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TRIM16 acts as a tumour suppressor by inhibitory effects on cytoplasmic vimentin and nuclear E2F1 in neuroblastoma cells

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The family of tripartite-motif (TRIM) proteins are involved in diverse cellular processes, but are often characterized by critical protein–protein interactions necessary for their function. TRIM16 is induced in different cancer types, when the cancer cell is forced to proceed down a differentiation pathway. We have identified TRIM16 as a DNA-binding protein with histone acetylase activity, which is required for the retinoic acid receptor β_2 transcriptional response in retinoid-treated cancer cells. In this study, we show that overexpressed TRIM16 reduced neuroblastoma cell growth, enhanced retinoid-induced differentiation and reduced tumourigenicity *in vivo*. TRIM16 was only expressed in the differentiated ganglion cell component of primary human neuroblastoma tumour tissues. TRIM16 bound directly to cytoplasmic vimentin and nuclear E2F1 in neuroblastoma cells. TRIM16 reduced cell motility and this required downregulation of vimentin. Retinoid treatment and enforced overexpression caused TRIM16 to translocate to the nucleus, and bind to and downregulate nuclear E2F1, required for cell replication. This study, for the first time, demonstrates 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.

Introduction

The tripartite motif (TRIM) protein family is composed of more than 70 highly conserved proteins, which display three similar structural motifs: N-terminal, RING-B box-coiled-coil domains and a variable C-terminus. TRIM proteins have been ascribed important roles in development, ubiquitination, retroviral immunity and cancer, but commonly depend on protein–protein interactions to fulfil their cellular functions (Le Douarin *et al.*, 1995; Cao *et al.*, 1998; Meroni *et al.*, 2005; Vichi *et al.*, 2005).

TRIM16, also called the oestrogen-responsive B box protein, is a member of the RING-B box-coiled-coil/TRIM protein family, and is constitutively expressed in many tissues in a nascent and post-translationally modified form (Liu *et al.*, 1998). In contrast to most B-box family members, TRIM16 has a predominantly cytoplasmic localization. TRIM16 is missing the RING finger domain, but has, in addition, a C-terminal Ret finger, or B30.2 (SPRY), domain of unknown function. This is shared with several other tripartite motif proteins, such as Midline 1 (MID1/TRIM 18) and TRIM 20, mutated in the human disorders Opitz syndrome and Familial Mediterranean fever, respectively (Trockenbacher *et al.*, 2001; Chae *et al.*, 2003).

In search for factors that mediate the retinoid anticancer signal, we have shown that the TRIM16 protein binds DNA and has histone acetyltransferase activity, which is required for the retinoid-induced retinoic acid (RA) receptor β_2 transcriptional response and growth inhibition in retinoid-treated cancer cells (Cheung *et al.*, 2006; Raif *et al.*, 2009). TRIM16 is a ubiquitously expressed protein that is predominantly cytoplasmic, and undergoes serine–threonine phosphorylation and increased protein stability following retinoid treatment. TRIM16 expression is also induced by oestrogen in breast cancer cells and keratinocyte growth factor in keratinocytes, as cells are entrained to undergo differentiation (Liu *et al.*, 1998; Beer *et al.*, 2002). TRIM16 reduced the cell viability in retinoid-sensitive neuroblastoma cells and retinoid-resistant breast and lung cancer cells, whereas the viability of non-cancer cells was unaffected by TRIM16 overexpression (Cheung *et al.*, 2006; Raif *et al.*, 2009). Importantly, exogenous TRIM16 overexpression in retinoid-resistant cancer cells induced retinoid sensitivity, making TRIM16 an important target for future differentiation therapy development in cancer.

Neuroblastoma is the most common solid tumour of early childhood, originating from cells that form the

normal sympathetic nervous system. The tumour cells have embryonic features, presumably as a consequence of an impaired capacity to respond to signals operating during normal differentiation. RA causes ganglionic differentiation of neuroblastoma cell lines *in vitro*, characterized by the appearance of long neurites and cell growth arrest (Thiele *et al.*, 1985). This observation led to successful clinical trials in children with advanced neuroblastoma, showing that retinoid therapy significantly improved survival in these patients (Matthay *et al.*, 1999, 2009).

The signalling pathways and mechanisms underpinning the effect of TRIM16 in retinoid-treated cancer cells are yet to be fully delineated. Owing to the profound effects of TRIM16 overexpression in the presence of retinoids, we examined the hypothesis that TRIM16 may act as a tumour suppressor in the absence of retinoids, and, moreover, that protein–protein interactions might explain its cellular effects. In this study, we show that TRIM16 is an important regulator of neuroblastoma cell differentiation, migration and tumourigenicity, via its interaction with cytoplasmic vimentin and nuclear E2F1 proteins in neuroblastoma cells.

Results

TRIM16 reduces neuroblastoma cell viability, clonogenicity and tumourigenicity

We have previously shown that TRIM16 can enhance the retinoid anti-proliferative effect on cancer cells through transcriptional regulation of retinoic acid receptor β_2. In this study, we sought to investigate whether TRIM16 had a tumour suppressor function in neuroblastoma, independent of its effects on the retinoid anticancer signal. We derived stable neuroblastoma BE(2)-C cell lines, which overexpressed TRIM16 (Figure 1a). In an Alamar Blue assay (Figure 1b), overexpression of TRIM16 in neuroblastoma tumour cells decreased the cell viability in two independently derived clones (TRIM16-4 and TRIM16-5), compared with empty vector clones (EV-1 and EV-8) ($P < 0.001$). Anchorage-independent growth in soft agar was also reduced in TRIM16-expressing clones, compared with control clones ($P < 0.01$) (Figures 1c and d). In a xenograft BALB/c nude mouse model, TRIM16 over-expression led to substantially decreased neuroblastoma tumour growth, as compared with the empty vector controls, 4 weeks post engraftment ($P < 0.01$) (Figure 1e). Together, these data indicated that TRIM16 inhibited the tumourigenic phenotype of neuroblastoma tumour cells in the absence of excess retinoid. Furthermore, we examined the effect of knockdown of TRIM16 expression on cell growth. The efficiency of TRIM16 small interfering RNA (siRNA) was evaluated after 24, 48 and 72 h of transfection by western blot (70% knockdown). The cell viability of BE(2)-C was significantly increased after transfection of 20 nM siRNA TRIM16 when compared with control siRNA ($P < 0.001$) (Figure 1f).

TRIM16 induces neuronal differentiation in neuroblastoma cells

TRIM16 expression is increased by oestrogen, keratinocyte growth factor and retinoic acid in breast cancer, skin and neuroblastoma cells, respectively (Liu *et al.*, 1998; Beer *et al.*, 2002; Cheung *et al.*, 2006). In order to investigate the mechanism of the TRIM16 tumour suppressor function, TRIM16-expressing BE(2)-C neuroblastoma cell clones were examined for neuritic differentiation *in vitro* in the presence of retinoid, and compared with control clones. Overexpression of TRIM16 led to a significant increase in the proportion of neurite-bearing cells after 10 μM all-*trans*-retinoic acid (aRA) treatment at days 4 and 6, compared with empty vector controls ($P < 0.001$) (Figures 2a and b).

Neuroblastoma tumour tissue is composed of primitive neuroblasts, more differentiated mature ganglion cells and interstitial neuropil. Moreover, a high degree of ganglion cell maturation within a tumour correlates with a better patient prognosis (Joshi, 2000). We therefore wanted to investigate whether TRIM16 was selectively expressed in particular cell types within the neuroblastoma tumour tissue. Paraffin-embedded, formalin-fixed human neuroblastoma tumour cross-sections were examined for the pattern of endogenous TRIM16 expression by immunohistochemical staining using a TRIM16-specific antibody in 21 patients from five clinical stages (stage I, II, III, IV and IVS). The expression of TRIM16 was stronger in the large ganglion cells and interstitial neuropil, which comprise the more differentiated component of the tumour, and is largely absent in the smaller primitive neuroblasts (Figure 2c). The proportion of TRIM16-positive cells was significantly higher in the differentiated region, compared with the poorly differentiated region ($P < 0.001$) across all clinical stages (Figure 2d). However, there was no statistically significant relationship between clinical stage and TRIM16 protein expression. We confirmed the differentiated nature of the region of the neuroblastoma tumour shown in Figure 2e, by demonstrating co-expression of the neuronal marker, class III β-tubulin isotype (Katsetos *et al.*, 1991). Furthermore, immunohistochemical staining revealed a strong nuclear and cytoplasmic TRIM16 expression in neonatal differentiated superior cervical ganglia cells, coeliac ganglia cells and normal adrenal gland. In contrast, TRIM16 expression is reduced in poorly differentiated neuroblastoma tumour cells and is predominately in the cytoplasm of 6-week-old TH-MYCN transgenic mouse tumours (Weiss *et al.*, 1997).

TRIM16 binds to and modulates vimentin protein expression in both neuroblastoma and lung cancer cells

TRIM proteins commonly bind to other proteins to affect cellular function (Urano *et al.*, 2002); thus, we investigated TRIM16-binding proteins in neuroblastoma cells. We examined protein lysates from BE(2)-C cells, transiently transfected with full-length TRIM16 tagged with an MYC oligopeptide for 48 h. As a negative control, we compared lysates from cells that
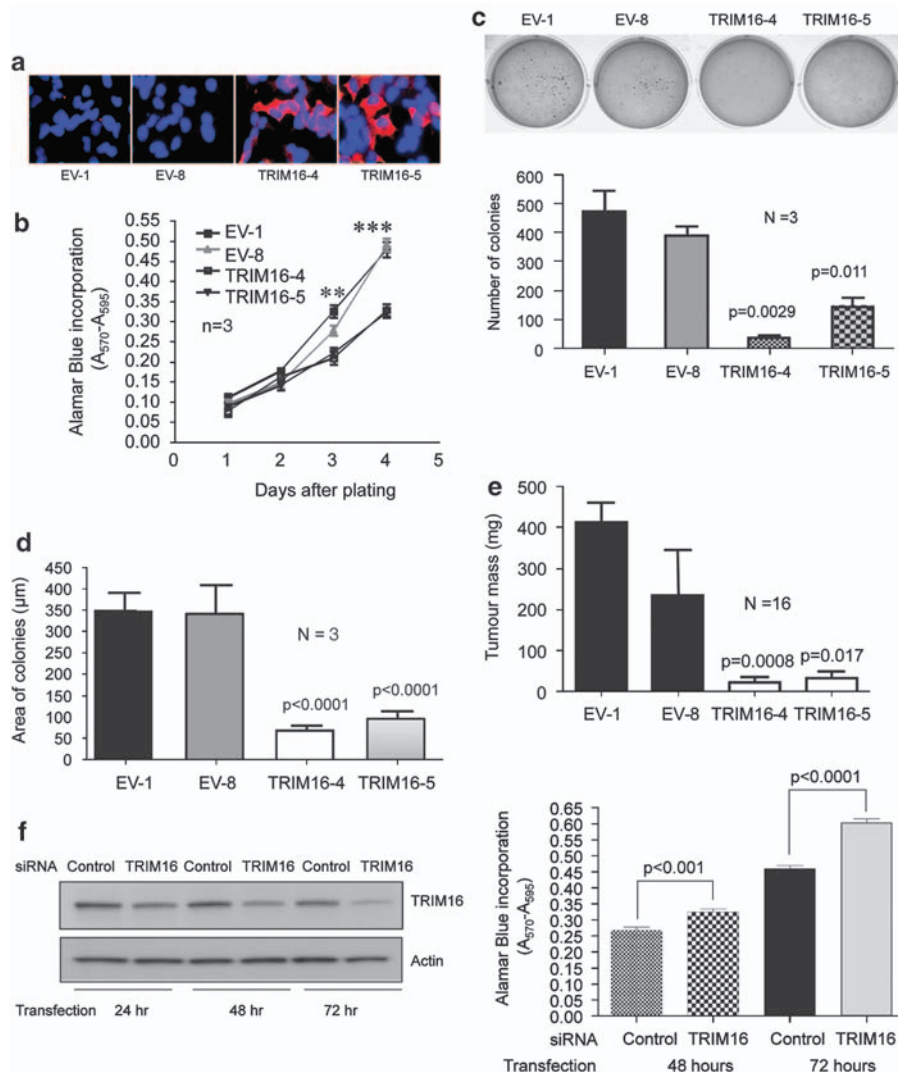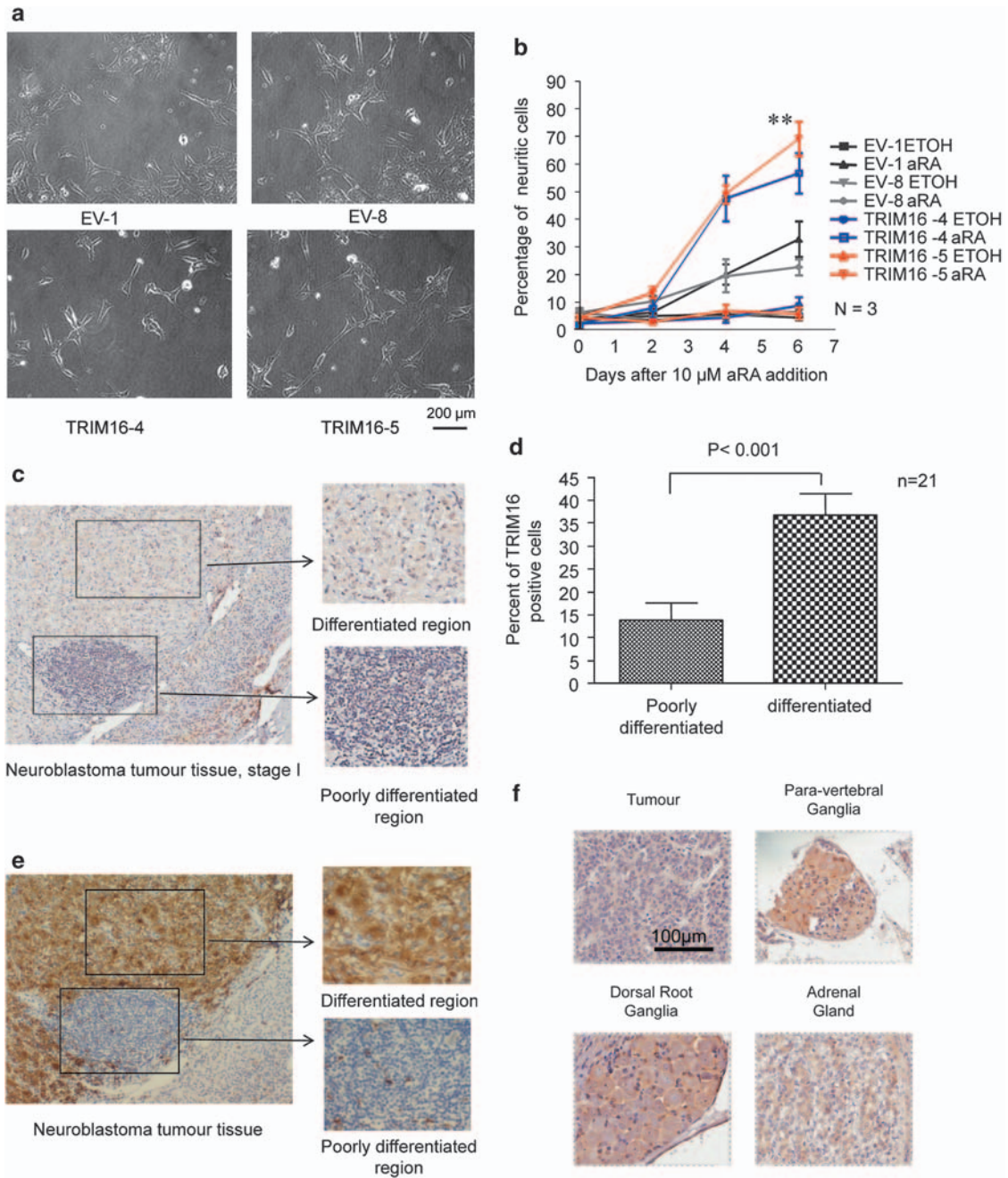
Figure 1 TRIM16 reduces neuroblastoma cell viability, clonogenicity and tumourigenicity. (**a**) The cells were stained with an antibody recognizing the Myc-tag epitope to confirm the TRIM16 plasmid transfection by immunofluorescence microscopy and immunoblot examination. (**b**) The viable cells were measured up to day 4 by Alamar Blue assay in BE(2)-C cells stably transfected with empty vector or with TRIM16 plasmid DNA. A statistically significant difference (**$P<0.01$ and ***$P<0.001$) compared with the empty vector control. (**c**) In the top panel, the clonogenicity of various BE(2)-C cell transfectants was determined using soft agar. In the bottom panel, number of colonies were compared between empty vector control (EV-1) and TRIM16 plasmid transfected BE(2)-C cell. (**d**) A colony-forming assay showed reduced colony size in clones overexpressing TRIM16. A statistically significant difference ($P<0.0001$) compared with empty vector control (EV-1). (**e**) The tumour volume of TRIM16 or empty vector transfected BE(2)-C cells injected into nude mice for 4 weeks. Each value shown in the figure represented data from 16 mice. A statistically significant difference ($P<0.01$) compared with empty vector control (EV-1). (**f**) In the left panel, western blotting analysis by TRIM16-specific antibody at 24, 48 and 72 h of BE(2)-C cells without or with TRIM16 siRNA transfection. Actin was used as an internal control for loading. In the right panel: the viable cells were measured up to 72 h by Alamar Blue assay in BE(2)-C cells transfected with control siRNA or TRIM16 specific siRNA.

were transfected with an empty vector. The supernatant was immunoprecipitated by an anti-MYC-Tag antibody. Protein bands were cut directly from the gel and micro-sequenced by the liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses. We have identified seven candidate TRIM16-binding partners (vimentin, E2F1, prohibitin, TRAP-1, EBNA2 co-activator p100, EPLIN and scaffold attachment factor A) in this way. We then performed immunoprecipitation and immunoblotting experiments on four of the candidate binding partners, and confirmed that vimentin, E2F1 and prohibitin indeed interact with TRIM16

in BE(2)-C cells. As vimentin expression is not only a marker associated with the invasive phenotype of cancer, but is also required for carcinoma cell motility, and E2F1 is a transcription factor that is central for cell survival and proliferation, vimentin (a cytoplasmic protein) and E2F1 (a nuclear protein) were chosen for further studies.

Vimentin is the predominant intermediate filament protein in mesenchymal cells, and is implicated in metastasis and cancer cell migration in colon and breast cancer cell lines (McInroy *et al.*, 2007). To determine whether TRIM16 interacted with vimentin, co-immunoprecipitation (co-IP) of vimentin with Myc-TRIM16

Figure 2 TRIM16 induces neuronal differentiation in neuroblastoma cells. (**a**, **b**) Up to 6 days of 10 μM all-*trans*-retinoic acid (aRA) treatment or ethanol control, cell differentiation was assessed by analysing neurite outgrowth under phase contrast microscopy. Cell images were captured and stored, and neurite outgrowth was quantified. Error bars indicate s.e. A statistically significant difference (**$P < 0.001$) compared with empty vector control. (**c**) Immunohistochemical staining for TRIM16 expression in a primary human neuroblastoma tumour (stage I). In the tumour tissue, endogenous TRIM16 was expressed (brown staining) higher in large mature ganglion cells and less in the neuroblasts (small blue round cells). (**d**) The percentage of TRIM16-positive cells were compared between differentiated regions and poorly differentiated regions in the tumour tissues from 21 neuroblastoma patients. (**e**) A formalin-fixed tissue section from the same patient of Figure 2c was subjected to immunohistochemical assessment of class III β-tubulin protein expression (brown staining). (**f**) Immunohistochemical staining for expression of TRIM16 (brown staining) in MYCN mice, using an anti-TRIM16 antibody (scale bar: 100 μm).

was performed on whole-cell lysates from transfected BE(2)-C neuroblastoma and SK-MES-1 lung cancer cells. Co-IP confirmed that TRIM16 and vimentin formed a complex in both cancer cell types (Figure 3a). TRIM16 markedly reduced vimentin protein expression or increased vimentin expression, as shown by TRIM16 overexpression or siRNA knockdown experiments, respectively (Figures 3b and c). The level of vimentin mRNA did not change in BE(2)-C and SK-MES-1 cells with overexpression of TRIM16 (data not shown). As previously published (Beer *et al.*, 2002), TRIM16 was predominantly found in the cytoplasm and to a lesser
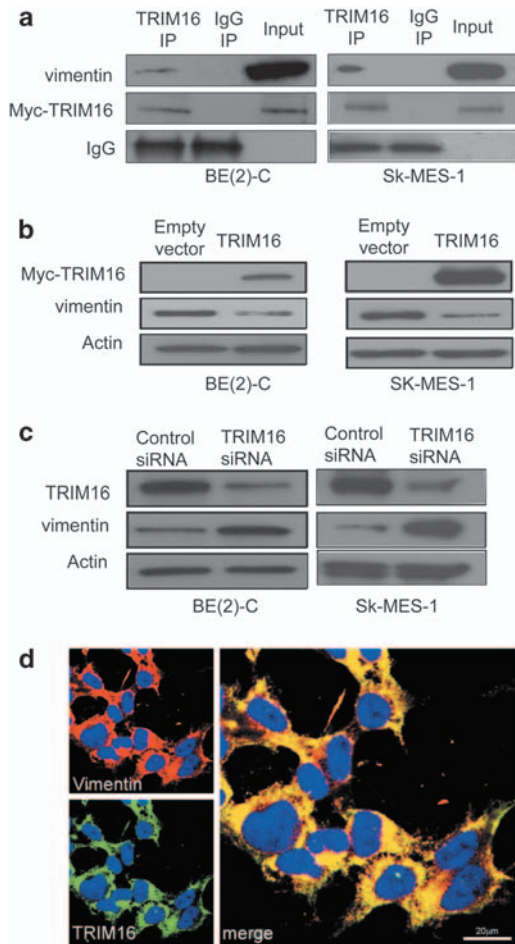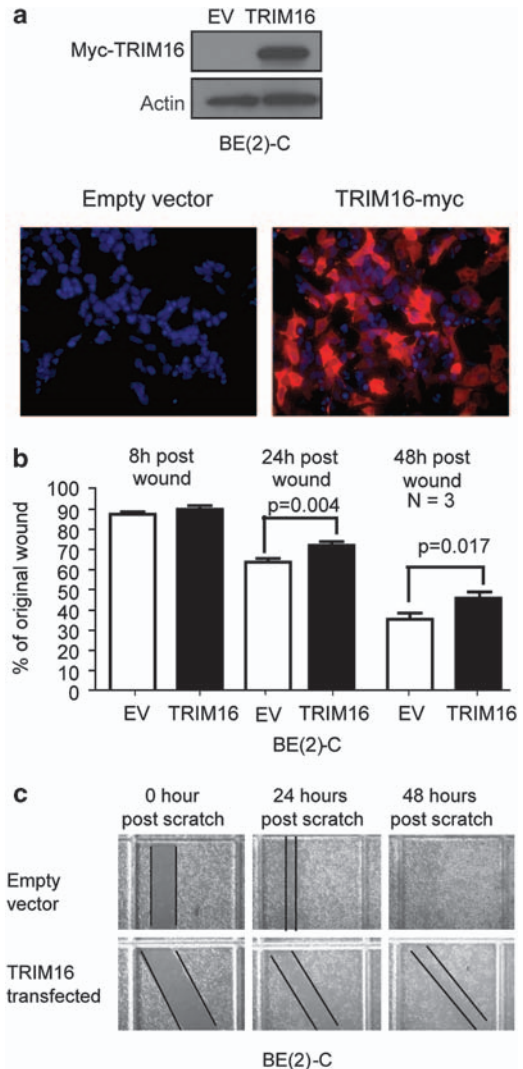
Figure 3 TRIM16 binds to and modulates vimentin protein expression in both neuroblastoma and lung cancer cells. (**a**) Interaction of TRIM16 with vimentin. Lysates of BE(2)-C and SK-MES-1 cells transfected with TRIM16 plasmids were analysed by immunoblotting using anti-vimentin and anti-Myc-tag antibodies (western blots: vimentin and TRIM16 proteins), combined without (input) or with immunoprecipitation using anti-TRIM16 antibody or anti-IgG antibody control. (**b**) TRIM16 overexpression downregulates exogenous vimentin. Lysates of transfected BE(2)-C and SK-MES-1 cells were analysed by anti-TRIM16 (top panel) or anti-vimentin (middle panel) or anti-actin as loading control (bottom panel). (**c**) BE(2)-C and SK-MES-1 cells were transfected with either control siRNA or siRNA specifically targeting TRIM16 for 72 h, followed by protein extraction and western blot. (**d**) Immunocytochemical expression patterns vimentin (orange/red fluorescence) and TRIM16 (green fluorescence) in BE(2)-C cells colocalized in the cytoplasm (visualized as yellow fluorescence in merge panel).

Figure 4 Downregulated vimentin is required for TRIM16 effects on cell motility. (**a**) Lysates of BE(2)-C cells transfected with TRIM16 plasmids were analysed by immunoblotting and an immunofluorescence assay using anti-Myc antibody. (**b**) Relative closure of BE(2)-C wounds. The average distance moved by the empty vector control and the average (and s.e.) movement of TRIM16 plasmid DNA transfected cells in three independent wounds is shown relative to the percentage of original wound. Three locations of three independent empty vector control and TRIM16 plasmid DNA transfected wounds were photographed at 8, 24 and 48 h post wound. (**c**) Representative phase contrast micrographs of closure of scratch-wounded confluent cultures of empty vector or TRIM16 plasmid DNA transfected BE(2)-C cells at time point immediately after wounding and 24 and 48 h post wounding.

extent in the nucleus of COS-1 and HaCaT cells. The localization of endogenous TRIM16 and vimentin proteins in neuroblastoma cells was determined by confocal microscopy. In Figure 3d, the merged image of vimentin and TRIM16 expression showed that the endogenous proteins were colocalized to the cytoplasm.
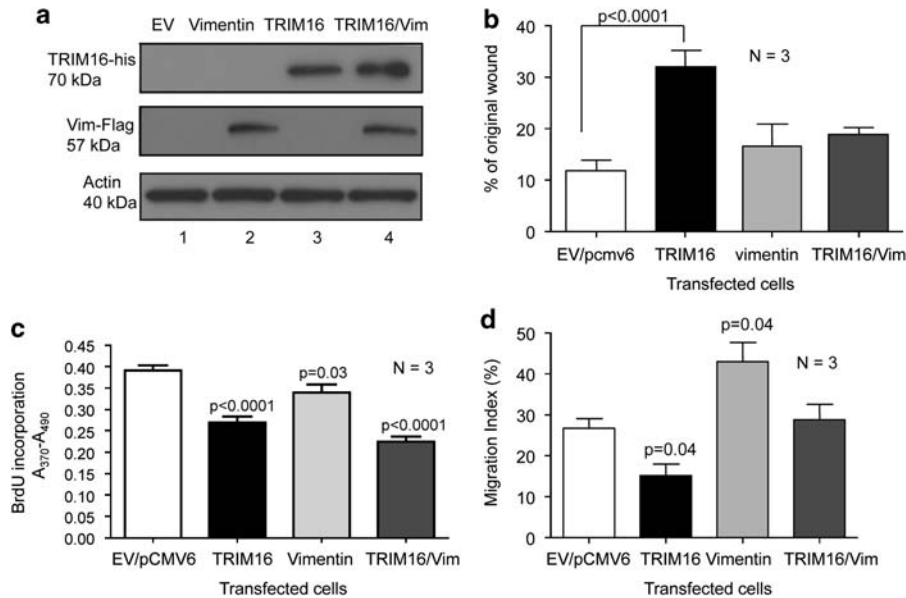
Downregulated vimentin is required for TRIM16 effects on cell motility and migration
We next tested the hypothesis that TRIM16 overexpression may reduce cell motility via its interaction

with vimentin in neuroblastoma cells. We transiently transfected TRIM16 plasmid DNA and empty vector control into BE(2)-C neuroblastoma cells. To optimize transfection conditions, we measured the effects of increasing amounts of plasmid DNA, while maintaining a constant volume of lipofectamine. Maximum efficiency was found to occur between 10 to 15 µg of plasmid per 1×10^6 cells in T75 flasks; 70% of cells were typically TRIM16-myc positive (Figure 4a). The scratch-

Figure 5 Downregulated vimentin is required for TRIM 16 reduction of cell motility and cell migration. (**a**) Lysates of BE(2)-C cells transfected with indicated plasmids (lane 1: pcDNA3.1 and pCMV-6 empty vectors; lane 2: vimentin; lane 3: TRIM16; and lane 4: TRIM16/vimentin) were analysed by immunoblotting using anti-His and anti-Flag antibodies. (**b**) The average distance migrated by the two empty vector controls (lane 1), TRIM16 (lane 2), vimentin (lane 3) and TRIM16/vimentin (lane 4) plasmid DNA-transfected cells in three independent wounds is shown relative to the percentage of original wound. (**c**) BE(2) cells were transfected with different plasmid DNA for 72 h, followed by incubation with 5-bromo-2-deoxyuridine (BrdU) for the last 6 h. BrdU incorporation was measured as OD units of absorbance. (**d**) Invasion assay of BE(2)-C cells through collagen-coated cell culture inserts. The percentage of the migrated cells divided by the total number of cells in the wells from three independent experiments is shown.

wound assays on confluent monolayers were used to investigate cell motility (McInroy *et al.*, 2007). The BE(2)-C cells, which overexpressed TRIM16, remained largely open at multiple time points, while empty vector control cells migrated particularly rapidly in this assay, filling the empty wound space (Figures 4b and c).

To investigate whether repression of vimentin expression by TRIM16 is required for cell motility and proliferation in neuroblastoma cells, we transiently transfected both TRIM16 and vimentin plasmid DNA into BE(2)-C cells for 48 h, and quantified wound closure at 32 h post-scratch. In Figure 5a, the anti-His-tag antibody and anti-Flag-tag antibody were used to confirm exogenous TRIM16 and vimentin overexpression, respectively. Overexpression of TRIM16 caused significantly slower wound closure at 32 h, compared with vimentin transfectants and empty vector control ($P < 0.0001$) (Figure 5b). Significantly, ectopic overexpression of both TRIM16 and vimentin into BE(2)-C cells blocked the inhibitory effect of TRIM16 on wound closure (Figure 5b). In contrast, overexpressed vimentin did not abrogate the inhibitory effect of TRIM16 on cell proliferation (Figure 5c). To investigate the role of TRIM16 and vimentin in neuroblastoma cell migration, we examined the invasiveness of BE(2)-C cells in a Transwell plate migration assay using 10% fetal calf serum as a chemoattractant. As shown in Figure 5d, overexpression of TRIM16 demonstrated slower cell migration at 24 h compared with the empty vector control ($P = 0.04$). In contrast, overexpression of vimentin demonstrated increased cell migration at 24 h compared with empty vector control ($P = 0.04$).

Significantly, ectopic overexpression of both TRIM16 and vimentin into BE(2)-C cells restored the cell migration index to control levels. These data indicate that repression of vimentin expression is specifically required for TRIM16 to exert its inhibitory effects on cell migration.

TRIM16 translocates to the nucleus and decreases nuclear E2F1 levels in neuroblastoma cells
Our LC–MS/MS analysis identified the nuclear transcription factor and cell cycle regulatory protein, E2F1 (Johnson *et al.*, 2006), as a potential TRIM16-binding protein. We hypothesized that TRIM16 might also translocate to the nucleus and directly interact with E2F1 to affect neuroblastoma cell proliferation. To test this hypothesis, BE(2)-C neuroblastoma cells were transiently transfected with TRIM16 plasmid DNA; then, 24 h later, nuclear and cytoplasmic protein fractions were subjected to co-IP with a Myc-tagged antibody, which recognized the transfected TRIM16. Co-IP confirmed that transfected TRIM16 indeed formed a complex with endogenous E2F1 in neuroblastoma cells (Figure 6a). To determine whether TRIM16 regulates E2F1 protein expression in the nucleus, BE(2)-C cells were transiently transfected with TRIM16 plasmid DNA, or treated with 10 μM aRA for 48 h. As shown in Figures 6b and c, the amount of TRIM16 protein in the nucleus was increased in cells after both aRA treatment and TRIM16 transfection, when compared with untreated control cells. Following aRA treatment, TRIM16 exhibited marked nuclear
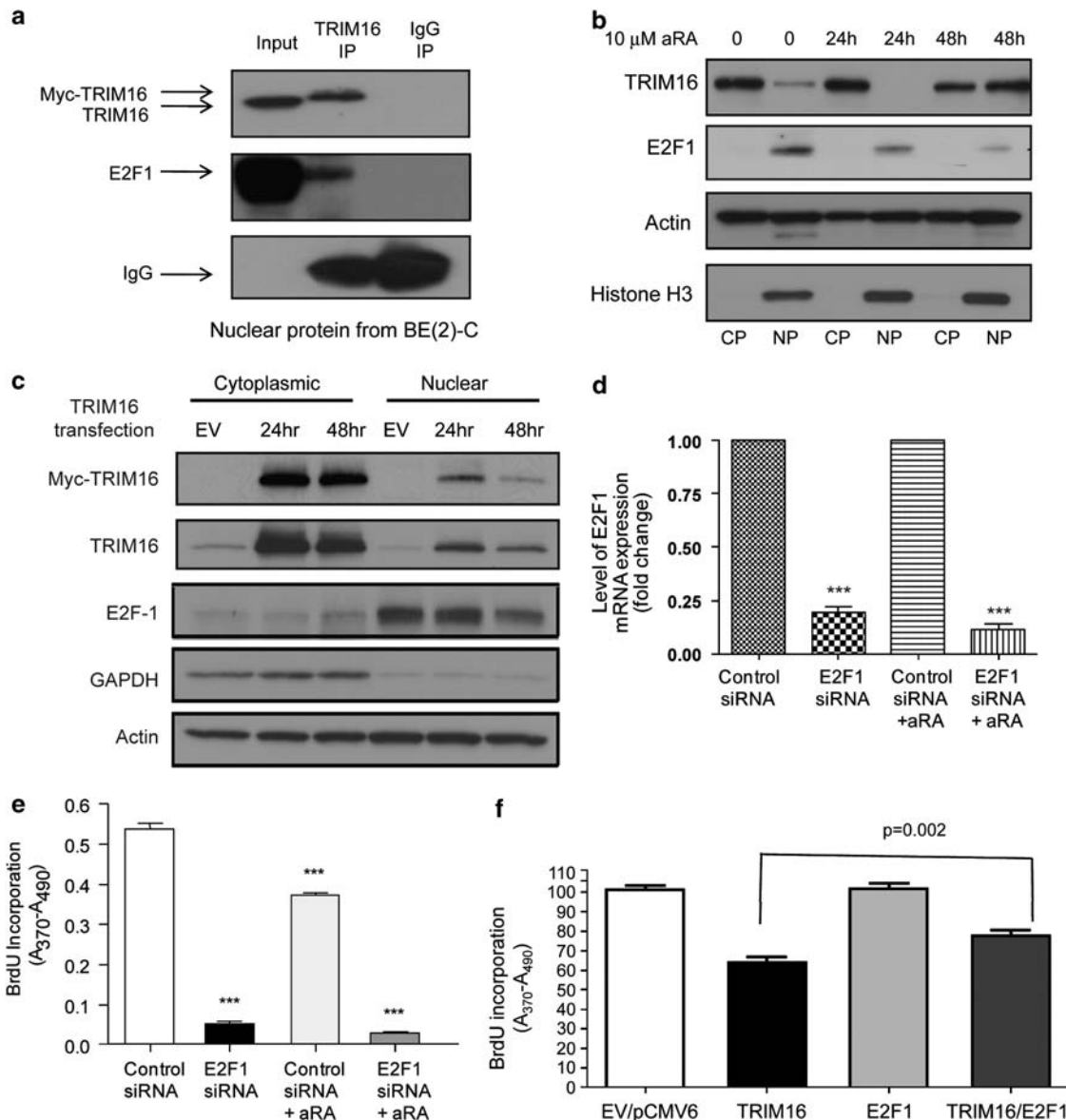
Figure 6 TRIM16 translocates to the nucleus and decreases E2F1 levels in BE(2)-C cells. (**a**) The nuclear protein extract from BE(2)-C cells transfected with TRIM16 plasmids was incubated with either a mouse IgG (right lane) or an anti-Myc antibody (middle lane). The purified IP complex was analysed by western blot using antibodies for TRIM16 and E2F1 proteins. (**b, c**) Immunoblotting analysis of expression of TRIM16 and E2F1 in BE(2)-C cells. Cytoplasmic (CP) and nuclear (NP) proteins of cells treated with $10\,\mu\text{M}$ all-*trans*-retinoic acid (aRA) for 24 and 48 h (Figure 6b) or transfected with TRIM16 plasmid DNA for 24 and 48 h (Figure 6c) were analysed by immunoblotting using anti-TRIM16 and anti-E2F1 antibodies. Anti-actin antibody is used as loading control, anti-histone H3 is used as control for nuclear protein and anti-GAPDH antibody is used as cytoplasmic protein control. (**d**, **e**) BE(2)-C cells were transfected with control siRNA or siRNA specifically targeting E2F1 for 72 h, followed by real-time PCR and 5-bromo-2-deoxyuridine (BrdU) cell proliferation assay. A statistically significant difference (***$P<0.001$) compared with control siRNA. (**f**) BE(2) cells were transfected with different plasmid DNA for 72 h, followed by incubation with BrdU for the last 6 h. BrdU incorporation was measured as OD units of absorbance.

translocation at 48 h, coinciding with a reduction in nuclear E2F1 (Figure 6b). In neuroblastoma cells transfected with TRIM16, there was an even greater increase in overall cellular TRIM16, with some of the excess TRIM16 moving to the nucleus. Interestingly, the transfected nuclear TRIM16 levels fell quickly to basal levels by 48 h post-transfection. Higher nuclear TRIM16 levels post-transfection again correlated with a twofold reduction in nuclear E2F1 levels at 48 h (Figure 6c). The

level of E2F1 mRNA did not change in BE(2)-C cells with overexpression of TRIM16 (data not shown).

To determine whether the higher basal E2F-1 levels in neuroblastoma cells affected cell proliferation, we transiently transfected siRNAs targeting E2F1 or control siRNA into BE(2)-C cells, in the presence or absence of $10\,\mu\text{M}$ aRA for 72 h. E2F1 siRNAs knocked down E2F1 expression and reduced cell proliferation by more than 90% in both aRA-treated and untreated cells
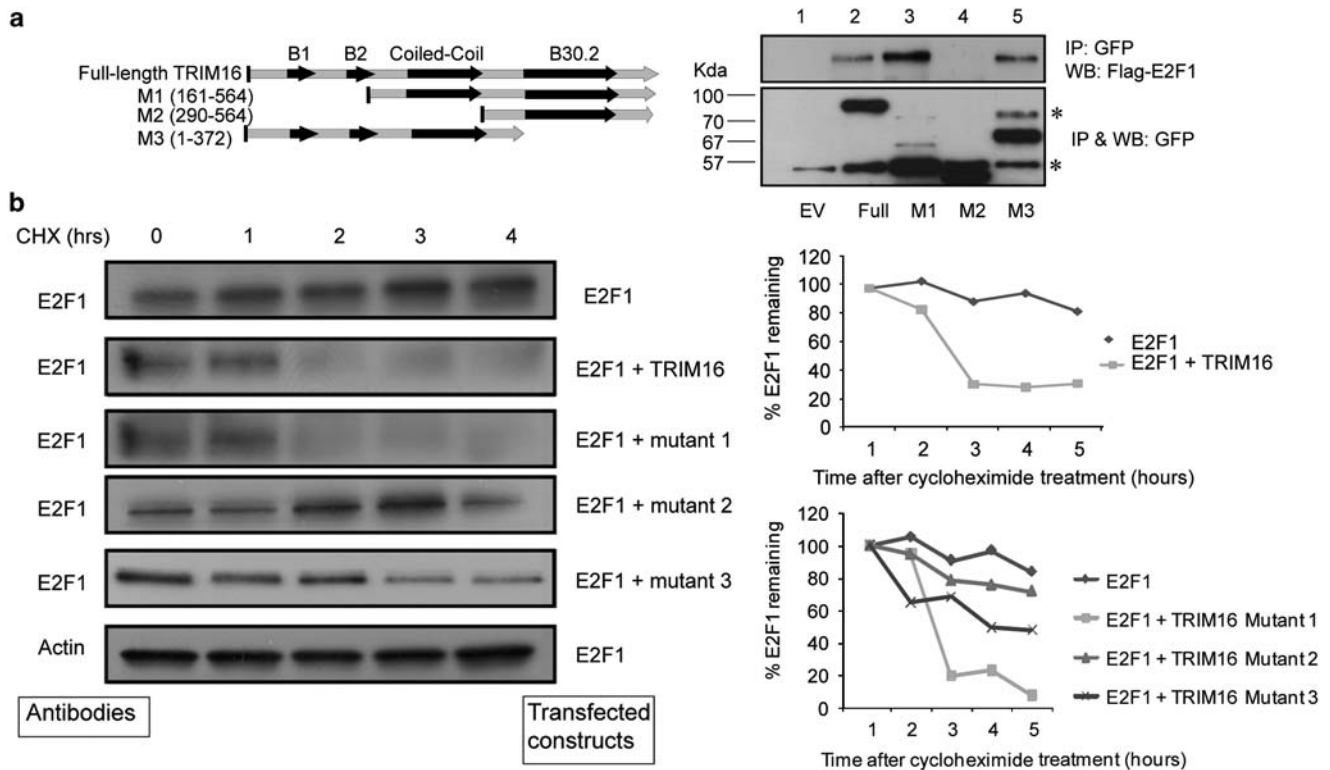
Figure 7 Identification of the interaction domains of TRIM16 and E2F1. (a) Interaction of individual TRIM16 domains with E2F1: Flag-tagged E2F1 and individual domains of TRIM16 were coexpressed in BE(2)-C cells. Flag- and GFP-tagged immunoprecipitates (IPs) were analysed for the presence of Flag- and GFP-tagged constructs (co-IPs) by western blots. Transfection of E2F1 together with the empty vector was used as a control. Non-specific bands are labelled by asterisks. (b) TRIM16 downregulates the protein E2F1 level by reducing the protein's half-life: BE(2)-C cells were transfected either with E2F1 or TRIM16, as well as E2F1 and TRIM16 mutants where indicated. After 16 h the transfected cells were trypsinized, pooled and then replated. After 24 h, cycloheximide was added at a final concentration of 25 μg/ml to the media. At the specified time points the cells were harvested and the protein extracted for analysis by western blots. The western blots were probed with polyclonal rabbit anti-E2F1 or anti-actin antibodies. Actin was used a protein loading control.

(Figures 6d and e). Significantly, ectopic overexpression of both TRIM16 and E2F1 into BE(2)-C cells partially blocked the inhibitory effect of TRIM16 on cell proliferation, compared with TRIM16-transfected cells alone ($P = 0.002$) (Figure 5f). These results suggested that TRIM16's effects on neuroblastoma cell growth required nuclear translocation of TRIM16 protein, binding to E2F1 protein with a consequent reduction in nuclear E2F1 levels.

To map the interaction domains of TRIM16 and E2F1, GFP-tagged TRIM16 domains and Flag-tagged E2F1 were used in co-IP experiments. The coiled-coil domain of TRIM16 is required for TRIM16 and E2F1 interaction, but not the two B-Box domains or the B30.2 domain (Figure 7a). Next, we used the cycloheximide-blocking method to determine whether TRIM16 expression induces proteolysis of E2F1. These experiments were performed by transfecting BE(2)-C cells with plasmids expressing E2F1 or TRIM16 full-length, or TRIM16 deletion mutants. As expected, E2F1 decayed at faster rates in the presence of full-length TRIM16 than in its absence (Figure 7b). The half-life of E2F1 was reduced from greater than 4 h to less than 2 h. We observed that the half-life of E2F1 was also reduced in the presence of TRIM16 mutant 1 and mutant 3, but not

TRIM16 mutant 2, which suggested that the effect of TRIM16 on the degradation of E2F1 was mediated by the coiled-coil domain of TRIM16.

Discussion

Arrested tissue differentiation is a key causal feature of many human cancer types. Differentiation therapy for cancer offers the significant advantages of tissue specificity, high efficacy and low toxicity. A better understanding of the molecular mediators of the differentiation response in cancer cells will lead to an improved therapeutic index compared with general cytotoxic drugs. TRIM16 has an important role in the differentiation response of neuroblastoma cells. As a general rule, TRIM proteins appear to function as a component of large multi-protein complexes (Short et al., 2006). Our previous study had shown that TRIM16 is a nuclear transcription factor with essential effects on the retinoid anticancer signal through transcriptional regulation of retinoic acid receptor-β_2. In this study we show that TRIM16 demonstrates the properties of a tumour-suppressor protein. Surprisingly,

TRIM16 bound key target proteins in both the cytoplasm and nucleus, which lead to reduced levels of cytoplasmic vimentin and nuclear E2F1, and have consequent effects on cell migration and growth, respectively.

Our data demonstrated that in neuroblasts, TRIM16 promoted neuritic differentiation. Neurogenesis involves both proliferation and differentiation. Human neuroblastoma cells show enhanced numbers of neurites and longer processes in response to aRA. The mechanism whereby retinoids regulate neurite outgrowth includes, but is not limited to, the regulation of the transcription of neurotrophin receptors (Encinas *et al.*, 2000). More recent evidence supports a role for aRA in regulating components of other signalling pathways or candidate neurite-regulating factors. Some of these effects, such as that on neuron navigator 2, may be direct, whereas others may be secondary to other aRA-induced changes in the cell (Muley *et al.*, 2008). In this study, our data have demonstrated that overexpression of TRIM16 led to a significant increase in the percentage of neurite-bearing cells during aRA treatment, and suggested that TRIM16 is a novel candidate neurite-regulating factor and may have an important role in retinoid-induced neuronal differentiation.

TRIM proteins form large protein complexes through self-interaction, which is most likely mediated via the B box and coiled-coil domain. However, they also bind to several other proteins (Kim *et al.*, 1996; Urano *et al.*, 2002). In this study, we identified vimentin as a novel binding partner of TRIM16 in neuroblastoma and lung cancer cells. At the protein level, TRIM16 expression correlates inversely with vimentin expression. Vimentin is initially expressed in nearly all neuronal precursors *in vivo*, and is replaced by neurofilaments shortly after the immature neurons become post-mitotic, and vimentin is essential for neuritogenesis in culture (Yabe *et al.*, 2003). In breast cancer cells, the transition from epithelial keratin to mesenchymal vimentin expression marks an important step in the malignant progression of breast cancer cells (Vora *et al.*, 2009). Our data showed that downregulation of vimentin was required for TRIM16's effects on cell motility, indicating that loss of TRIM16 function or expression may increase the invasiveness of neuroblastoma cells. Because overexpression of TRIM16 did not change the vimentin mRNA expression level, it is probable that TRIM16 regulated vimentin at the post-translational level. In the scratch wound assay, overexpression of vimentin alone did not affect wound filling due to the presence of abundant endogenous vimentin in the cells. When both proteins are co-expressed, overexpression of TRIM16 downregulates endogenous vimentin expression, and therefore, overexpressed vimentin interferes with TRIM16's effect on cell mobility, but not with its effect on cell proliferation.

Similar to PML and hemopoietic lineage switch 5, which are members of the TRIM protein family (Mu *et al.*, 1997; Lalonde *et al.*, 2004), TRIM16 was able to reduce the growth of neuroblastoma cells, inhibit colony formation and suppress tumour development, indicating that TRIM16 is a tumour suppressor protein. Elevated

levels of TRIM16 can reduce cell growth, indicating that TRIM16 might directly regulate the cell cycle machinery. As a step towards understanding the role of TRIM16 in cell cycle progression, E2F1 was identified as a novel TRIM16 binding partner and its expression is inversely correlated with TRIM16 expression in the nucleus. The E2F family of transcription factors are centrally important in mammalian cell division. E2Fs are also targets of a variety of oncogenes and tumour suppressor genes (Trimarchi *et al.*, 2002). Therefore, it is likely that the TRIM16 and E2F1 interactions are linked to TRIM16's tumour suppression function. Furthermore, the E2F transcription factor is best known for its ability to regulate cell cycle progression by coordinating a large group of genes involved in G1-to-S phase transition. The hypophosphorylated retinoblastoma protein (pRb) repressed E2F activity by shielding the E2F transactivation domain, leading to restriction of cell cycle progression (Weinberg, 1995). Our previous data provided evidence that TRIM16 overexpression correlated with inhibited cell growth through its effects on cyclin D1 and phospho-Rb (Raif *et al.*, 2009). As deregulation of E2F transcriptional activity due to alterations in the p16(INK4a)–cyclin D1–Rb pathway is a hallmark of human cancer (Johnson *et al.*, 2006), our findings suggest that TRIM16 may have a critical role in cell cycle progression, and that the effect of TRIM16 on cell proliferation may be through translocation to the nucleus, interaction with E2F1 and downregulation of nuclear E2F1 in neuroblastoma cells.

The TRIM/RBCC proteins self-associate, mainly through their coiled-coil region. Homo-interaction results in the formation of large protein complexes, which identify different discrete nuclear and/or cytoplasmic sub-cellular compartments (Reymond *et al.*, 2001). The presence of the RING domain and its strong association with ubiquitination suggest a role for this protein family in the ubiquitin-mediated proteolysis. The RING finger domain has been found to have a critical role in mediating the transfer of ubiquitin both to heterologous substrates, as well as to the RING proteins themselves. This domain is therefore a characteristic signature of many E3 ubiquitin ligases (Joazeiro and Weissman, 2000). TRIM16 is missing the RING finger domain, but it has both B-box1 and B-box2, which share a similar but distinct pattern of cysteine and histidine residues (Borden *et al.*, 1993). Accumulating evidence suggests that B-boxes mediate protein–protein interactions specific to each TRIM class (Peng *et al.*, 2000; Short *et al.*, 2002; Mrosek *et al.*, 2008). It is unclear how overexpressed TRIM16 reduced vimentin and E2F1 protein levels. It is possible that TRIM16 acts as an E3 ubiquitin ligase, and targets these proteins for proteolysis and promotes growth inhibition in neuroblastoma cells.

TRIM16 is expressed in most normal and cancer cells, but is mainly localized to the cytoplasmic component of cancer cells. In this study we show that TRIM16 acts as a tumour suppressor by its inhibitory effects on proteins in both the nuclear and cytoplasmic compartments. These data suggest that TRIM16 may have transactiva-

tion-dependent effects and transactivation-independent effects. In agreement with our observations are the recent findings that p53 can act as a tumour suppressor in both cytoplasmic and nuclear compartments of cancer cells. In addition to being a nuclear transcription factor regulating genes involved in apoptosis and cell cycle, p53 has additional activities in the cytoplasm, where it triggers apoptosis and inhibits autophagy (Green *et al.*, 2009).

As TRIM16 has been demonstrated to suppress tumour growth, cell migration and sensitize cancer cells to retinoids, our findings highlight TRIM16 as a potential drug development target for the treatment of cancers overexpressing vimentin and E2F1 proteins. Agents that increase the cytoplasmic and/or nuclear levels of TRIM16 might be expected to inhibit cell migration and replication, respectively, and, moreover, enhance the anticancer effects of differentiation therapies, such as retinoids.

Materials and methods

Plasmid construction and stable transfection
The TRIM16 full-length cDNA was subcloned into the pcDNA3.1(−)/myc.His vector at the *Eco*RI multi-cloning site (Cheung *et al.*, 2006). The TRIM16-overexpressed BE(2)-C stable cell lines were established with transfection of pcDNA3.1(−)/TRIM16/myc-His vector or the pcDNA/myc-His empty vector. The two clones of overexpression of TRIM16 (TRIM16-4 and TRIM16-5) and two empty vector clones (EV-1 and EV-8) were selected for further studies.

The TRIM16-GFP deletion mutants were produced by OriGene (Rockville, MD, USA). The deletion mutants were synthesized by PCR of full-length TRIM16 cDNA (NM_006470.3). The final mutants were in the pCMV6-AC-GFP vector. The mutant products correspond to the following amino acid (AA) sequences; AA 1–564 (full-length), AA 161–564 (M1), AA 290–564 (M2) and AA 1–372 (M3).

Immunohistochemistry
Paraffin-embedded section of neuroblastoma patients' tissue was obtained from Paediatric Anatomical Pathology, South Eastern Area Laboratory Services (SEALS). The slides were incubated with rabbit anti-TRIM16 (Bethyl Laboratories, Montgomery, TX, USA) at $1\,\mu g/ml$ or rabbit anti-class III β-tubulin at 1 in 1000 dilution overnight at $4\,^{\circ}C$. The secondary antibody used was goat anti-rabbit immunoglobulin/biotinylated (Dako, Glostrup, Denmark) at 1 in 500 dilutions for 1 h at room temperature. The biotinylated antibody was then labelled with streptavidin-HRP (Dako) for 45 min at room temperature. The sections were developed with 3,3′-diamino-benzidine tetrahydrochloride (Dako) and counterstained with haematoxylin.

Immunohistochemistry quantification
Among the 21 neuroblastoma tumours, there were three in stage I, six in stage II, four in stage III, seven in stage IV and one in stage IVS, by the Evans staging system (Evans *et al.*, 1971). The positive staining of TRIM16 was analysed on digitally captured images using the Aperio ScanScope XT slide scanner (Aperio Technologies, Vista, CA, USA) (Brennan *et al.*, 2008; Rexhepaj *et al.*, 2008). At least three of each differentiated ($\geqslant 5\%$ of neuroblasts toward ganglion cells) and

poorly differentiated regions ($\leqslant 5\%$ of neuroblasts toward ganglion cells) with an area of minimum $10\,000\,\mu m^2$ were selected and analysed using colocalization algorithms with ImageScope (Aperio Technologies) (Joshi, 2000).

Tumourigenicity assays
Tumourigenicity was determined by injecting 5×10^6 of TRIM16 or empty vector stably tranfected BE(2)-C cells subcutaneously into 6–8-week-old female BALB/c nude mice. Tumour volumes were measured twice a week using callipers, and the tumour mass was determined after 4 weeks post engraftment.

Transient transfection with plasmid DNA or siRNA
Cells (1×10^6) were transfected with either $15\,\mu g$ of TRIM16 expression vector or empty vector for 72 h before harvesting for transient transfection experiments. Cells were transfected with either 20 nM control siRNA (Dhamarcon, Lafayette, CO, USA), or siRNA specific to TRIM16 (Ambion, Foster City, CA, USA) or E2F1 (Dhamarcon) before harvesting for siRNA gene knockdown experiments.

Confocal fluorescence microscopy images
The BE(2)-C cells were fixed in 10% formalin, and then non-specific staining was blocked in 10% fetal calf serum/phosphate-buffered saline. The cells were incubated in 5% fetal calf serum/phosphate-buffered saline with goat anti-vimentin antibody (1:400) and custom TRIM16 rabbit antibody (1:100). The cells were then incubated with Alexa Fluor 555 anti-goat IgG (1:1000) and Alexa Fluor 488 anti-rabbit IgG (1:1000), the cells were then mounted in VectaShield with 4′,6-diamidino-2-phenylindole. Images were collected with a Zeiss LSM 510 META confocal fluorescence microscope (Zeiss, Jena, Germany) using a $\times 63$ oil Plan-apochromat objective (NA, 1.4; Zeiss).

Scratch wound assays and migration assay
For scratch wound assays, BE(2)-C cells were transfected with either $15\,\mu g$ of TRIM16 expression vector or empty vector with Lipofectamine 2000. For the double transfection assays, $10\,\mu g$ of TRIM16 expression vector and $10\,\mu g$ of vimentin expression vector were transfected into the cells. After 48 h from transfection, cells were grown to 90–100% confluence and wounded using $20\,\mu l$ pipette tips, and the distance between the wound was measured with Image J software (National Institutes of Health, Bethesda, MD, USA) at various time points.

For migration assay, BE(2)-C cells were serum starved for 2 h and then 4×10^4 cells were seed on Transwell inserts pre-coated with collagen IV on the underside of the insert. Transwell inserts were placed in companion plates containing media with 10% fetal calf serum and the cells were allowed to migrate at $37\,^{\circ}C$. The migration index is the percentage of the migrated cells divided by the total number of cells in the wells.

Immunoprecipitation assays and western blots
Lysates were immunoprecipitated with anti-TRIM16 (Bethyl Laboratories) or anti-myc tag antibody (Cell Signalling Technology, Danvers, MA, USA). Anti-vimentin antibody, anti-E2F1 antibody and anti-Myc tag antibody 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.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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