TRIM16 levels in the cytoplasm and nucleus of MET-1 cells in the presence of retinoic acid.

Figure 6: Figure 6A (cell viability measured via Alamar blue incorporation) and B (cell proliferation as measured by BrdU incorporation) illustrate the reduced cell growth in cells transiently transfected with empty vector (EV) or with TRIM16 plasmid DNA at 72 hours post transfection. C. Cytoplasmic (CE) and nuclear (NE) proteins of MET-1 cells transfected with TRIM16 plasmid DNA for 24 and 48 hours were analysed by immunoblotting using anti-TRIM16 and anti-E2F1 antibodies. D. phospho-pRb (ser807/811) protein was analysed by Western blot with samples from MET-1 and MET-4 cells, transiently transfected with TRIM16 cDNA plasmid or empty vector plasmid. Actin was used as a loading control. E. Lysates of MET-1 cells transfected with indicated plasmids (Lane 1 and 3: GFP-TRIM16 and pCMV-6 empty vectors; Lane 2 and 4: GFP-TRIM16 and pCMV-6-E2F1) were immunoprecipitated by anti-GFP antibody and analysed by immunoblotting using anti-Flag and anti-GFP antibodies.

Figure 7: Schematic illustration of TRIM16 deletion-mutants (Fig 7C) M1-M4 together with empty vector (EV) and wild-type TRIM16 and their ability to transactivate βRARE in retinoid sensitive cells- Figure 7A is pre-RA treatment; Figure 7B is 48 hours post- RA treatment.

Figure 8: Schematic illustrations of the M1-M4 deletion-mutant constructs and the results of those constructs on cell proliferation, as measure by BrdU incorporation.

Figure 9: A: Results of Scratch- Wound assays on confluent monolayers of MET-1 and MET-4 SCC cells transiently transfected with TRIM-16 expression vectors or empty vector controls.

Figure 9: B and C: TRIM16 binds to and modulates vimentin protein expression in SCC cells: B. Interaction of TRIM16 with vimentin. Lysates of MET-1 cells transfected with TRIM16-GFP and vimentin-Flag plasmid DNA and immunoprecipitated with GFP antibody, were then analysed by immunoblotting using anti-GFP and anti-Flag tag antibodies. C. TRIM16 overexpression downregulates exogenous vimentin. Lysates of TRIM16 or empty vector transfected MET-1 cells were analysed by anti-GFP (top panel) or anti-vimentin (Middle panel) or anti-Actin as loading control (Bottom panel).

Figure 10: The average distance moved by the empty vector (EV) control and the average (and standard error) movement of TRIM16 and 4 deletion mutant transfected cells in three independent wounds is shown relative to percentage of original wound. Schematic representations of TRIM16 full-length, M1, M2, M3 and M4 constructs used are shown below.

Figure 11: (A) Gene expression of TRIM16 in melanoma cells (G361 and A375) compared to normal melanocytes (NHEM). (B) corresponding protein expression of TRIM16 with β-Actin as a loading control and (C) the quantitated output of protein expression normalised to β-Actin.

Figure 12: (A) TRIM16 over-expression induces growth arrest and (B) apoptosis. (C) Western blot of TRIM16 over-expression at 24, 48 and 72 hours. (EV= empty vector), β-Actin used as a loading control. Illustrative comparison of G361 cell numbers at 48 hours following transfection with empty vector (top – Figure 12D) or TRIM16 expressing vector (bottom – Figure 12D).

Figure 13: Results of Scratch-Wound assays on confluent monolayers of G361 cells transiently transfected with TRIM-16 expression vectors or empty vector controls.

Figure 14: Representative slides of immunohistochemical TRIM16 staining for increasing melanoma stages: dysplastic compound naevus (DCN), in situ melanoma (IM), dermal invasive melanomas (DIM), lymph node metastasis (LNM) and melanoma distant metastasis (MDM)

Figure 15: Tabulation of the grade of TRIM16 staining between melanoma stages

Detailed description of the embodiments

Reference will now be made in detail to certain embodiments of the invention. While the invention will be described in conjunction with the embodiments, it will be understood that the intention is not to limit the invention to those embodiments. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents, which may be included within the scope of the present invention as defined by the claims. All of these different combinations constitute various alternative aspects of the invention.

As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to exclude further additives, components, integers or steps.

The words "treat" or "treatment" refer to therapeutic treatment wherein the object is to slow down (lessen) an undesired physiological change or disorder. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Treatment may not necessarily result in the complete clearance of an infection but may reduce or minimise complications and side effects of infection and the progression of infection. The success or otherwise of treatment may be monitored by physical examination of the individual, cytopathological, DNA, or mRNA detection techniques.

The words “prevent” and “prevention” refer to prophylactic or preventative measures for protecting or precluding an individual not having a skin cancer from developing skin cancer, or for protecting or precluding an individual having skin pre-cancer from progressing to skin cancer.

The phrase "therapeutically effective amount" means an amount of a compound of the present invention that (i) treats the particular disease, condition, or disorder, (ii)

attenuates, ameliorates, or eliminates one or more symptoms of the particular disease, condition, or disorder, or (iii) delays the onset of one or more symptoms of the particular disease, condition, or disorder described herein.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

The phrase "pharmaceutically acceptable" indicates that the substance or composition must be compatible chemically and/or toxicologically, with the other ingredients comprising a formulation, and/or the mammal being treated therewith.

Composition

TRIM16 (tripartite motif-containing protein 16), also known as EBBP (estrogen-responsive B box protein) was first identified by Liu et al (Mol endo 12(11): 1733-1748 (1998)) and is a member of the RING-B box-coiled-coil (RBCC) or tripartite motif (TRIM) protein family. The nucleotide sequence and corresponding protein sequence of TRIM16 (GenBank Accession no AF096870) is reproduced in Figure 1.

The inventors made the surprising discovery that while TRIM16 is highly expressed in normal skin cells, its expression is significantly downregulated or undetectable in skin cancer cells (Figure 3), potentially resulting in the unrestrained proliferation of keratinocytes. During the early stages this may manifest as actinic keratoses which can then possibly progress to cutaneous SCC. This data strongly points to TRIM16 having a tumor suppressor function, and its presence can be protective of cells susceptible to developing skin cancer.

The invention therefore provides a pharmaceutical composition for minimising the risk of an individual developing skin cancer, the composition including TRIM16 (tripartite motif-containing protein 16) together with a pharmaceutically acceptable diluent, carrier or excipient.

Moreover, administration of TRIM16 to skin cancer cells lacking detectable TRIM16 expression has a therapeutic effect by restoring TRIM16 activity. Therefore, in another embodiment of the invention, there is provided a pharmaceutical composition for treating an individual having a skin cancer, the composition including TRIM16 (tripartite motif-containing protein 16) together with a pharmaceutically acceptable diluent, carrier or excipient.

A cancer is generally defined as an abnormal proliferation of cells. Epithelial cancers of the skin (or skin cancer) are the most common malignant tumours in humans and have an increasing incidence. Epithelial cancers are commonly (but not always) referred to as carcinomas. The most common types of skin cancer are squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). These are usually local carcinomas. Melanomas are a cancer of the melanocytes in the skin and are a more aggressive skin cancer with a greater propensity to metastasise. Other types of skin cancers that are less common include dermatofibrosarcoma protuberans, merkel cell carcinoma and Kaposi's sarcoma.

A "pre-cancer" is a neoplasm that is not invasive but has the potential to progress to a cancer if not identified and/or if it is left untreated. These lesions are in order of increasing potential for progressing to cancer atypia, dysplasia and carcinoma in situ. Thus, in this specification skin pre-cancers (or pre-malignant conditions) may develop into skin cancers if not identified and treated early. For example, Actinic keratosis (also called solar keratosis) is a premalignant condition of thick scaly, or crusty patches of skin that can potentially progress to SCC.

Usually, signs of skin cancer or skin pre-cancer include changes in the skin that do not heal, ulcers in the skin, discoloration, and changes to existing moles or appearance of new ones during adulthood.

The TRIM16 of the composition of the invention may be provided as a full length polypeptide or a via an expression vector including the nucleotide sequence that encodes the full length polypeptide. In one embodiment, the full length TRIM16 polypeptide has a sequence according to SEQ ID NO: 2, and is encoded by a nucleotide sequence

according to SEQ ID NO: 1. It is envisaged that either the protein itself according to SEQ ID NO: 2 may be included in the composition of the invention, or the nucleotide sequence of SEQ ID NO: 1 may be included in the composition of the invention, wherein the nucleotide sequence is delivered to the cells via an expression vector.

Alternatively, the TRIM16 of the composition of the invention may be provided as a fragment or variant of the full length polypeptide. A preferred fragment of TRIM16 is the tumor suppressor domain of TRIM16. Accordingly, in a preferred embodiment of the invention, there is provided a pharmaceutical composition for minimising the risk of an individual developing skin cancer, or for treating an individual having a skin cancer, the composition including a tumor suppressor domain of TRIM16 (tripartite motif-containing protein 16) together with a pharmaceutically acceptable diluent, carrier or excipient. The tumor suppressor domain (TSD) preferably has a sequence according to SEQ ID NO: 3. In another embodiment, the TRIM16 is not in the form of a full length polypeptide.

Whereas the TRIM16 of the invention may comprise a TSD as part of other TRIM16 sequence, in particular embodiments the TRIM16 provided in the form of the TSD (the TSD being a fragment of TRIM16) may consists only of the TSD. In other words, the TSD is not part of a larger TRIM16 polypeptide. In such embodiments, the TSD may be defined consisting of an N terminal coiled-coil domain, a linker, a C terminal Ret finger protein domain and a nuclear localisation signal with the linker. Alternatively, it may be defined as consisting of the sequence SEQ ID NO:3.

The TSD comprises a "Coiled-Coil Domain" (bold text of SEQ ID NO: 3), and a linker region (underlined text). Within the linker region, the inventors have identified a putative nuclear localisation signal of YKKKL (bold and underlined text). As will be described in more detail throughout the specification, and without being bound by any theory, the Coiled-Coil Domain is required for TRIM16 retinoid-dependent tumour suppressor function and the linker is required for both retinoid-dependent tumor suppressor function and TRIM16-induced growth inhibition in retinoid-sensitive cells. Within the linker is a putative nuclear localisation signal of "YKKKL", critical for TRIM16's translocation from the cytoplasm to the nucleus after retinoid treatment.

The TSD may optionally include a RFP domain (Ret finger protein), also referred to as a B30.2 domain.

Accordingly, in another embodiment of the invention, there is provided a pharmaceutical composition for minimising the risk of an individual developing skin cancer, the composition including a tumor suppressor domain of TRIM16 together with a pharmaceutically acceptable diluent, carrier or excipient, wherein the tumor suppressor domain comprises an N terminal coiled-coil domain, a linker, a C terminal Ret finger protein domain and a nuclear localisation signal with the linker.

In an alternative embodiment, the tumor suppressor domain consists of an N terminal coiled-coil domain, a linker, a C terminal Ret finger protein domain and a nuclear localisation signal with the linker.

In yet another alternative, a protein having functional homology to TRIM16 may be administered in the composition of the invention. By functional homology it is meant that the protein achieves the same outcome as TRIM16, whether it is via the same pathway or not. The protein having functional homology with TRIM16 also exhibits at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% ,99% or 100% sequence homology across the TSD. Percent sequence identity is determined by conventional methods, by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, US 53711) AS DISCLOSEDIN Needleman, s.b. and Wunsch, C.D., (1970) Journal of Molecular Biology, 48, 443-453, which is hereby incorporated by reference in its entirety. GAP is used with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Sequence identity of polynucleotide molecules is determined by similar methods using GAP with the following setting for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3.

Examples of proteins that have functional homology and the required level of sequence homology across the TSD are TRIM16 isotypes including TRIM16-002 (protein ID: ENSP00000399918 at human e!Ensembl database and accession AKØ56026), and TRIM16-202 (protein ID: ENSP00000402644 and accession Q59EB2).

The TSD of TRIM16 may optionally be part of a fusion protein. For example, with a protein containing a nuclear localisation signal. This is a preferred embodiment where only the coiled coil domain of the TSD is to be administered, the coiled coil domain being a TRIM16 fragment.

Alternatively, the TSD of TRIM16 may be linked to a targeting protein for targeting, for example, a retinoic acid receptor. The TSD of TRIM16 may also be provided in a non-TRIM16 protein backbone. Alternatively, the linker domain of the TSD may be a non-TRIM16 protein backbone but with the TRIM16 nuclear localisation signal of YKKKL.

The inventors have also discovered that the anti-tumor effects of TRIM16 may be retinoid-dependent or retinoid independent. The inventors have discovered that TRIM16 acts as a tumour suppressor, affecting neuritic differentiation, cell migration and replication through interactions with cytoplasmic vimentin and nuclear E2F1 in neuroblastoma cells without retinoid treatment. Over-expression of TRIM16 caused growth inhibition in multiple cancer cell lines including: SCC, melanoma, breast, lung cancer and neuroblastoma cells without retinoid treatment.

Retinoids are a group of synthetic and natural Vitamin A analogs known to have anti-cancer activity. The anti-cancer effects of retinoids are mediated by their nuclear receptors which are heterodimers formed between the retinoic acid (RA) receptors including RARα, RARβ and RARγ and retinoid X receptors RXRα, RXRβ and RXRγ. Retinoid binding to these receptors can promote apoptosis and inhibit growth in skin cancer cells. RARβ2 in particular is a critical factor in conveying the growth inhibitory component of the signal. It has been shown that TRIM16 is a RARβ binding partner and an important co-regulator in the retinoid anti-cancer signal (Cheung et al, *J Biol Chem.* 281: 18246-56).

The composition of the invention may therefore further include a retinoid capable of binding to a retinoic acid receptor and/or novel small molecule ligands which bind to retinoic acid receptors. The retinoid may be selected from one or more of retinol, retinal, retinoic acid, isotretinoin, alitretinoin, etretinate, acitretin, tazorotene, bexarotene and adapalene.

Alternatively, retinoids such as all-trans-retinoic acid, 13-cis-reinoic acid, and N-(4-hydroxyphenyl) retinamide (4-HPR) may be selected. Low doses of these retinoids are capable of including differentiation and apoptosis. 9-cis-retinoic acid, a pan retinoid agonist has shown up-regulation of retinoic acid receptor β (RARβ), and the rexinoid bexarotene may also be included in a composition of the invention.

In yet another embodiment, the composition of the invention may further include UVA and UVB blocking ingredients including one or more of cinnamates, OMC (Octyl methoxycinnamate), ethylhexyl p-Methoxycinnamate, salicylates, OCS (Octyl Salicytate), Homomenthyl Salicylate, Triethanolamine, PABA (Para Aminobenzoic Acid), Padimate O, Padimate A, Glyceryl Aminobenzoate, Octyl Dimethyl Paba, Octocrylene, Zinc Oxide, Titanium Oxide, Benzoophenones, Oxybenzone, Dioxybenzone and Avobenzone. It would be within the skill of the person in the art to identify other suitable UVA and UVB blocking agents suitable for inclusion in the composition of the invention.

The composition of the invention may further comprise a pharmaceutically acceptable diluent, carrier, excipient or like compound. Acceptable diluents, carriers, excipients, and stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as plasma albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine;

monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

Sustained-release preparations may be prepared. Suitable, examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the N-acylated dipeptide proline boronate compound, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or polyCvinylalcohol)), polylactides, copolymers of L-glutamic acid and gamma- ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

The composition may be prepared for various routes and types of administration. The TRIM16 is optionally mixed with pharmaceutically acceptable diluents, carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences (1980) 16th edition, Osol, A. Ed.), in the form of a lyophilized formulation, milled powder, or an aqueous solution. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and if necessary, shaping the product. Formulation may be conducted by mixing at ambient temperature at the appropriate pH, and at the desired degree of purity, with physiologically acceptable carriers, i.e., carriers that are non-toxic to recipients at the dosages and concentrations employed. The pH of the formulation depends mainly on the particular use and the concentration of compound, but may range from about 3 to about 8. Formulation in an acetate buffer at pH 5 is a suitable embodiment. The inhibitory compound for use herein is preferably sterile. The compound ordinarily will be stored as a solid composition, although lyophilized formulations or aqueous solutions are acceptable.

Thus in certain embodiments there is provided a use of a composition as described above in the manufacture of a medicament for minimising the risk of an individual developing skin cancer, or for treating an individual having a skin cancer, or for minimising the risk of metastasis of skin cancer cells.

In alternative embodiments, there is provided TRIM16 as described above in compositions for use in minimising the risk of an individual developing skin cancer, or for treating an individual having a skin cancer, or for minimising the risk of metastasis of skin cancer cells.

It is especially advantageous to formulate the compositions of the present invention for topical application. Compositions for topical application may be formulated as an ointment, cream, gel or lotion. Ointments and creams may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Lotions and gels may be formulated with an aqueous or oily base, and will in general also contain one or more emulsifying agents, stabilising agents, dispersing agents, suspending agents, thickening agents, or colouring agents.

The composition of the present invention is preferably formulated for percutaneous administration. Percutaneous, or transdermal, administration of compounds may be achieved by a number of routes including supersaturated drug solutions, eutectic systems, complexation, liposomes, vesicles and particles. Alternatively or in addition to, the stratum corneum of the skin can be modified by hydration, or by chemical enhancers acting on the structure of the stratum corneum lipids and keratin (Benson, Current Drug Del, 2005: 2, 23-33). Other methods would be known to the skilled person. Once the TRIM16 has passed through the skin surface into the target cells, it may be translocated to the nucleus passively during cell division, or actively via its NLS.

In a preferred embodiment, the composition is formulated for percutaneous administration as a topical formulation, the topical formulation comprising TRIM16, or a tumor suppressor domain of TRIM16, one or more lower alcohols, such as ethanol or isopropanol; a penetration enhancing agent such as isopropyl myristate; a thickener; and water. Additionally, the present invention may optionally include salts, emollients,

stabilizers, antimicrobials, fragrances, and propellants. The topical formulation may be a gel, an ointment, a cream or a patch and is comprised of TRIM16, or a tumor suppressor domain of TRIM16; a penetration enhancing agent, such as isopropyl myristate; a thickening agent, such as a neutralized carbomer; a lower alcohol, such as ethanol or isopropanol; and water.

The penetration enhances in the topical formulation increase skin permeability by reversibly damaging or altering the physiochemical nature of the stratum corneum to reduce its diffusional resistance (Benson, Current Drug Del, 2005: 2, 23-33).

The viscosity of the topical formulation is preferably about 13,000 cps to about 33,000 cps. Accordingly, the viscosity of the topical formulation of the present invention may be any amount between about 13,000 cps and 33,000 cps, such as, e.g., 14,000, 15,000, 16,000, 17,000, 18,000, 19,000, 20,000, 21,000, 22,000, 23,000, 24,000, 25,000, 26,000, 27,000, 28,000, 29,000, 30,000, 31,000, 32,000, or 33,000 cps.

The composition of the invention may also be formulated in unit dosage form for ease of administration and uniformity of dosage. The specifications for the dosage unit forms of the present invention may be determined by a person skilled in the art depending on, for example (a) the characteristics of the adjuvant and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active ingredient for the particular treatment.

In the context of percutaneous administration, the concentration of TRIM16 administered is such that in the composition it results in a therapeutic level of drug delivered over the term that the drug is to be used. Such delivery is dependent on a number of variables including the time period for which the individual dosage unit is to be used, the flux rate of the drag from the composition, the surface area of application site, etc.

The topical formulation for percutaneous administration may be rubbed or placed onto an area of skin of the subject and allowed to dry. It is particularly preferred that the formulation is rubbed or placed on a skin cancer lesion, or an area of skin suspected of

being or developing in to a skin cancer lesion. Application of the topical formulation results in an increased TRIM16 level in the target cells and is effective to minimise an individual's risk of developing skin cancer, treating a skin cancer lesion, or minimising the risk of metastasis of skin cancer cells in the subject. The composition is thus useful for treating a number of conditions or diseases.

In one embodiment, the pharmaceutical composition of the present invention is administered once, twice, or three times a day, or as many times necessary to achieve the desired therapeutic effect. In another embodiment the composition of the present invention is administered once, twice, or three times a day on alternate days. In another embodiment the composition of the present invention is administered once, twice, or three times a day on a weekly, biweekly, or monthly basis.

The composition may be packaged in a variety of ways depending upon the method used for administering the drug. Generally, a kit or article for distribution includes a container having deposited therein the pharmaceutical formulation in an appropriate form. Suitable containers are well-known to those skilled in the art and include materials such as bottles (plastic and glass), sachets, ampoules, plastic bags, metal cylinders, and the like. The container may also include a tamper-proof assemblage to prevent indiscreet access to the contents of the package. In addition, the container has deposited thereon a label that describes the contents of the container. The label may also include appropriate warnings.

Methods using TRIM16

Work by the inventors has shown that loss of TRIM16 expression promotes keratinocyte migration, invasion and proliferation (Fig. 2), while over-expression of TRIM16 correlates with an increase in p53 expression, reduced cell proliferation and induced differentiation-associated markers. Accordingly the invention seeks to provide a method of minimising the risk of an individual developing skin cancer including the step of applying TRIM16 (tripartite motif-containing protein 16) or a composition comprising TRIM16 to the skin of an individual.

As detailed above, the TRIM16 to be administered to an individual may be provided as a full length polypeptide (such as the polypeptide of SEQ ID NO:2) or via an expression vector including a nucleotide sequence that encodes the full length polypeptide (such as SEQ IN NO:1). Alternatively, the TRIM16 of the composition to be administered may be provided as a fragment or variant of the full length polypeptide. A preferred fragment of TRIM16 is the tumor suppressor domain of TRIM16. Accordingly the invention seeks to provide a method of minimising the risk of an individual developing skin cancer including the step of applying a composition including a tumor suppressor domain of TRIM16 (tripartite motif-containing protein 16). The tumor suppressor domain preferably has a sequence according to SEQ ID NO: 3.

In an alternative embodiment, the tumor suppressor domain consists of an N terminal coiled-coil domain, a linker, a C terminal Ret finger protein domain and a nuclear localisation signal with the linker.

TRIM16, or any of the TRIM16 fragments and variants described throughout the specification, administered to minimise the risk of an individual developing skin cancer is preferably formulated as a topical composition.

Preferably the individual's risk of developing SCC or melanoma is minimised.

TRIM16 administered as part of the method of the invention may be co-administered with a retinoid or small molecule compound capable of binding to a retinoic acid receptor or activating retinoid acid receptor expression and transactivation. The retinoid may be administered prior to the TRIM16, simultaneously with TRIM16, or following TRIM16 administration. It is particularly advantageous if the TRIM16 and retinoid are formulated in the one composition for simultaneous administration, but it is envisaged that administration of TRIM16 prior to or following administration of the retinoid.

In a preferred embodiment, there is provided a method to prevent or at least reduce the risk of an individual developing skin cancer by administering TRIM16 together with one or more of UVA and UVB blocking agents described above.

Without being bound by any particular theory or mode of action, the level of expression of TRIM16 may be indicative of the presence or absence of a pre-cancer or cancer in a cell. The method of minimising the risk of an individual developing skin cancer may therefore further include the step of first determining the individual's susceptibility to skin cancer or pre-cancer by either:

a) determining the level of expression of TRIM16 in a cell; or

b) determining the level of expression of a compound, the expression of which is regulated by TRIM16.

The level of expression of TRIM16 may be determined by contacting a biological sample from the individual with a TRIM16 binding agent. In the specification the term "TRIM binding agent" refers to any agent that can bind to TRIM16 or to a nucleic acid encoding TRIM16.

An individual may have a cancer or pre-cancer cell if the expression level of TRIM16 is reduced or less than the expression level of that protein in normal skin tissue. In the context of the present invention the term "reduced expression" means that less TRIM16 can be found in the cell compared to a healthy control cell. Individuals with low expression of TRIM16 may be particularly susceptible to the development of skin cancer and pre-cancers. Similarly, individuals whose cells have been exposed to, for example, UV damage, but have not yet developed skin cancer or have only developed skin pre-cancer will be particularly benefited by having their risk determined.

The invention therefore seeks to provide a method of diagnosing skin cancer in an individual, comprising the steps of:

a) determining the level of expression of TRIM16 in a cell suspected of being a skin cancer cell from the individual; and

b) determining the level of expression of TRIM16 in a normal skin cell from the individual;

wherein lower levels of TRIM16 in the cell suspected of being a skin cancer cell compared to the normal skin cell are indicative of skin cancer.

When the levels of TRIM16 in the cell suspected of being cancerous, the cell may only be pre-cancerous, whereas the complete absence of TRIM16 is indicative of cancer. The diagnostic methods of the invention may be accompanied by other complementary tests for malignancy, including immunohistochemistry for cancer markers, and histopathological examination of cells for characteristics of aberrant cell morphology characteristic of cancer (e.g. nuclear size and shape, cell size and shape) to assist in distinguishing between cancerous and pre or early cancerous cells.

The biological sample may be a tissue biopsy taken directly from a patient. The tissue biopsy is typically taken from the region of a patient that shows the warning signs of a cancer or pre-cancer. Preferably the biopsy is a skin biopsy. The biopsy is taken under normal conditions and prepared suitably for analysis by standard techniques. Typically, the specimens are embedded in paraffin wax or are frozen unfixed tissue to be sectioned for analysis. The analysis of the tissue sections may be by well known techniques, including immunohistochemistry, immunofluorescence or in situ hybridisation.

Alternatively, the biological sample may be derived from the tissue biopsy taken from the patient. It is envisaged that RNA or protein may be extracted from the tissue sample for analysis. Such analysis is well known to the relevant skilled persons and would include without limitation Northern and Western blot analysis to determine the expression levels of TRIM16 in that sample. Protein and RNA extracts of the tissue sample are prepared by standard procedures.

It is also envisaged that the expression of TRIM16 may be determined without having to obtain a tissue biopsy from the patient. In this circumstance, techniques may be used to visualise the expression of TRIM16 in the relevant area of concern on the patient. For example, a cream containing an agent that can be visualised under UV light may be applied to the skin of the patient.

TRIM16 binding agents may bind directly or indirectly to TRIM16 and may include protein binding molecules such as antibodies, antiserum (both monoclonal and polyclonal), and antigen-binding fragments of antibodies. Alternatively, the expression of TRIM16 in a cell may be determined by means of a TRIM16 binding agent that binds to a TRIM16 nucleic acid. Down-regulation of expression of a protein is usually accompanied by the down-regulation of the corresponding transcript. Accordingly, the down-regulation of expression of TRIM16 may be analysed indirectly by measuring the amount of corresponding transcripts. Methods of detecting transcripts are within the skill set of the person in the art.

Alternatively, the step of determining the individuals susceptibility to skin cancer or pre-cancer may include the step of determining the level of expression of a compound that regulates the level of expression of TRIM16 in a cell, or determining the level of expression of a compound, the expression of which is regulated by TRIM16. A compound whose expression is regulated by TRIM16 may be for example βRARE, a retinoic acid response element that is essential for retinoid-induced expression of retinoic acid receptor β (RARβ).

A further embodiment of the invention provides a kit for carrying out the step of determining the individual's susceptibility to skin cancer or pre-cancer. Preferably, instructions for its use are also included in the kit.

With regards to existing skin cancer and pre-cancer treatments surgical excision is currently the best available treatment of the disease. In some instances, radiation therapy or cryotherapy may provide an alternative to surgery. Further surgery or chemotherapy may be required in the case of skin cancers that have metastasised. However, these methods are invasive and treatment of metastasised cancers is not always successful. Consequently, there is a need for alternative or complementary treatments.

Moreover, based on findings by the inventors that loss of TRIM16 expression promotes keratinocyte migration, invasion and proliferation, while over-expression of TRIM16 correlates with an increase in p53 expression, reduced cell proliferation and induced

differentiation-associated markers, administration of TRIM16 to skin cancer cells lacking detectable TRIM16 expression is thought to have a therapeutic effect by restoring TRIM16 activity. There is therefore provided a method of treating a skin cancer lesion including the step of applying a therapeutically effective amount of TRIM16 (tripartite motif-containing protein 16) to the skin cancer. Application of TRIM16 may provide the therapeutic effect by one or more of increasing p53 expression, reducing cell proliferation and inducing differentiation-associated markers.

Preferably the skin cancer lesion is a SCC lesion or a melanoma lesion.

As detailed above, the TRIM16 to be administered to an individual may be provided as a full length polypeptide (such as the polypeptide of SEQ ID NO:2) or an expression vector that includes a nucleotide sequence that encodes the full length polypeptide (such as SEQ IN NO:1). Alternatively, the TRIM16 to be administered may be provided as a fragment or variant of the full length polypeptide. A preferred fragment of TRIM16 is the tumor suppressor domain of TRIM16. Accordingly, in a preferred embodiment of the invention, the invention seeks to provide a method of treating a skin cancer lesion including the step of applying a tumor suppressor domain of TRIM16 (tripartite motif-containing protein 16). The tumor suppressor domain preferably has a sequence according to SEQ ID NO: 3.

In an alternative embodiment, the tumor suppressor domain consists of an N terminal coiled-coil domain, a linker, a C terminal Ret finger protein domain and a nuclear localisation signal with the linker.

Regardless of the form of TRIM16 administered, it must be able to translocate to the nucleus of the cell. This may be a passive process during cell division, or an active process via the nuclear localisation signal.

A method of treating a skin cancer lesion including the step of applying TRIM16 is also particularly advantageous if the skin cancer is retinoid resistant, or promotion of the retinoid anticancer signal in retinoid sensitive cancer cells is desired. Administration of retinoids is a common first line treatment for skin cancers. However, retinoid resistance is very common. The present invention is therefore particularly advantageous in that

skin cancer lesions in individuals which were previously unresponsive to retinoid treatment can be rendered retinoid sensitive by the presence of TRIM16 in the nucleus of the cell, such that retinoid treatment becomes effective.

Retinoid-induced expression of $RAR\beta_2$ is a necessary component of the retinoid anticancer signal in cancer cells. The inventors have previously shown that TRIM16 is a novel $RAR\beta_2$ transcriptional regulator in the retinoid signal and can upregulate the level of $RAR\beta_2$ (Raif et al. Cancer Letters 227: 82-90 (2009)). Therefore, without being bound by any theory, direct or indirect administration of TRIM16 to the nucleus of retinoid-resistant skin cancer cells can restore retinoid sensitivity to the cells. Alternatively, the direct or indirect administration of TRIM16 the nucleus of retinoid-sensitive skin cancer cells can promote the retinoid anti-cancer signal. There is therefore provided a method of conferring or enhancing retinoid sensitivity to a retinoid-treatment resistant skin cancer including the steps of:

(a) identifying an individual with a retinoid resistance skin cancer; and

(b) applying TRIM16 to cells of the skin cancer;

wherein TRIM16 confers or enhances retinoid sensitivity of the cells of the skin cancer.

In an alternative embodiment, the invention seeks to provide a method of restoring retinoid sensitivity of a skin cancer cell that is retinoid resistant, by administering a composition of the invention including TRIM16.

It is particularly advantageous if the TRIM16 administered in the above mentioned methods of the invention is co-administered with a retinoid or small molecule compound capable of binding to a retinoic acid receptor or activating retinoid acid receptor expression and transactivation.

TRIM16, or any of the TRIM16 fragments and variants described throughout the specification, administered to treat a skin cancer is preferably formulated as a topical composition for percutaneous administration of the TRIM16.

Further to the above, the presence or absence of TRIM16 in the nucleus of a cell, or the presence of non-functional TRIM16, may be an indicator as to the retinoid sensitivity of a cell. In turn, this can indicate whether retinoid treatment for patients with skin cancer is likely to have any therapeutic effect. As TRIM16 is an important co-regulator in the retinoid anti-cancer signal, its absence or non-functionality can indicate that the cell is resistant to retinoid therapy. Therefore, there is provided a method of identifying a retinoid resistant skin cancer cell comprising the identification of a non-functional form of TRIM16, or the absence or low levels of TRIM16 in the cell. This is advantageous as it means the individual is not subjected to timely and costly treatments that have no effect. Such a pre-screening step has not previously been available. Administration of TRIM16 to a skin cancer identified as being retinoid resistant can restore retinoid sensitivity of the skin cancer cell.

By 'non-functional form of TRIM16' it is meant that TRIM16 expression is either absent or markedly reduced compared to the levels on normal cells or retinoid sensitive cells, or that the TRIM16 on the cells is non-functional due to, for example, a mutation as the coding level. The mutation may be in the NLS, resulting in the TRIM16 not being able to translocate to the nucleus where it is required for sensitivity of the cells to retinoids, or the mutation may mean it is not be able to form the correct conformation required for binding and signalling. The mutation may also result in truncation of the coding sequence.

The non-functional form of TRIM16 may be detected by TRIM16 binding agents. In the specification the term "TRIM16 binding agent" refers to any agent that can bind to TRIM16 or to a nucleic acid encoding TRIM16.

The determination of the expression of TRIM16 may be performed using a TRIM16 binding agent that binds directly to TRIM16 (ie, a protein binding molecule). In this embodiment, the amount of TRIM16 in the sample is measured.

Such protein binding molecules may be an antibody or an antigen-binding fragment thereof. Antiserum may be polyclonal or monoclonal. In addition the antibodies may be single chain, chimeric, CDR-grafted or humanized. Polyclonal and monoclonal antiserum may be raised against TRIM16 using conventional techniques.

The protein binding agent may be conjugated to a detectable label such as a radioisotope, an enzyme (such as peroxidase, alkaline peroxidase or alkaline phosphatase), an ultrasonic probe, a nuclear resonance (NMR) probe and the like.

Alternatively, the expression of TRIM16 in a cell is determined by means of a TRIM16 binding agent that binds to a TRIM16 nucleic acid. Down-regulation of expression of a protein is usually accompanied by the down-regulation of the corresponding transcript. Accordingly, the down-regulation of expression of TRIM16 may be analysed indirectly by measuring the amount of corresponding transcripts. This can be done by the use of nucleic acid binding molecules binding to the transcript of TRIM16 (ie mRNA). Such nucleic acid molecules are for instance, oligonucleotides such as DNA-heterodimers or RNA-heterodimers consisting of at least 7 monomeric units that can bind to the nucleic acid sequence encoding TRIM16 as shown in SEQ ID NO: 1 in Figure 1. The determination of the expression of TRIM16 in a cell can be performed using well known methods, e.g. Polymerase Chain Reaction (PCR) such as RT-PCR or a hybridisation technique such as northern blot, employing an oligonucleotide probe that hybridises to the mRNA transcripts. Preferably, the probe consists of at least 7 contiguous nucleotides of the TRIM16 nucleic acid sequence shown in SEQ ID NO: 1 of Figure 1.

Alternatively, detection of antibodies directed against TRIM16 or TRIM16 gene mutations. Antibodies may be detected by assays such as ELISAs or RIAs.

The role of TRIM16 in cell proliferation and cell migration means that TRIM16 may play an important role as a regulator of metastasis. Accordingly, in a further aspect of the invention, there is provided a method of minimising metastasis of skin cancer cells including the step of applying TRIM16. In the presence of TRIM16 skin cancer cells exhibit reduced cell motility, and are not as invasive as skin cancer cells lacking TRIM16.

Moreover, the inventors have discovered a relationship between the aggressiveness of a skin cancer and the level of TRIM16 expression. An aggressive skin cancer has a higher rate of cell proliferation as well as a greater propensity metastasis. Detecting the level of TRIM16 may therefore provide important prognostic information, such that skin

cancer cells lacking TRIM16 are likely to be more aggressive. The method of minimising metastasis of skin cancer cells may therefore include a first step of determining the level of expression of TRIM16 in a skin cancer cell from the individual. The recognition of this relationship by the inventors means that for the first time, individuals with skin cancer can have their treatment tailored accordingly, based on a more informed prognosis.

There is also provided a use of TRIM16 as described above in the manufacture of a medicament for minimising the risk of an individual developing skin cancer, or for treating an individual having a skin cancer, for minimising the risk of metastasis of skin cancer cells, a method of conferring or enhancing retinoid sensitivity to a retinoid-treatment resistant skin cancer, accelerating healing of burns, decreasing granulation of skin tissue, or reducing acne.

In alternative embodiments, there is provided TRIM16 as described above in compositions for use in minimising the risk of an individual developing skin cancer, or for treating an individual having a skin cancer, for minimising the risk of metastasis of skin cancer cells, a method of conferring or enhancing retinoid sensitivity to a retinoid-treatment resistant skin cancer, accelerating healing of burns, decreasing granulation of skin tissue, or reducing acne.

The role of TRIM16 in cell proliferation and migration also indicates that the compositions of the invention may be useful for accelerating healing of burns, decreasing granulation of skin tissue, reducing acne and other cosmetic applications where decreased cell proliferation and/or migration may be advantageous. The invention therefore provides methods for accelerating healing of burns, decreasing granulation of skin tissue, or reducing acne, comprising the step of administering TRIM16.

Materials and Methods

Immunohistochemistry of patient tissue samples: Archival samples of tumour and non tumour full-thickness skin tissue were collected from surgical excisions performed at Royal Prince Alfred Hospital, Sydney, Australia. Samples were anonymized before processing

and approval for the study was obtained from the Sydney South West Area Health Service ethics committee. Slides were dried in a 60°C oven for 1 hour. The tissue slides were then immersed in xylene to deparaffinise the section. Slides were then re-hydrated with 100%, 90%, 70% ethanol and MilliQ H2O sequentially. Antigen retrieval was done by immersing the slides in 0.01M tri-sodium citrate buffer with 0.05% Tween-20, pH6, at 104°C for 15 minutes. The endogenous peroxidases were inactivated by immersing slides in 3% hydrogen peroxide. 10% goat serum was used to block non-specific binding of immunoglobulin. The slides were incubated with a custom made rabbit anti-TRIM16 (the sequence of the peptides: Ac-CTNTTPWEHPYPDLPS-amide) (Invitrogen, Victoria, Australia) or Rabbit IgG (I-1000, Vector Laboratories, Burlingame, CA) at 1.91µg/µl at 4°C overnight. Slides were then incubated with secondary goat anti-rabbit immunoglobulin/biotinylated (E0432, Dako, Glostrup, Denmark) at 1 in 500 dilutions for 1 hour at room temperature. The biotinylated antibody was then labelled with streptavidin-HRP (Dako, K1016) for 45 minutes at room temperature. The sections were developed with 3, 3'-diaminobenzidine tetrahydrochloride (Dako, K3468) for 1 minute at room temperature and counterstained with haematoxylin.

Analysis of Immunohistochemistry skin tissue samples: 8 tissues were selected based on their TRIM16 staining intensity to construct a reference range for intensity. An arbitrary score was assigned to each level of staining intensity, 0 for negative staining, 1 for weak staining, 2 for weak-moderate staining, 3 for moderate-strong staining, and 4 for strong staining. Each patient slide was then scored by a blind researcher base on the intensity reference range. 4 fields per patient sample were observed and the average score were recorded. Statistical analysis was done using one-way analysis of variance.

Cell culture: Primary human keratinocytes (PHK) were purchased from Gibco and cultured in Defined Keratinocyte-SFM. The PHK were a pool from 4 neonatal foreskins. Tumorigenic keratinocytes (SCC-15), derived from squamous-cell carcinoma, were purchased from ATCC and cultured in DMEM/F12 (1:1) medium containing 10% fetal calf serum and 0.4 µg/ml hydrocortisone. MET-1 and MET-4, spontaneously derived SCC from a primary cutaneous tumour, were cultured in DMEM/F12 (3:1) medium containing 10% fetal calf serum and a selection of growth hormones as described (Proby, 2000).

Authentication of cell lines were done through CellBank Australia in 2009 or provided by the supplier.

PCR and sequencing of genomic TRIM16 DNA: Genomic DNA was isolated from cell lines using DNA Extraction Kit (Promega, Madison, WI) while extractions of DNA from patients' samples were performed with TRIzol (Invitrogen, Victoria, Australia). The DNA was amplified using High Fidelity taq polymerase (Roche, NSW, Australia) and primers designed inside the introns directly beside the exons (Table 1). The parameters of the PCR consisted of a denaturing step of 95°C for 5 minutes followed by 35 cycles of 58°C annealing, 72°C extension and 95°C denaturation with each step running for 45 seconds (on the GeneAmp PCR system 9700, Applied Biosystems, Mulgrave, Australia). ExoSAP-IT (UBS, Ohio, USA) was used to clean up the PCR product. Sequencing of the TRIM16 DNA was performed by Australian Genomic Research Facility (Westmead, NSW, Australia) using capillary separation on AB3730xl sequencer. Sequences were analysed on Mutational Surveyor 3.97V-Demo (Soft Genetics, USA).

Transient Transfection with Plasmid DNA: Cells (5×10^5) were transfected with 3 µg of plasmid DNA per 6-well plate for 24 hours before harvesting for transient transfection experiments. SCC-15 cells were transfected using Lipofectamine 2000 (Invitrogen, Victoria, Australia) according to manufacture protocol. MET-1 and MET-4 cells were transfected with Lipofectamine LTX (Invitrogen) according to manufacture protocol.

Scratch wound assays: For scratch wound assays, MET-1 cells were transfected with either 6 µg of TRIM16 full-length or TRIM16 deletion mutants expression vector or empty vector with Lipofectamine LTX, as previously described (Marshall, 2010). For the double transfection assays, 4 µg of TRIM16 expression vector and 4 µg of vimentin expression vector were transfected into the cells. After 24 hours from transfection, cells were grown to 90–100% confluence and re-seeded in culture inserts (Ibidi, Martinsried, Germany) at a density of 4.2×10^4 per chamber. After 24 hours, the insert was lifted and the distance between the wound was measured with Image J software (National Institutes of Health, Bethesda, MD, USA) at various time points.

Immunoprecipitation assays and western blots: Lysates were immunoprecipitated with anti-Turbo GFP (Evrogen, Moscow, Russia). Anti-vimentin antibody (Santa Cruz, CA), anti-E2F1 antibody (Cell Signaling, Danvers, MA), anti-cyclin E2 anti-Flag tag antibody (Cell Signaling) were used in immunoblots. Rabbit polyclonal actin antibody (Sigma, St Louis, MO, USA), and Histone H3 antibody and anti-GAPDH antibody were used to normalize for differences in whole cell lysates, nuclear or cytoplasmic protein loading, respectively.

Examples

1. TRIM16 is a tumour suppressor and its expression is reduced in human skin pre-cancer and cancer

It was investigated whether TRIM16 expression was lost during skin tumorigenesis *in vivo*. TRIM16 expression by immunohistochemical staining of skin tissue samples collected from 128 patients was assessed. Archival paraffin-embedded skin specimens were classified into the following six categories: normal skin (NS): 16 patients; recent scars: <30days old, 28 patients; old scars: (>30days old, 27 patients; Actinic keratosis: 27 patients; squamous cell carcinoma (16 patients) and basal cell carcinoma (14 patients) and scored according to grading of staining for TRIM16 expression using a semi-quantitative method (Figure 3).

TRIM16 was most strongly expressed in the cytoplasm and nucleus of the normal stratum in the stratum spinosum and stratum granulosom of normal skin tissue, recent scars and old scars. Conversely, TRIM16 expression was reduced in Actinic keratosis ($p < 0.05$) and further diminished in SCC ($P < 0.01$)(3-fold) and BCC ($p < 0.01$) (4-fold) tissue. Analysis of staining intensity showed that TRIM16 expression is markedly reduced during the progression from normal skin to SCC and BCC (Figure 3).

2. TRIM16 cellular localization is indicative of retinoid response in cancer cells

When increasing concentrations of retinoic acid (in this case, in the form if 13-cis-RA) is applied to non-cancerous human keratinocytes, cell proliferation is reduced (Fig. 5A).

However, it was shown that the response was dependent on TRIM16 translocation from the cytoplasm to the nucleus.

TRIM16 was found to be expressed in most normal and cancer cells, but mainly localized in the cytoplasmic component of cells. TRIM16 protein was found to translocate to the nucleus after retinoid treatment from the cytoplasm in normal (figure 5B) and retinoid-sensitive cancer cells. In retinoid-resistant cancer cells however TRIM16 does not translocate to the nucleus (Figure 5D). TRIM16 cellular localization therefore may be considered an important indicator of retinoid responsiveness in skin cancer cells, and therefore an indicator of treatment success with retinoids.

It can further be seen in Figure 5C however that administration of TRIM 16 does still inhibit cell growth regardless of the presence of retinoic acid, indicating a role for TRIM16 in a retinoic-acid independent pathway.

3. Enforced over-expression causes TRIM16 to translocate to the nucleus, reduces cell growth and decreases nuclear E2F1 and pRb phosphorylation.

To determine whether TRIM16 plays a role in skin cancer cell growth, human TRIM16 cDNA was transiently transfected into three different SCC cell lines. Transient over-expression of TRIM16 led to a significant decrease in viable SCC-15, MET-1 and MET-4 cells when compared with empty vector control ($p < 0.0005$), as measured by Alamar blue assay at 48 hours after transfection (Figure 6A). In the cell proliferation study, BrdU incorporation in SCC-15, MET-1 and MET-4 cells was decreased in cells transfected with TRIM16 plasmid DNA, compared with the empty vector control ($p < 0.0005$) (Figure 6B). Most importantly, the amount of TRIM16 protein in the nucleus was significantly increased in cells after 24 and 48 hours TRIM16 transfection, as measured by immunoblot (Figure 6C).

To determine whether the effects of TRIM16 on cell growth are mediated by effects on the cell cycle regulatory proteins E2F1 and pRB in retinoid-resistant skin cancer cells, immunoblot analysis of transfected cell lines referred to above showed that overexpression of TRIM16 decreased nuclear E2F1 protein expression and also

markedly reduces Phospho-pRb (ser807/811), compared with empty vector (Figure 6C and 6D).

MET-1 cells were transiently transfected with TRIM16-GFP and E2F1-Flag plasmid DNA to determine whether TRIM16 directly interacts with E2F1 to affect SCC cell proliferation. After 24 hours, the whole cell lysates were subjected to co-immunoprecipitation with a GFP-tagged antibody which recognized the transfected TRIM16, and probed with anti-Flag antibody for E2F1. Co-IP confirmed that transfected TRIM16 indeed formed a complex with E2F1 in SCC cells (Figure 6E).

These results suggest that TRIM16 effects on SCC cell growth require nuclear translocation of TRIM16 protein and interaction of TRIM16 with E2F1, with a consequent reduction in nuclear E2F1 and pRb phosphorylation levels. These cellular interactions occur naturally on administration of TRIM16 to the nucleus.

4. The TRIM16 Coiled-coil Domain is necessary for retinoic acid receptor beta transactivation

A series of TRIM16 deletion-mutant expression vectors M1-M4 in pcDNA3.1 were generated, (Figure 7C) constitutively expressed with 6xHis and Myc protein epitopes. The wild type TRIM16 expression vector contains nucleotides 1 to 564 of TRIM16. Mutant 1 (M1) lacks B1 and B2, and contains nucleotides 161 to 564. Mutant 2 (M2) lacks B1 and B2 as well as the coiled-coil domain and contains nucleotides 290 to 564. Mutant 3 (M3) lacks the RFP domain, and contains nucleotides 1 to 372, and mutant 4 (M4) lacks the coiled-coil domain and RFP domain, and contains nucleotides 1 to 165.

Using these mutants, the coiled-coil domain of TRIM16 was shown to be necessary for transactivation of the βRARE in retinoid-sensitive cancer cells. As can be seen, construct M2 and M4 which lack the coiled-coil domain, show lower levels of transactivation in the presence of retinoic acid. This result suggests that the coiled-coil domain is required for TRIM16 retinoid-dependent tumour suppressor function in cancer cells.

5. Function of TRIM16 "linker" region:

The 82 amino acid "linker" region between the coiled-coil domain and RFP domain is necessary for the effect of TRIM16-induced growth inhibition in retinoid-sensitive cells (Figure 8). Figure 8 shows the results of BrdU incorporation, which is indicative of actively proliferating cells. Analysis of the human TRIM16 sequence further identified a candidate nuclear localization signal (NLS)-YKKKL which appears to be required for TRIM16's nuclear localisation, and therefore, for TRIM16 retinoid-independent tumour suppressor function. The results for M1-M3 and TRIM16 are statistically significant.

6. TRIM16 effects on cell motility

The effect of TRIM16 over expression cell motility in skin cells was investigated. TRIM16 plasmid DNA and empty vector control was transiently transfected into MET-1 and MET-4 SCC cells. The scratch-wound assays on confluent monolayers were utilised to investigate cell motility (McInroy et al., 2007). The SCC cells which over-expressed TRIM16 remained largely open at multiple time points, while empty vector control cells migrated particularly rapidly in this assay, filling the empty wound space. This data indicates that loss of TRIM16 function or expression may increase invasiveness of skin cancer cells (Figure 9A).

It was further determined that TRIM16 reduces cell motility by binding to and reducing vimentin protein expression in SCC cells. Vimentin is the predominant intermediate filament protein in cells, and, is implicated in metastasis and cancer cell. Co-immunoprecipitation (co-IP) of vimentin with GFP-TRIM16 was performed on whole cell lysates from transfected MET-1 cells. Co-IP confirmed that TRIM16 and vimentin formed a complex (Figure 9B). TRIM16 markedly reduced vimentin protein expression, as shown by TRIM16 overexpression experiment in MET-1 SCC cells (Figure 9C). This finding suggests that TRIM16 overexpression may reduce cell motility through its interaction with vimentin in SCC cells.

7. Overexpression of TRIM16 reduces cell motility through its RFP domain.

Having shown that TRIM16 affects cell motility in SCC cells, the TRIM16 deletion-mutant expression vectors M1-M4 in pcDNA3.1 were transiently transfected into MET1 cells for 24 hours to determine which domain of TRIM16 is required for reducing cell motility. TRIM16 full-length, M1 and M2 facilitated repair of cell injury slower than empty vector, but not M3 and M4, which suggests the effect of TRIM16 on cell motility is mediated by the RFP domain of TRIM16 (Figure 10).

8. Homologs of TRIM16

It is important that any TRIM16 homolog has a high degree of homology with the TSD of TRIM16. Alignment of human TRIM16 (SEQ ID NO:2) (Accession AF096870.1; Swiss Prot 095361.3), human TRIM16-002 (SEQ ID NO:4) (Accession Ø526026) and human TRIM16-202 (SEQ ID NO:5) (Accession Q59EB2) in Figure 4 shows:

i) Coiled-coil domain of TSD (first boxed sequence)

TRIM16-002 homology to TRIM16: 94.4%

TRIM16-202 homology to TRIM16: 100%

ii) Linker region of TSD (second boxed sequence)

TRIM16-002 homology to TRIM16: 100%

TRIM16-202 homology to TRIM16: 100%

9. TRIM16 in melanoma cell lines.

The activity of TRIM16 was analysed in the melanoma cell lines G361 and A375, compared to the normal human epidermal melanocyte cell line (NHEM). TRIM16 mRNA expression was found to be decreased in G361 compared to the normal melanocyte cell line (Figure 11A). In good accordance with the mRNA expression results, TRIM16 protein expression levels were also reduced (Figure 11B and 11C).

Further, a TRIM16 expressing plasmid was transiently transfected into the melanoma cell lines G361 and A375 to over-express (as illustrated by the western blot results of Figure 12C). Over-expression of TRIM16 was subsequently shown to induce growth arrest (Figure 12A) and cell death (Figure 12B). The effect on cell numbers is also demonstrated in Figure 12D, where it can be seen that G361 cells over expressing TRIM16 are significantly reduced in number at 48 hours post introduction of the TRIM16 expressing plasmid.

10. TRIM16 effects on cell motility

Further to the example investigating the effect of TRIM16 on the motility (migration) of SCCs, TRIM16 plasmid DNA and empty vector control were transiently transfected into G361 cells using the same scratch-wound assays. The cells which over-expressed TRIM16 remained largely open at multiple time points, while empty vector control cells migrated particularly rapidly in this assay, filling the empty wound space (Figure 13B and quantitated at 8 hours in Figure 13A). This data indicates that loss of TRIM16 function or expression may increase invasiveness of melanoma skin cancer cells as well as SCC skin cancer cells.

11. TRIM16 in patient melanoma samples

Analysis of TRIM16 expression by immunohistochemical staining of melanoma samples collected from 18 patients was assessed. The paraffin-embedded tissue sections were classified into five categories: Dysplastic compound naevus (DCN), in situ melanoma (IM), dermal invasive melanoma (DIM), melanoma lymph node metastasis (MLM), and melanoma distant metastasis (MDM) (Figure 14). TRIM16 expression was significantly reduced between DCN and LNM ($p<0.05$) and MDM ($p<0.01$). Stages of melanoma were scored according to a grade of staining for TRIM16 expression using a semi-quantitative method, and the results tabulated in Figure 15. TRIM16 staining decreases as the stage of melanoma progresses.

The claims defining the invention are as follows:

1. A pharmaceutical composition for minimising the risk of an individual developing skin cancer, or for treating an individual having a skin cancer or skin pre-cancer, the composition including TRIM16 (tripartite motif-containing protein 16) and a pharmaceutically acceptable diluent, carrier or excipient.

2. The composition according to claim 1 wherein TRIM16 is provided as a tumor suppressor domain of TRIM16.

3. The composition according to claim 2, wherein the tumor suppressor domain comprises an N terminal coiled-coil domain, a linker, a C terminal Ret finger protein domain and a nuclear localisation signal within the linker.

4. The composition according to claim 2, wherein the tumor suppressor domain consists of the sequence of SEQ ID NO: 3.

5. The composition according to any one of claims 1 to 4, further including a retinoid capable of binding to a retinoic acid receptor.

6. The composition according to claim 5 wherein the retinoid is selected from one or more of retinol, retinal, retinoic acid, isotretinoin, alitretinoin, etretinate, acitretin, tazorotene, bexarotene and adapalene.

7. The composition according to any one of claims 1 to 6 wherein the skin cancer is melanoma or squamous cell carcinoma.

8. The composition according to any one of claims 1 to 7, wherein the composition is formulated with one or more lower alcohols, a penetration enhancing agent, a thickener and water for percutaneous administration to skin.

9. A method of minimising the risk of an individual developing skin cancer comprising the step of applying an effective amount of TRIM16 to skin cells on an area of the

individual's skin, wherein the amount of TRIM16 applied is effective to minimise the risk of skin cancer developing in the skin cells.

10. The method according to claim 9, wherein the TRIM16 is formulated with one or more lower alcohols, a penetration enhancing agent, a thickener and water for percutaneous administration to skin.

11. The method according to claim 9 or 10, wherein a retinoid capable of binding to a retinoic acid receptor is applied with the TRIM16.

12. The method according to any one of claims 9 to 11 further including the step of determining the level of expression of TRIM16 in a cell, thereby determining the susceptibility of the individual to developing skin cancer.

13. The method according to any one of claims 9 to 11 further including the step of determining the level of expression of a compound in a cell, the expression of which is regulated by TRIM16, thereby determining the susceptibility of the individual to developing skin cancer.

14. The method according to any one of claims 9 to 13 wherein the skin cancer is melanoma or squamous cell carcinoma.

15. A method for treating a skin cancer on an individual including the step of applying a therapeutically effective amount of TRIM16 to cells of the skin cancer.

16. The method according to claim 15 wherein the skin cancer is resistant to retinoids prior to the application of a therapeutically effective amount of TRIM16.

17. The method according to claim 15 or 16, wherein a retinoid capable of binding to a retinoic acid receptor is co-administered with TRIM16.

18. The method according to any one of claims 15 to 18 wherein TRIM16 is formulated with one or more lower alcohols, a penetration enhancing agent, a thickener and water for percutaneous administration to the skin cancer.

19. A method for determining the suitability of retinoid treatment of skin cancer in an individual having skin cancer, the method including the step of determining the level of expression of TRIM16 in cells from the skin cancer, wherein an absence of TRIM16, or reduced levels of TRIM16 compared to a non-cancerous skin cell from the individual, is indicative of the skin cancer being resistant to retinoid treatment of the skin cancer.

20. A method of conferring or enhancing retinoid sensitivity to a retinoid-treatment resistant skin cancer including the steps of:
(a) identifying an individual with a retinoid resistance skin cancer; and
(b) applying TRIM16 to cells of the skin cancer;
wherein TRIM16 confers or enhances retinoid sensitivity of the cells of the skin cancer.

SEQ ID NO:1 Homo sapiens TRIM16 mRNA, complete cds

LOCUS AF096870 2568 bp mRNA linear PRI 24-NOV-1998

```
   1 gctgtcctga gcctgagtac tctagctgcc ttgtcgccat cgcatctggc tgccatccag
  61 cgccagcaca cagtaatgag tggccgagct tcctctggga gggaggaaac agttaaaatc
 121 ttgcagcagc tgcaatcatc taggcgtggt tctcttgtct gacttgggct gcacagatcc
 181 tgggccaagg gacagaagaa agacagccta ggagcagagc ctcccagatg gctgagttgg
 241 atctaatggc tccagggcca ctgcccaggg ccactgctca gcccccagcc cctctcagcc
 301 cagactctgg gtcacccagc ccagattctg ggtcagccag cccagtggaa gaagaggacg
 361 tgggctcctc ggagaagctt ggcagggaga cggaggaaca ggacagcgac tctgcagagc
 421 agggggatcc tgctggtgag gggaaagagg tcctgtgtga cttctgcctt gatgacacca
 481 gaagagtgaa ggcagtgaag tcctgtctaa cctgcatggt gaattactgt gaagagcact
 541 tgcagccgca tcaggtgaac atcaaactgc aaagccacct gctgaccgag ccagtgaagg
 601 accacaactg gcgatactgc cctgcccacc acagcccact gtctgctttc tgctgccctg
 661 atcagcagtg catctgccag gactgttgcc aggagcacag tggccacacc atagtctccc
 721 tggatgcagc ccgcagggac aaggaggctg aactccagtg cacccagtta gacttggagc
 781 ggaaactcaa gttgaatgaa aatgccatct ccaggctcca ggctaaccaa aagtctgttc
 841 tggtgtcggt gtcagaggtc aaagcggtgg ctgaaatgca gtttggggaa ctccttgctg
 901 ctgtgaggaa ggcccaggcc aatgtgatgc ccttcttaga ggagaaggag caagctgcgc
 961 tgaaccaggc caacggtatc aaggcccacc tggagtacag gagtgccgag atggagaaga
1021 gcaagcagga gctggagagg atggcggcca tcagcaacac tgtccagttc ttggaggagt
1081 actgcaagtt taagaacact gaagacatca ccttccctag tgtttacgta gggctgaagg
1141 ataaactctc gggcatccgc aaagttatca cggaatccac tgtacactta atccagttgc
1201 tggagaacta taagaaaaag ctccaggagt tttccaagga agaggagtat gacatcagaa
1261 ctcaagtgtc tgccgttgtt cagcgcaaat attggacttc caaacctgag cccagcacca
1321 gggaacagtt cctccaatat gcgtatgaca tcacgtttga cccggacaca gcacacaagt
1381 atctccggct gcaggaggag aaccgcaagg tcaccaacac cacgccctgg gagcatccct
1441 acccggacct ccccagcagg ttcctgcact ggcggcaggt gctgtcccag cagagtctgt
1501 acctgcacag gtactatttt gaggtggaga tcttcggggc aggcacctat gttggcctga
1561 cctgcaaagg catcgaccgg aaaggggagg agcgcaacag ttgcatttcc ggaaacaact
1621 tctcctggag cctccaatgg aacgggaagg agttcacggc ctggtacagt gacatggaga
1681 ccccactcaa agctggccct ttccggaggc tcggggtcta tatcgacttc ccgggaggga
1741 tcctttcctt ctatggcgta gagtatgata ccatgactct ggttcacaat tttgcctgca
1801 aattttcaga accagtctat gctgccttct ggctttccaa gaaggaaagc gccatccgga
1861 ttgtagatct gggagaggaa cccgagaagc cagcaccgtc cttggtggtg actgctccct
1921 agactccagg agccatatcc cagacctttg ccagctacag tgatgggatt tgcatttttag
1981 ggtgatttgt gggcagaaat aactgctgat ggtagctggc ttttgaaatc ctatggggtc
2041 tctgaatgaa aacattctcc agctgctctc ttttgctcca tatggtgctg ttctctatgt
2101 gtttgcagta attctttttt ttttttgaga cggagtctcg cactgttgcc caggctggag
2161 agcagtggcg cgatctcggc tcactgcaag ctccgcctcc cgagttcaag caattctcct
2221 gcctcagcct cccgagtagc tgggattaca ggtgcctgcc accacaccca gctaatgttt
2281 tgtattttta gtagagatgg ggtttcacca tgttggccag gcagatctca aactcctgac
2341 ctcgtgatgc acccacctcg gcctcccaaa gtgctgggat tacatgcgtg agccactgcg
2401 ccctgcctgt ttgtagtaat ttttaggcac caaatctccc tcatcttcta gtgccattct
2461 cctctctgtt caggtaaatg tcacactgtg cccagaatgg atgaccaggg accttaaaga
2521 gtggctgaaa agattgcaga gttatcataa taaattgcta acttgcgt
```

Figure 1A

SEQ ID NO:2

MAELDLMAPGPLPRATAQPPAPLSPDSGSPSPDSGSASPVEEEDVGSSEKLGRETEE
QDSDSAEQGDPAGEGKEVLCDFCLDDTRRVKAVKSCLTCMVNYCEEHLQPHQVNIKL
QSHLLTEPVKDHNWRYCPAHHSPLSAFCCPDQQCICQDCCQEHSGHTIVSLDAARRD
KEAELQCTQLDLERKLKLNENAISRLQANQKSVLVSVSEVKAVAEMQFGELLAAVRKA
QANVMLFLEEKEQAALSQANGIKAHLEYRSAEMEKSKQELERMAAISNTVQFLEEYCK
FKNTEDITFPSVYVGLKDKLSGIRKVITESTVHLIQLLENYKKKLQEFSKEEEYDIRTQVS
AVVQRKYWTSKPEPSTREQFLQYAYDITFDPDTAHKYLRLQEENRKVTNTTPWEHPY
PDLPSRFLHWRQVLSQQSLYLHRYYFEVEIFGAGTYVGLTCKGIDRKGEERNSCISGN
NFSWSLQWNGKEFTAWYSDMETPLKAGPFRRLGVYIDFPGGILSFYGVEYDTMTLVH
KFACKFSEPVYAAFWLSKKENAIRIVDLGEEPEKPAPSLVGTAP

Figure 1B

SEQ ID NO:3 SEQUENCE OF TUMOR SUPPRESSOR DOMAIN OF TRIM16

DAARRDKEAELQCTQLDLERKLKLNENAISRLQANQKSVLVSVSEVKAVAEMQFGE LLAAVRKAQANVMLFLEEKEQAALSQANGIKAHLEYRSAEMEKSKQELERMAAISN TVQFLEEYCKFKNTEDITFPSVYVGLKDKLSGIRKVITESTVHLIQLLEN**YKKKL**QEFSK EEEYDIRTQVSAVVQRKYWTSKPEPSTREQFLQYA

NLS

Figure 1C

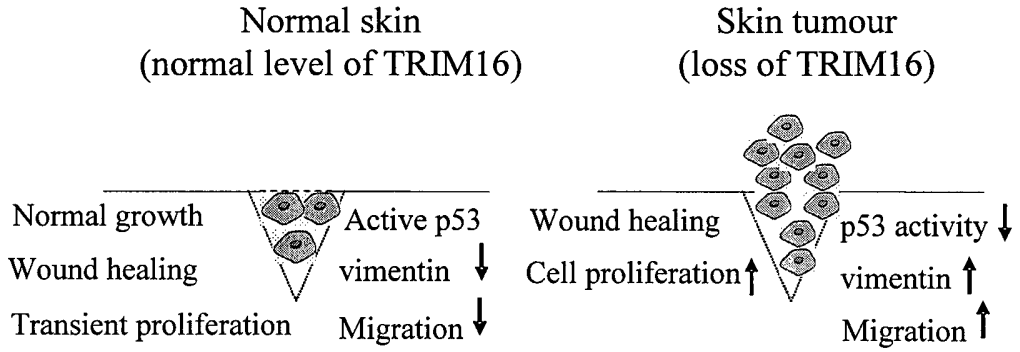

Normal skin
(normal level of TRIM16)
Skin tumour
(loss of TRIM16)
Normal growth
Wound healing
Transient proliferation
Active p53
vimentin
Migration
Wound healing
Cell proliferation
p53 activity
vimentin
Migration

Figure 2

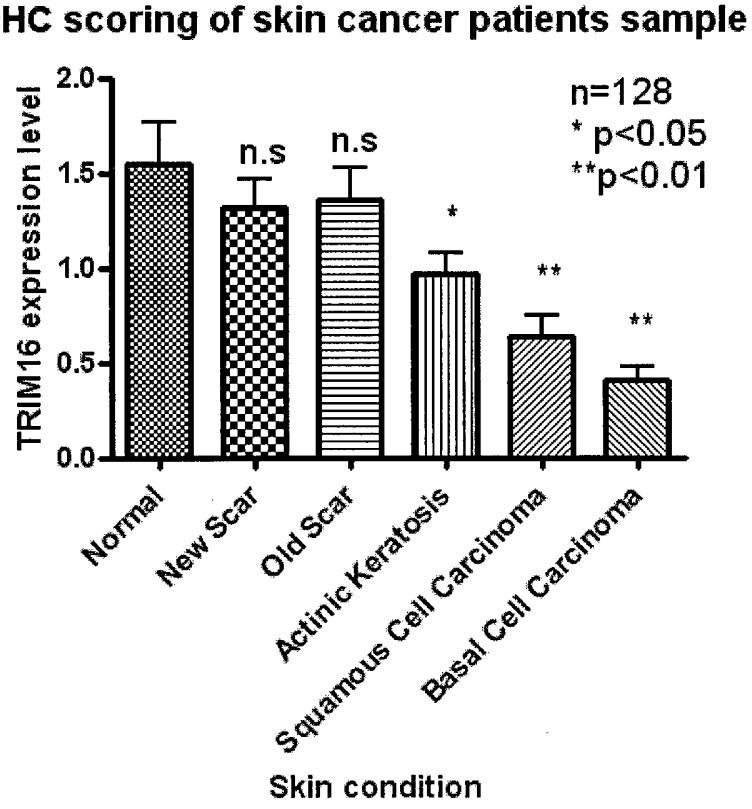

IHC scoring of skin cancer patients sample
TRIM16 expression level
2.0
1.5
1.0
0.5
0.0
n=128
* p<0.05
**p<0.01
n.s
n.s
*
**
**
Normal
New Scar
Old Scar
Actinic Keratosis
Squamous Cell Carcinoma
Basal Cell Carcinoma
Skin condition

Figure 3

O95361=SEQ ID NO:2; B3KP96=SEQ ID NO:4; Q59EB2=SEQ ID NO:5

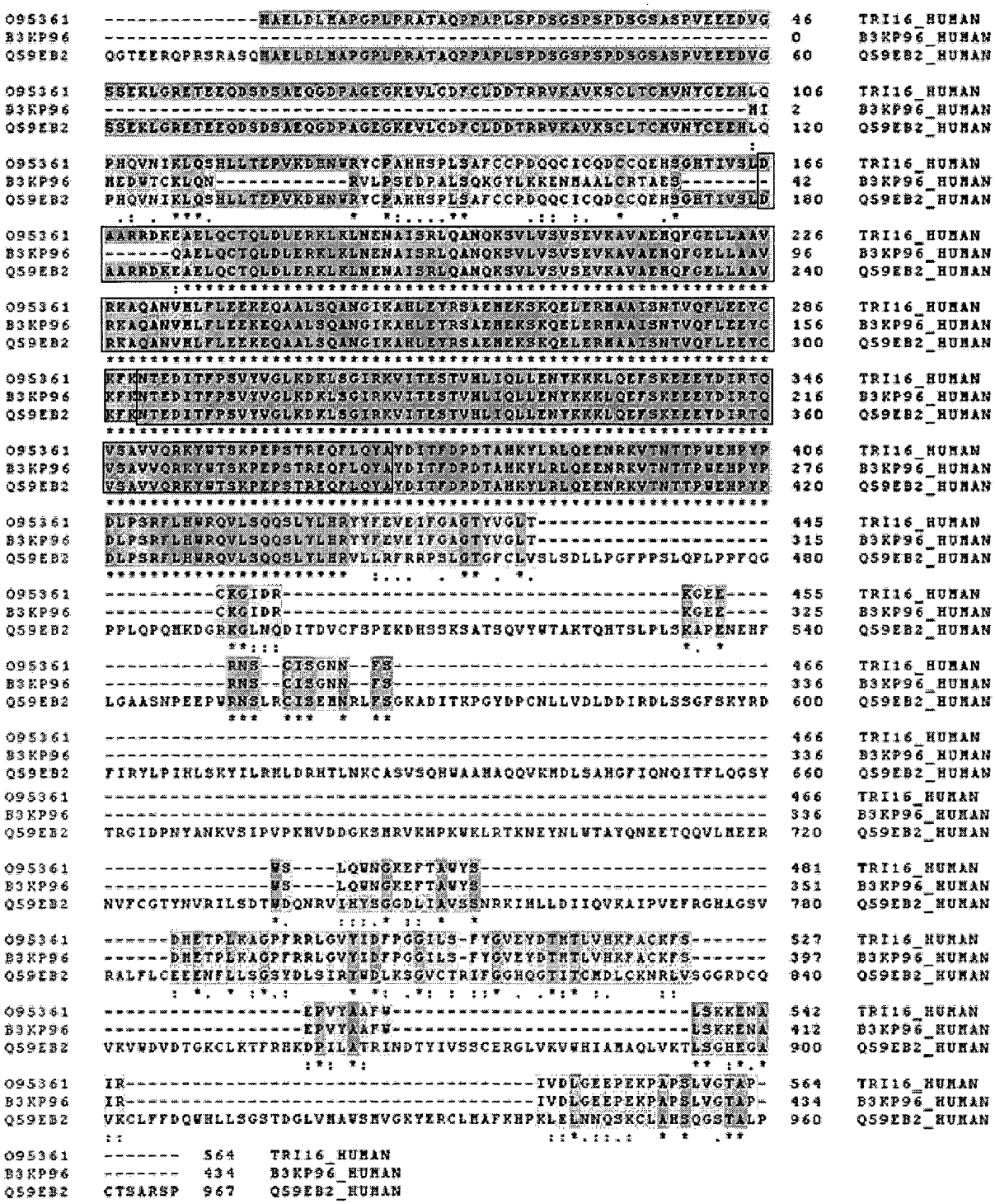
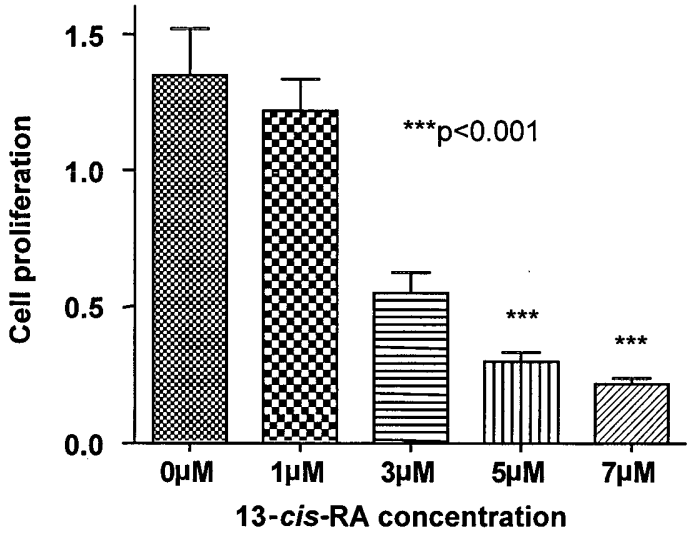


Figure 4

A


1.5
1.0
0.5
0.0
Cell proliferation
***p<0.001
***
***
0μM
1μM
3μM
5μM
7μM
13-cis-RA concentration

B

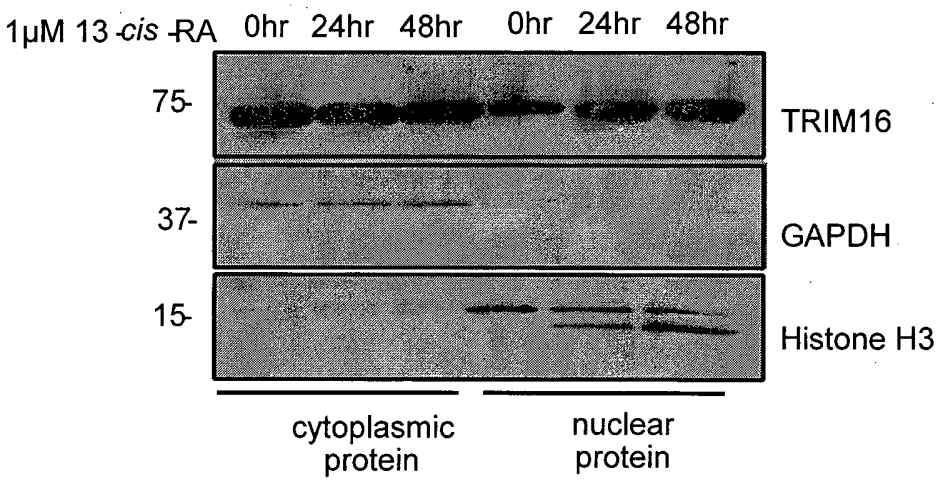
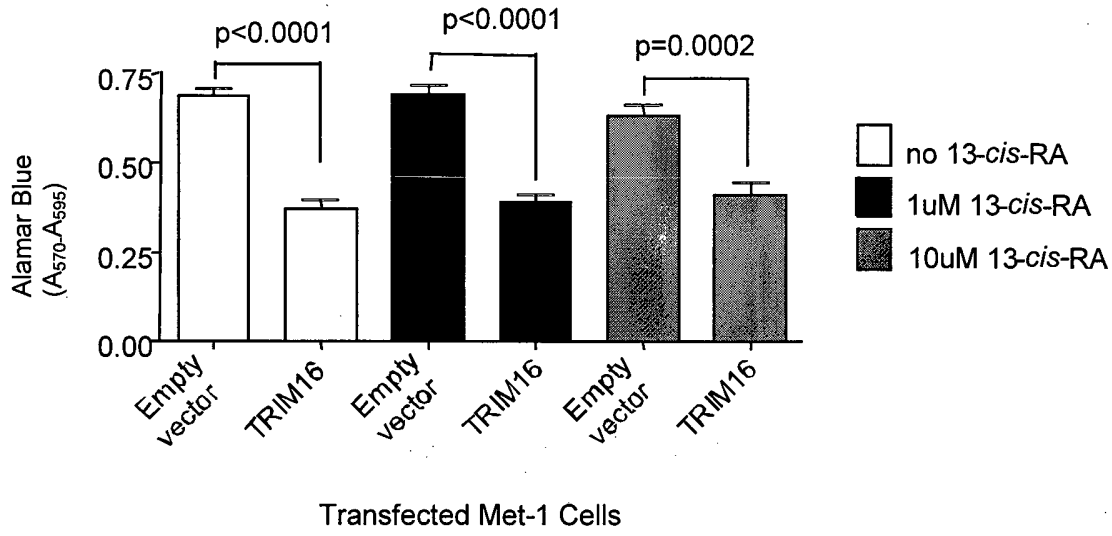

1μM 13-cis-RA
0hr 24hr 48hr 0hr 24hr 48hr
75-
37-
15-
TRIM16
GAPDH
Histone H3
cytoplasmic protein
nuclear protein

Figure 5

C



D

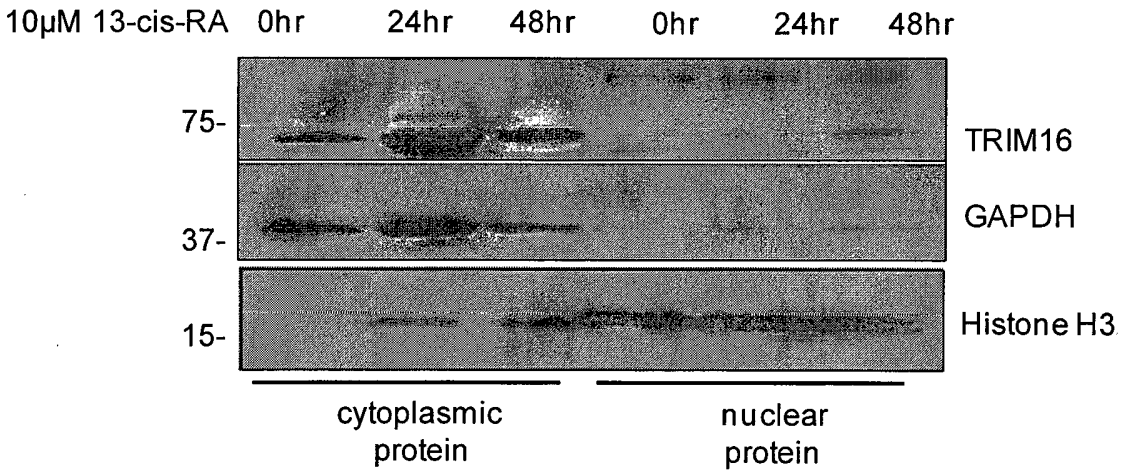


Figure 5 continued

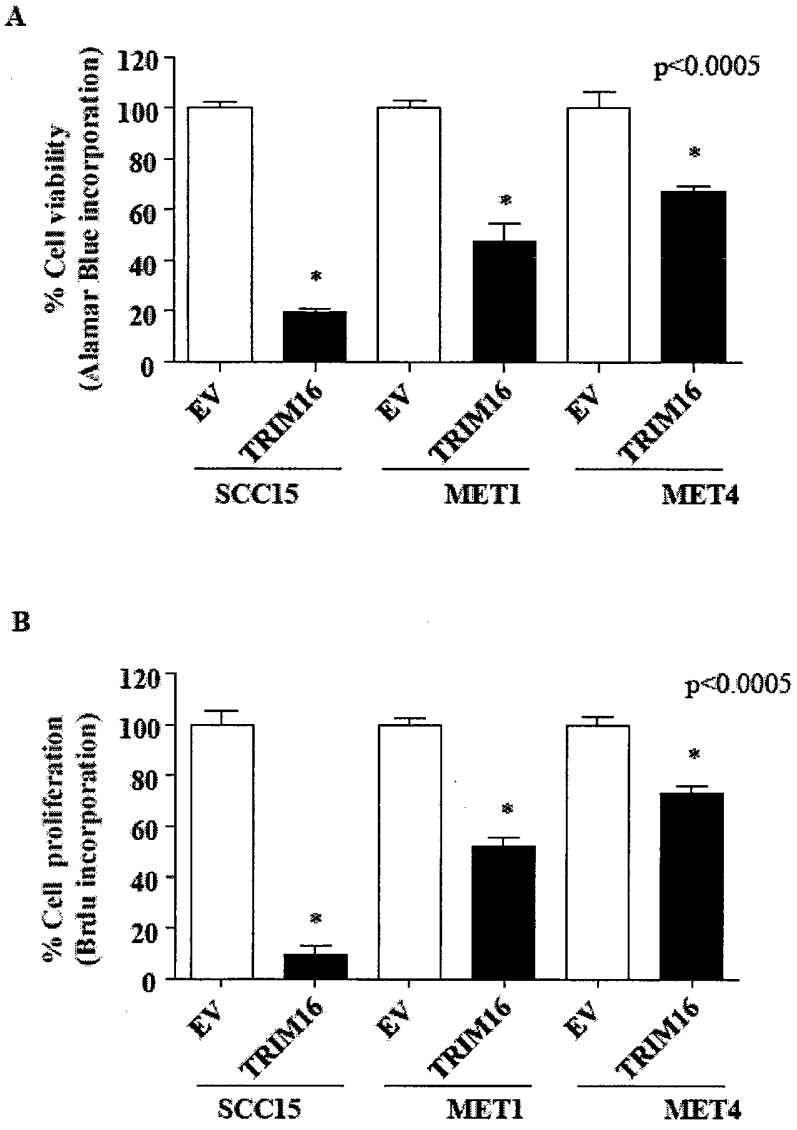
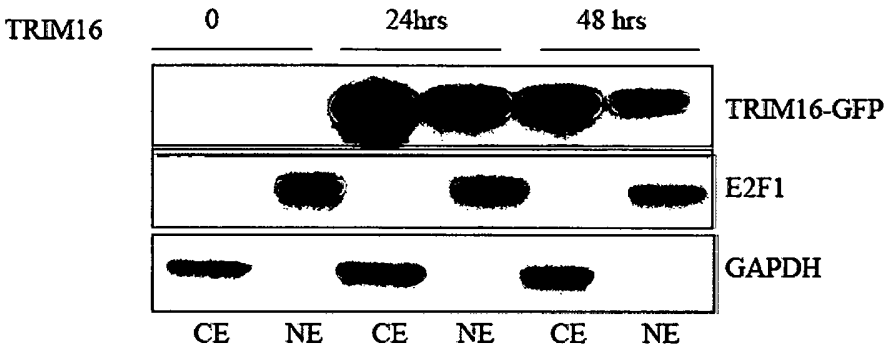
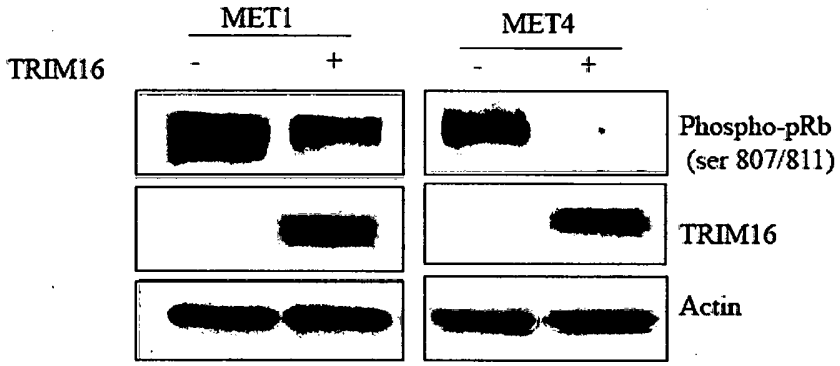

A
% Cell viability
(Alamar Blue incorporation)
120
100
80
60
40
20
0
p<0.0005
*
*
*
EV
TRIM16
EV
TRIM16
EV
TRIM16
SCC15
MET1
MET4
B
% Cell proliferation
(Brdu incorporation)
120
100
80
60
40
20
0
p<0.0005
*
*
*
EV
TRIM16
EV
TRIM16
EV
TRIM16
SCC15
MET1
MET4

Figure 6

C



D



E

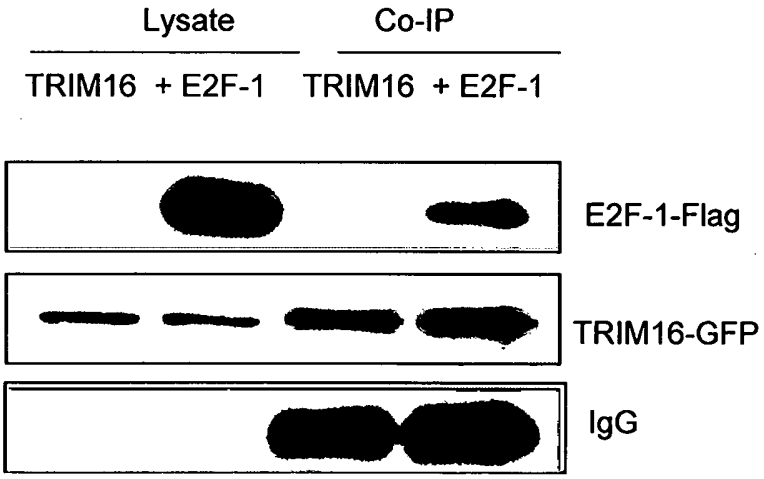


Figure 6 continued

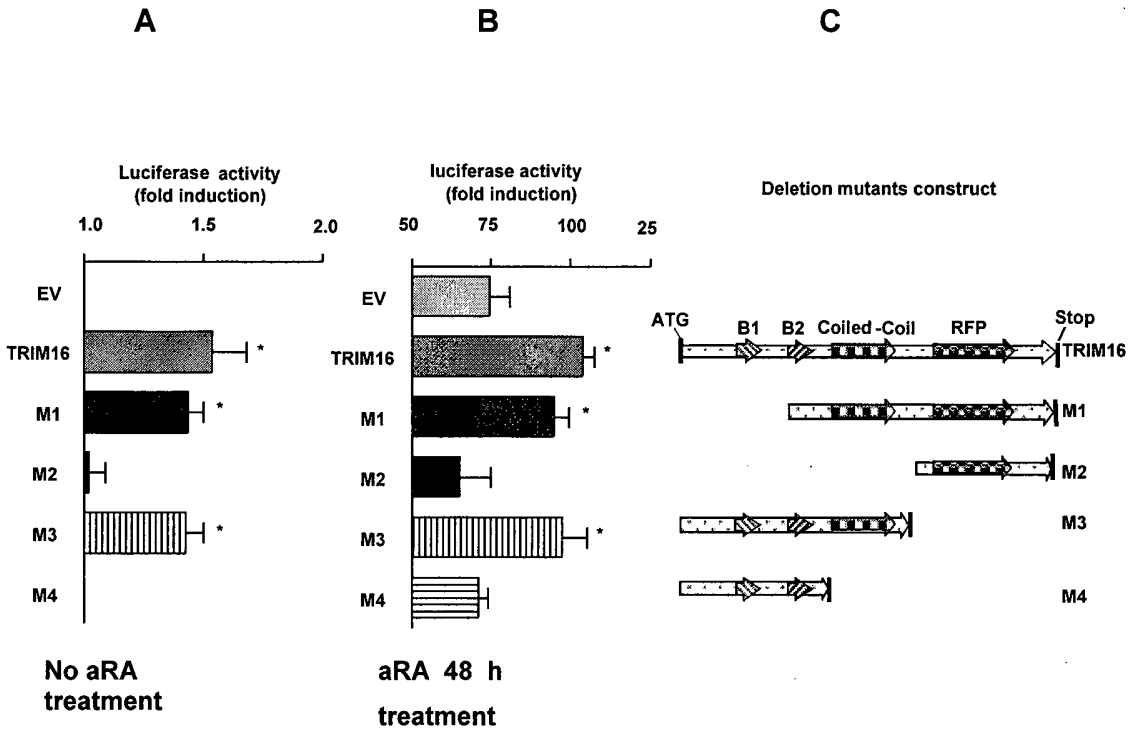
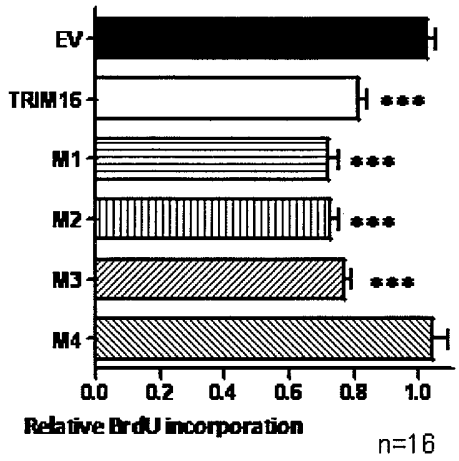

A
B
C
Luciferase activity
(fold induction)
1.0
1.5
2.0
EV
TRIM16
M1
M2
M3
M4
No aRA
treatment
luciferase activity
(fold induction)
50
75
100
25
aRA 48 h
treatment
Deletion mutants construct
ATG
B1
B2
Coiled-Coil
RFP
Stop
TRIM16

Figure 7


EV
TRIM16
M1
M2
M3
M4
***
***
***
***
0.0
0.2
0.4
0.6
0.8
1.0
Relative BrdU incorporation
n=16

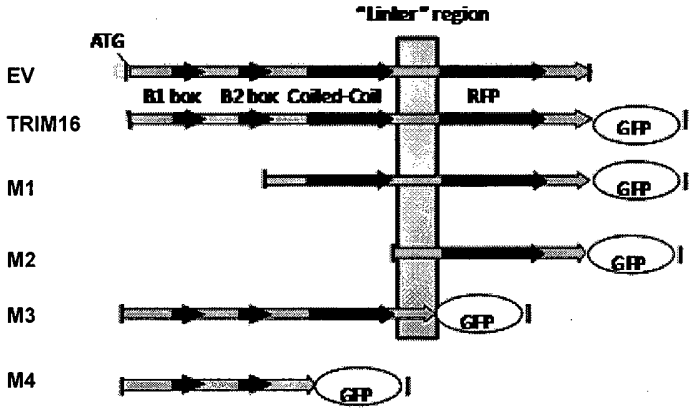
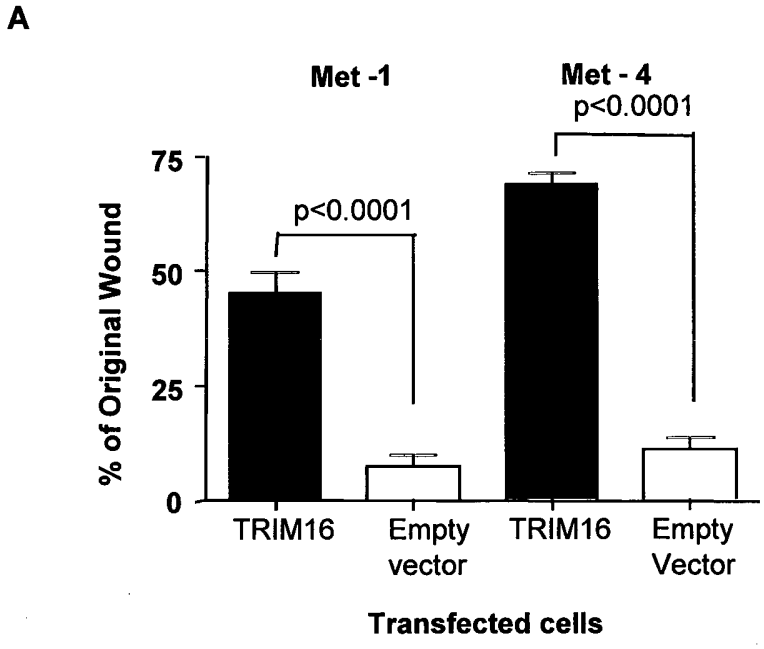

"Linker" region
ATG
EV
TRIM16
M1
M2
M3
M4
B1 box
B2 box
Coiled-Coil
RFP
GFP
GFP
GFP
GFP
GFP

Figure 8


A
Met -1
Met - 4
p<0.0001
p<0.0001
75
50
25
0
% of Original Wound
TRIM16
Empty vector
TRIM16
Empty Vector
Transfected cells

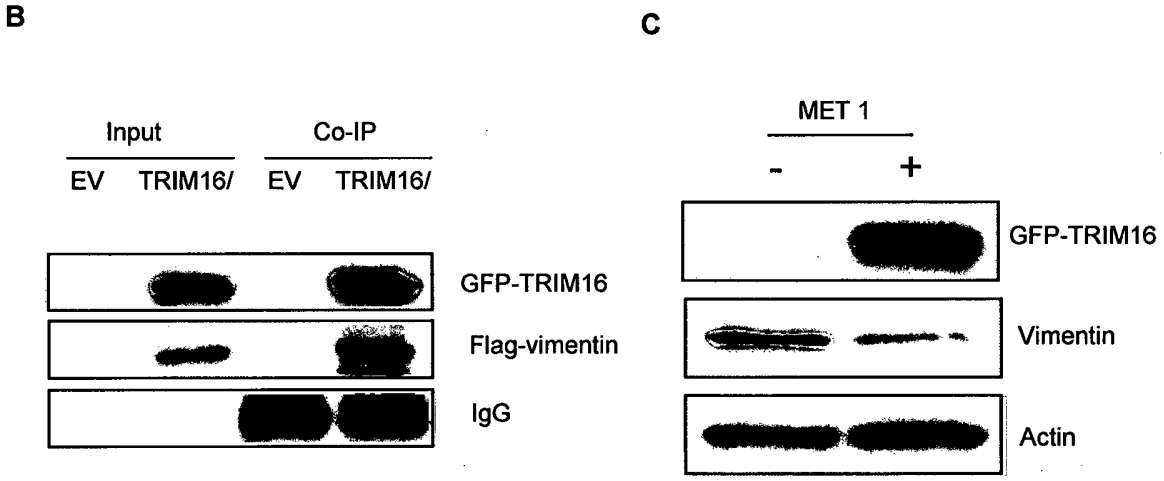

B
Input
Co-IP
EV
TRIM16/
EV
TRIM16/
GFP-TRIM16
Flag-vimentin
IgG
C
MET 1
-
+
GFP-TRIM16
Vimentin
Actin

Figure 9

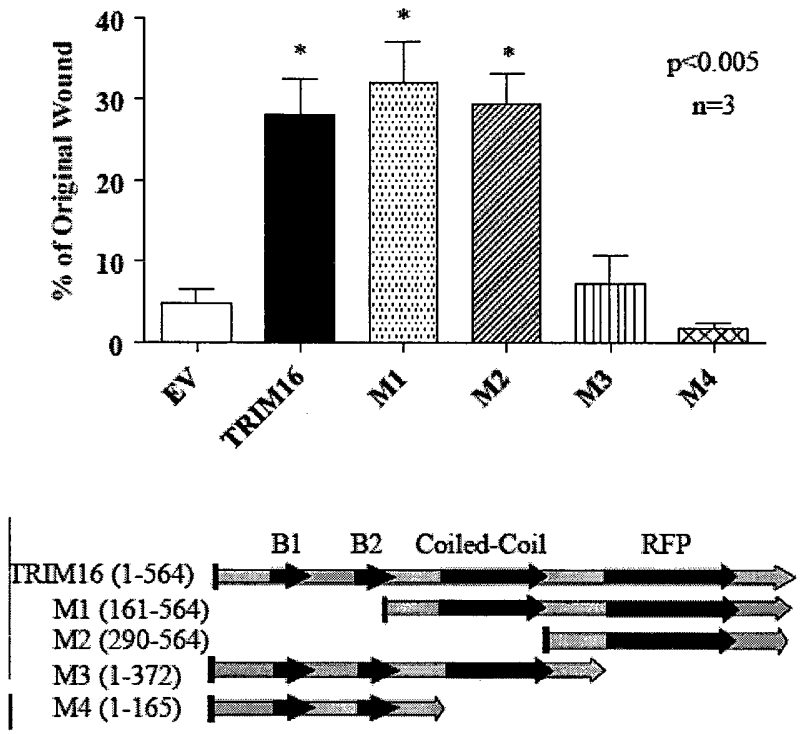
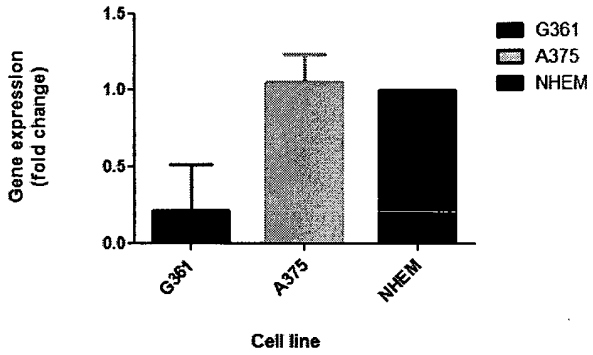
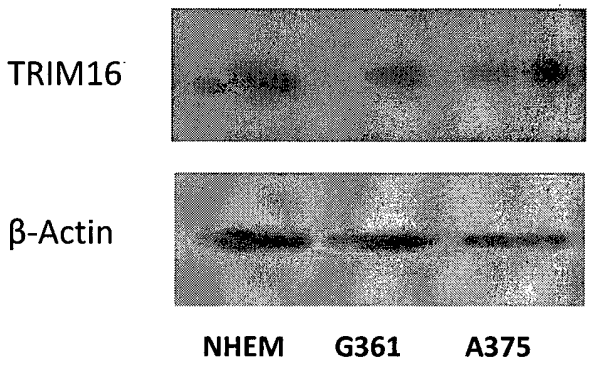

% of Original Wound
40
30
20
10
0
*
*
*
p<0.005
n=3
EV
TRIM16
M1
M2
M3
M4
B1
B2
Coiled-Coil
RFP
TRIM16 (1-564)
M1 (161-564)
M2 (290-564)
M3 (1-372)
M4 (1-165)

Figure 10

A



B



C

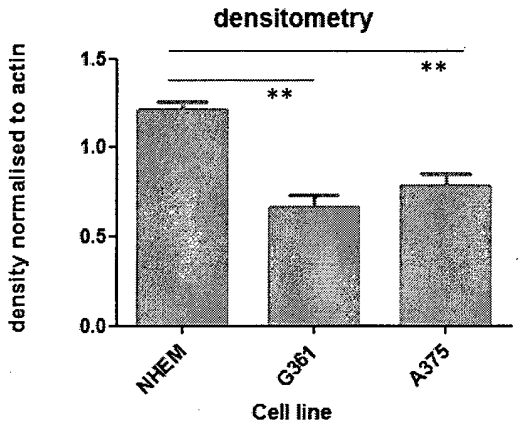
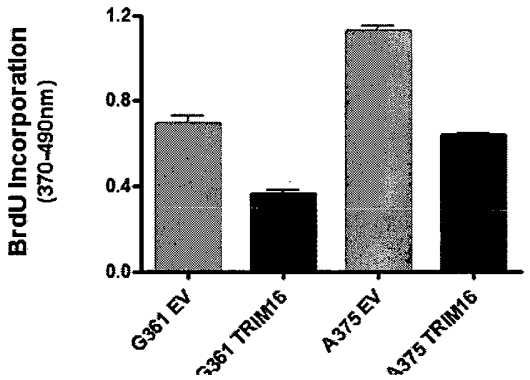


Figure 11

A


BrdU Incorporation
(370-490nm)
1.2
0.8
0.4
0.0
G361 EV
G361 TRIM16
A375 EV
A375 TRIM16

B

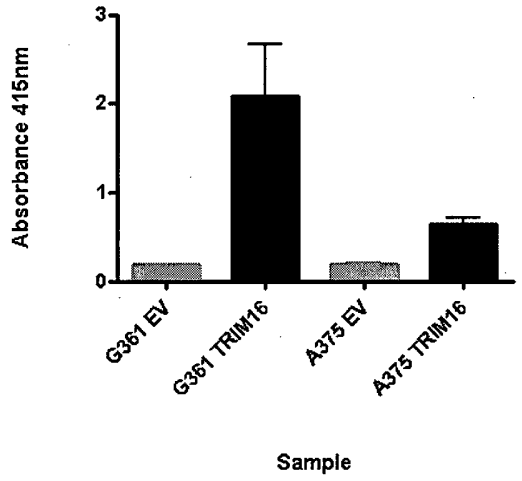
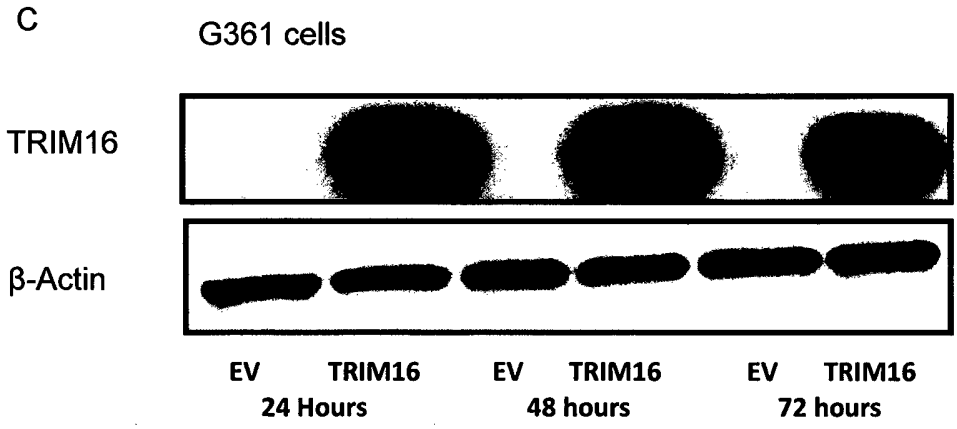

Absorbance 415nm
3
2
1
0
G361 EV
G361 TRIM16
A375 EV
A375 TRIM16
Sample

Figure 12

C



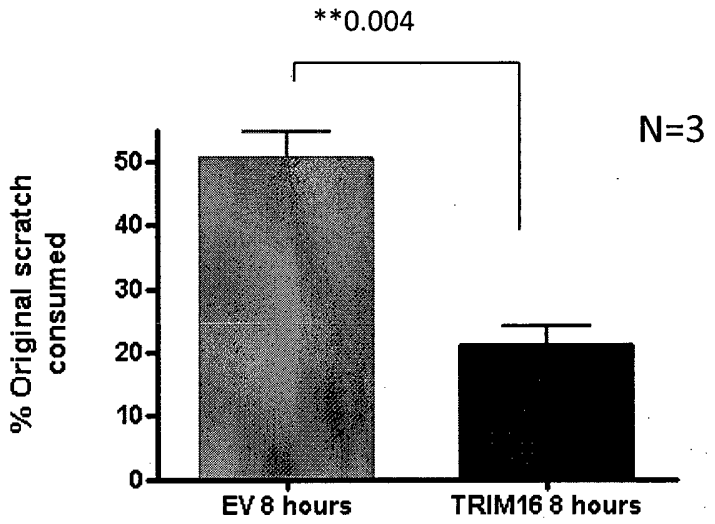
D

G361 Empty Vector at 48 hours

G361 TRIM16 at 48 hours

Figure 12 continued:

A


**0.004
N=3
% Original scratch consumed
50
40
30
20
10
0
EV 8 hours
TRIM16 8 hours

B

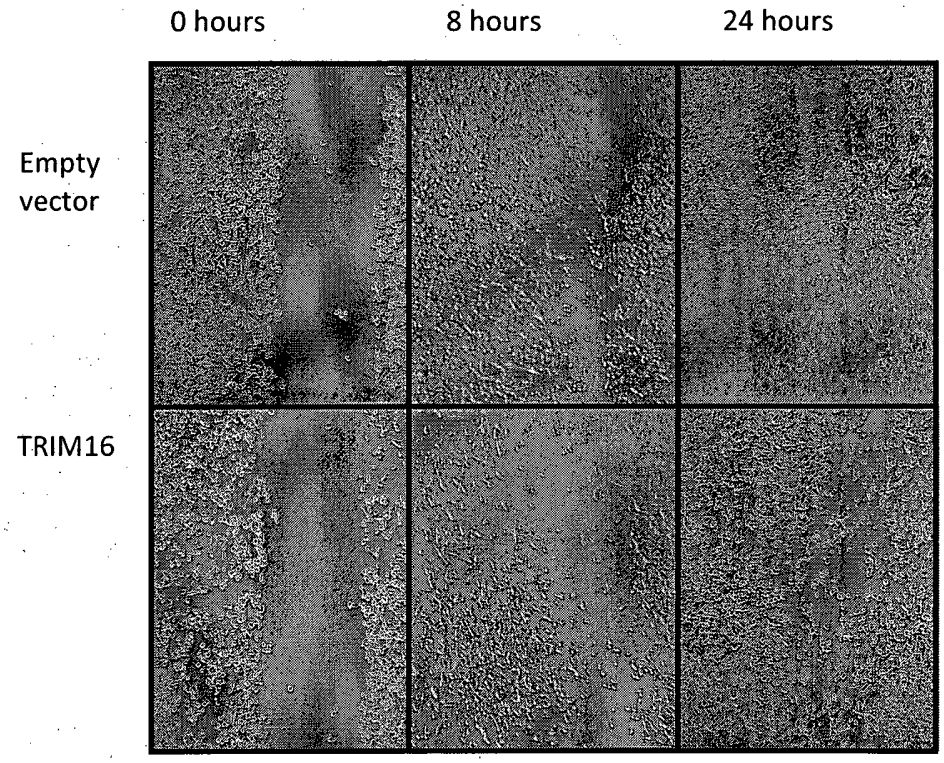
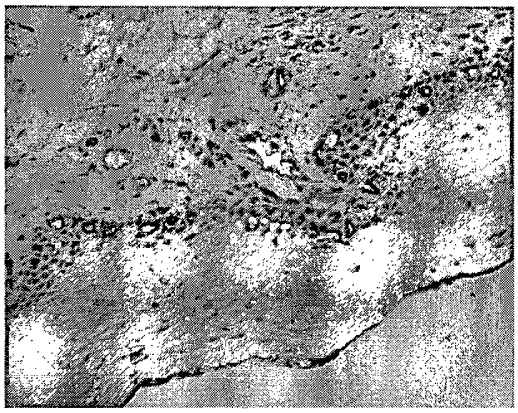
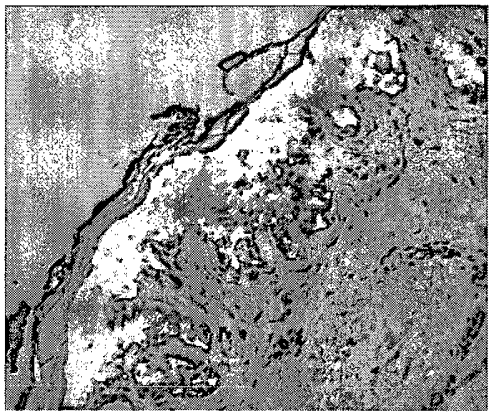

0 hours
8 hours
24 hours
Empty vector
TRIM16

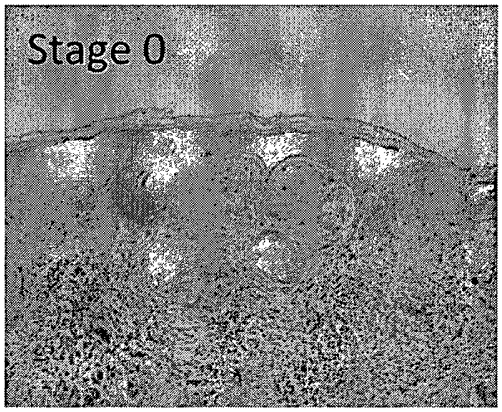
Figure 13



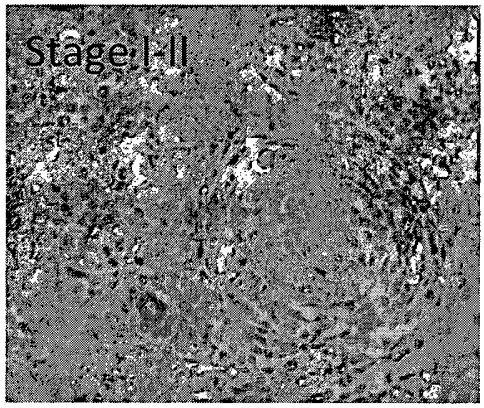
Normal skin



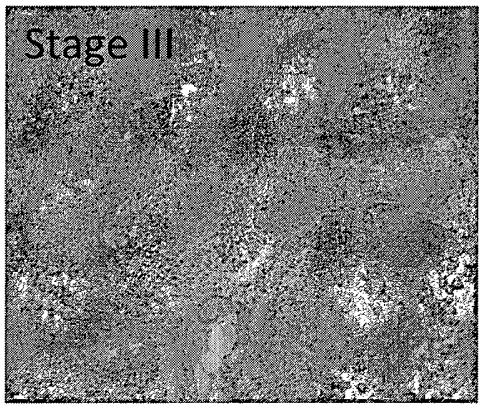
Dysplastic compound naevus



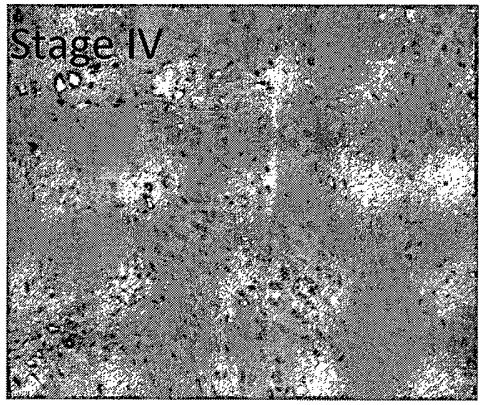
Melanoma in situ



Dermal invasive melanoma



Melanoma lymph node metastasis



Melanoma distant metastasis

Figure 14

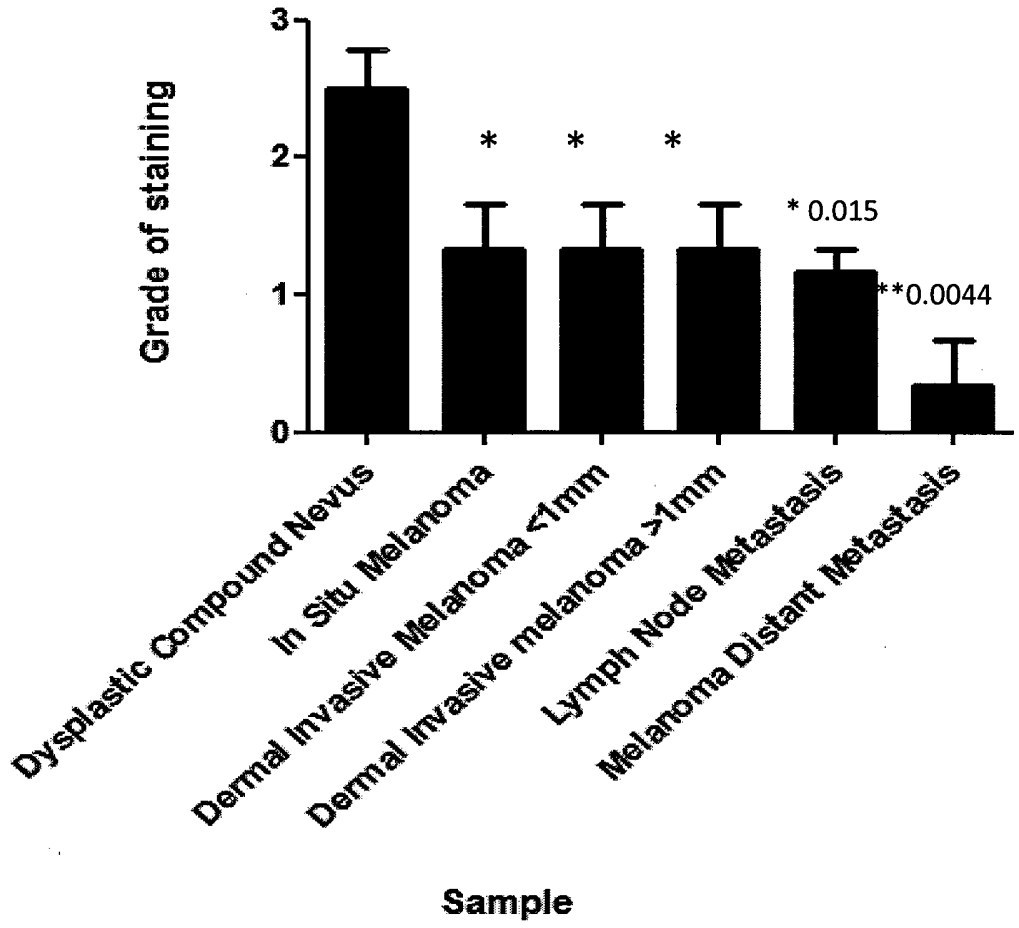

Grade of staining
3
2
1
0
*
*
*
* 0.015
**0.0044
Dysplastic Compound Nevus
In Situ Melanoma
Dermal Invasive Melanoma <1mm
Dermal Invasive melanoma >1mm
Lymph Node Metastasis
Melanoma Distant Metastasis
Sample

Figure 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2011/000236

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

A61K 38/17 (2006.01) ***A61P 35/00*** (2006.01)
A61K 31/07 (2006.01) ***C07K 14/435*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPODOC, WPI, MEDLINE, HCA, BIOSIS, GOOGLE (tripartate motif-containing 16, TRIM16, EBBP, estrogen responsive B box protein, cancer, tumor, melanoma, neuroblastoma, carcinoma, glioma)
GENOMEQUEST (SEQ ID NO: 3))

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2009/0238832 A1 (BODARY-WINTER et al) 24 September 2009 (see 0009, 0011, 0054, 0488 and Figure 432)	1-3, 7-8
X	CHEUNG, B. B *et al.* "The Estrogen-Responsive B Box Protein is a Novel Regulator of the Retinoid Signal" The Journal of Biological Chemistry. 2006 Vol. 281, No. 26, pages 18246-18256. (see page 18254 LHS, page 18255 RHS)	1-20

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 April 2011	2 MAY 2011
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **Neal Dalton** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 3 9935 9615

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RAIF, A. *et al.* "The Estrogen-Responsive B Box Protein (EBBP) Restores Retinoid Sensitivity in Retinoid-Resistant Cancer Cells via Effects on Histone Acetylation" Cancer Letters. 2009 Vol. 277, Iss. 1, pages 82-90 (see page 88 RHS, 89 RHS)	1-20
X	CHEUNG, B. *et al.* "The Molecular Mechanism of the Estrogen-Responsive B Box Protein (EBBP)-Mediated Anticancer Signal" [abstract]. In: Proceedings of the 99th Annual Meeting of the American Association for Cancer Research. 2008 Apr 12-16: San Diego California (CA) Abstract No. 108. [retrieved on 1 December 2010]. Retrieved from Internet < URL: http://www.aacrmeetingabstracts.org/cgi/content/meeting_abstract/2008/1_Annual_Meeting/108?maxtoshow=&hits=10&RESULTFORMAT=&author1=cheung&fulltext=EBBP&andorexactfulltext=and&searchid=1&FIRSTINDEX=0&sortspec=relevance&resourcetype=HWCIT > (see whole document)	1-20
X	CHEUNG, B. B. *et al.* "Characterization of a Novel Regulator of the Retinoid Signal: The Estrogen-Responsive B Box Protein" [abstract]. In: Proceedings of the American Association for Cancer Research. 2006. Vol. 47. Abstract No. 5331 [retrieved on 1 December 2010]. Retrieved from Internet < URL: http://www.aacrmeetingabstracts.org/cgi/content/abstract/2006/1/1250-b?maxtoshow=&hits=10&RESULTFORMAT=&author1=cheung&fulltext=EBBP&andorexactfulltext=and&searchid=1&FIRSTINDEX=0&sortspec=relevance&resourcetype=HWCIT > (see whole document)	1-20
P,X	MARSHALL, G. M. *et al.* "TRIM16 Acts as a Tumour Suppressor by Inhibitory Effects on Cytoplasmic Vimentin and Nuclear E2F1 in Neuroblastoma Cells" Oncogene. 2010, Vol. 29, pages 6172–6183. Published online 23 August 2010 (see abstract, introduction, page 6173 RHS, discussion)	1-20
P,X	CHEUNG, B. B. "TRIM16 Acts as a Tumour Suppressor in Neuroblastoma and Skin Cancer" BIT's 3rd World Cancer Congress (WCC)-2010. June 22-25, 2010. Singapore. [retrieved on 1 December 2010]. Retrieved from Internet < URL: http://www.bitlifesciences.com/cancer2010/Program.asp > (see Track 2-7)	1-12, 14-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2011/000236

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
See Supplemental Box

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Supplemental Box
(To be used when the space in any of Boxes I to IV is not sufficient)

Continuation of Box No: III

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

In assessing whether there is more than one invention claimed, I have given consideration to those features which can be considered to potentially distinguish the claimed combination of features from the prior art.

This International Searching Authority has found that there are different inventions as follows:

- Claims 1-18 are directed to: 1) compositions for minimising the risk of developing skin cancer, or treating an individual with skin cancer, comprising TRIM16 (or the tumour suppressor domain fragment thereof), and 2) methods for minimising the risk of developing skin cancer, or treating an individual with skin cancer, comprising applying an effective amount of TRIM16 (or the tumour suppressor domain fragment thereof) to skin cells/cancer cells. It is considered that this group of claims, wherein TRIM16 is used to treat skin cancer, define a first invention.
- Claim 19 is directed to a method of determining the suitability of retinoid treatment of skin cancer by detecting the level of expression of TRIM16 in the cancer cells, wherein reduced levels of expression compared to non-cancerous skin cells is indicative of the cancer cells being resistant to retinoid treatment. It is considered that this claim, wherein TRIM16 expression is indicative of retinoid resistance, defines a second invention.
- Claim 20 is directed to a method of conferring or enhancing retinoid sensitivity to a.retinoid-treatment resistant skin cancer, comprising applying TRIM16 to the skin cancer cells. It is considered that this claim, wherein TRIM16 confers/enhances retinoid sensitivity to skin cancer cells, defines a third invention.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

The only feature common to all of the claims is the association of TRIM16 with cancer. However this concept is not novel in light of:

D2: CHEUNG, B. B *et al.* "The Estrogen-Responsive B Box Protein is a Novel Regulator of the Retinoid Signal" The Journal of Biological Chemistry. 2006 Vol. 281, No. 26, pages 18246-18256. (see page 18254 RHS).

This means that the common feature can not constitute a special technical feature within the meaning of PCT Rule 13.2, second sentence, since it makes no contribution over the prior art.

Because the common feature does not satisfy the requirement for being a special technical feature it follows that it cannot provide the necessary technical relationship between the identified inventions. Therefore, the claims do not satisfy the requirement of unity of invention *a posteriori.*

Additionally, a feature common to the second and third inventions is the association of TRIM16 levels with retinoid sensitivity in cancer cells. However this concept is also disclosed in D2.

As this concept is known in the prior art, the common feature of the second and third inventions can not constitute a special technical feature within the meaning of PCT Rule 13.2, second sentence, since it makes no contribution over the prior art.

Because the common feature of the second and third invention does not satisfy the requirement for being a special technical feature it follows that it cannot provide the necessary technical relationship between the identified inventions. Therefore, the claims do not satisfy the requirement of unity of invention *a posteriori*

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2011/000236

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	2009/0238832	AU	2003279084	AU	2010203031	CA	2499843
		EP	1585482	JP	2010162016	US	2007/0042945
		WO	2004/028479				

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX