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Overexpression of TRIB2 in human lung cancers contributes to tumorigenesis through downregulation of C/EBP α

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Abstract

Lung cancer is the most common cause of cancer-related mortality worldwide. Here, we report elevated expression of tribbles homolog 2 (TRIB2) in primary human lung tumors and in non-small cell lung cancer cells that express low levels of differentiation-inducing transcription factor CCAAT/enhancer-binding protein alpha (C/EBP α). In approximately 10–20% of cases, elevated *TRIB2* expression resulted from gene amplification. TRIB2 knockdown was found to inhibit cell proliferation and *in vivo* tumor growth. In addition, TRIB2 knockdown led to morphological changes similar to C/EBP α overexpression and correlated with increased expression and activity of C/EBP α . TRIB2-mediated regulation of C/EBP α was found to occur through the association of TRIB2 with the E3 ligase TRIM21. Together, these data identify TRIB2 as a potential driver of lung tumorigenesis through a mechanism that involves downregulation of C/EBP α .

Keywords

tribbles; lung; C/EBP α ; TRIM21

Introduction

Lung cancer is currently the leading cause of death from all cancers in the United States (Jemal *et al.*, 2010). Despite the use of aggressive medical therapy, non-small cell lung cancers (NSCLCs), a major subset of lung cancers, have an overall 5-year survival rate of less than 15% (Jemal *et al.*, 2007). Recently, the cancer stem cell hypothesis has been applied extensively to solid tumors, such as lung tumors, in addition to hematopoietic cancers (Al-Hajj *et al.*, 2003; Hemmati *et al.*, 2003; Singh *et al.*, 2003; Galli *et al.*, 2004; Bapat *et al.*, 2005; Collins *et al.*, 2005; Fang *et al.*, 2005; Dalerba *et al.*, 2007; O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007; Eramo *et al.*, 2008; Schatton *et al.*, 2008). As such, much

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Conflict of interest

The authors declare no conflict of interest.

research has gone into uncovering the different populations of progenitor cells within the lung. These are considered to be a rare population of undifferentiated cells with enhanced ability to proliferate, self-renew, and eventually differentiate into phenotypically diverse and heterogeneous cell populations (Kim *et al.*, 2005).

The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors is known to affect different aspects of development and differentiation in the lung (Cassel and Nord 2003). In particular, expression of C/EBP α has been shown to be crucial for lung development, as knockout mice exhibit massive hyperproliferation of type II pneumocytes and do not survive after birth (Flodby *et al.*, 1996). C/EBP α may act as a tumor suppressor in the lung, as its overexpression in lung tumor cells results in a dramatic decrease in cell proliferation (Halmos *et al.*, 2002), and it is often downregulated in NSCLC (Halmos *et al.*, 2002; Costa *et al.*, 2007). Work done by Pear and colleagues showed that the human homolog of the *Drosophila* tribbles protein, TRIB2, associates with and inhibits the transcriptional activity of C/EBP α (Keeshan *et al.*, 2006; Dedhia *et al.*, 2010). Additionally, overexpression of TRIB2 induces acute myelogenous leukemia in mice through inactivation of C/EBP α (Keeshan *et al.*, 2006), and downregulation of the tumor suppressor gene *FOXO3a* is mediated by TRIB2 in human melanoma cells (Zanella *et al.*, 2010).

Based, in part, on these reports, we examined the roles of tumor-initiating cells and TRIB2 in lung tumor oncogenesis. Our results show that TRIB2 is overexpressed and often amplified in lung cancer. Upon short hairpin RNA (shRNA)-mediated knockdown of this putative oncogene, tumor cells lines ceased proliferation and failed to produce tumors *in vivo* when injected into non-obese diabetic/severe combined immunodeficient mice (NOD/SCID) mice. Finally, we show that TRIB2-mediated downregulation of C/EBP α requires the presence of the E3 ligase TRIM21.

Results

TRIB2 is overexpressed and amplified in a subset of human lung cancers

It has been established that growth of cell lines under non-adherent spheroid conditions can increase their tumor-initiating capabilities (for example Ricci-Vitiani *et al.*, 2007; Todaro *et al.*, 2007). To confirm that this was also true for lung cancer cell lines, NCI-H1650 cells that had been grown as monolayers or as spheroids were injected into NOD/SCID mice and tumor growth was measured over time. Cells grown as spheroids were more efficient at initiating tumorigenesis than the corresponding number of cells grown as monolayers (Supplementary Figure S1A). From this data and supporting literature, we reasoned that a gene upregulated in cancer cells grown as spheroids might be instrumental in lung tumorigenesis. To identify potential drivers of lung tumorigenesis, microarray expression analysis was performed comparing the transcriptional profile of eight NSCLC cell lines grown either as monolayers or as spheroids. From this transcriptional analysis, TRIB2 was identified as significantly overexpressed in the spheroid population in eight out of eight NSCLC cell lines examined ($P < 0.05$; Supplementary Table S2). TRIB2 overexpression in the spheroid population ranged from 2 to 76-fold over that of the corresponding monolayer culture. Using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis, TRIB2 expression levels in the NSCLC cell lines propagated either as monolayers or non-adherent spheroids were confirmed. There was a significant increase in TRIB2 expression levels in all cell lines grown as spheroids relative to monolayer conditions (Figure 1a).

Based upon these results, we hypothesized that TRIB2 may have a role in human lung tumorigenesis. The expression pattern of TRIB2 in 68 human primary lung tumor samples was subsequently examined using microarray-based mRNA expression analyses. mRNA

expression levels were normalized to the corresponding control lung tissue before comparison of TRIB2 levels between tumors. TRIB2 was two- to six-fold overexpressed in 29.4% (20 of 68) of bulk, unsorted primary lung tumor samples (Figure 1b and Supplementary Table S1). The variation in TRIB2 expression levels between primary tumor samples could partially be attributed to the percentage of stem-like cells present within the tumor tissue. Immunohistochemical examination of the tumor samples revealed that for some samples, TRIB2 was overexpressed in a high percentage of cells throughout the tumor, whereas for other samples, high TRIB2 expression was localized to a subset of cells within the tumor tissue, and was relatively low in the surrounding tissues (Figure 1c and Supplementary Figure S1B). In the latter instances, overall TRIB2 expression appeared to be relatively low, but in fact, there were localized regions with intense staining. The subset of cells exhibiting high TRIB2 expression levels may represent tumor-initiating cell populations (Figure 1c and Supplementary Figure S1B).

The *TRIB2* gene resides at 2p24, a chromosomal locus prone to frequent breaks and amplifications (Durkin and Glover, 2007). We examined *TRIB2* gene copy number in primary human samples to determine whether increased gene copy number could provide an additional explanation for increased TRIB2 expression. Using single-nucleotide polymorphism analysis, gene amplification surrounding the TRIB2 locus was observed in 8 of the 68 samples (12%) previously analyzed (Supplementary Figure 1C). In addition, quantitative PCR analysis of an independent cohort of tumor samples revealed significant and specific gene amplification of 7- to 10-fold at the TRIB2 locus when normalized to non-cancerous counterparts for 3 of 14 (21%) tumor samples analyzed (LT4, LT6 and LT12; Figure 1d). Further, *TRIB2* gene amplification was confirmed by fluorescence *in situ* hybridization analysis in both lung cancer cell lines and in LT12 (Figure 1e). Specific amplification of the *TRIB2* locus was observed in LC-1/sq-SF lung cancer cells, whereas the elevated level of TRIB2 in H2228 cells was found to be due to polyploidy. The lung tumor samples examined were generally diploid, and *TRIB2* gene copy numbers roughly matched the values obtained by quantitative PCR (Figure 1d). These results indicate that TRIB2 is overexpressed in a subset of lung tumors (around 30%), and that in approximately 10–20% of cases, the overexpression stems from gene amplification.

Depletion of TRIB2 gene expression inhibits proliferation, sphere formation and *in vivo* tumorigenicity

Based upon observations that overexpression of TRIB2 has a role in cellular transformation (Keeshan *et al.*, 2006; Dedhia *et al.*, 2010; Zanella *et al.*, 2010), the effect of modulating TRIB2 levels using shRNA constructs specific for TRIB2 (sh1–4) or a non-targeting control (shNT) was examined. TRIB2 expression was efficiently depleted using independent shRNA constructs (Figure 2a and Supplementary Figure S2A) in multiple cell lines. Two shRNA constructs were chosen for further analysis, as they consistently exhibited the greatest level of TRIB2 knockdown (Figure 2a). TRIB2 sh1 is directed against the 3' non-coding region of TRIB2, whereas TRIB2 sh2 is directed against the coding sequence of TRIB2. Both constructs consistently decreased mRNA levels by ~75% (Supplementary Figure S2A).

Depletion of TRIB2 in two NSCLC lines, NCI-H226 and NCI-H1650 (H226 and H1650, respectively, hereafter), resulted in significant inhibition of cell proliferation (Figure 2b). This was accompanied by a modest increase in apoptosis (Supplementary Figure S2B). The effect of loss of TRIB2 expression on the ability of cells to grow under low-adherence conditions was also examined. Following knockdown of TRIB2 expression, the ability of cells to quickly re-form spheroids was slightly impaired, and there was marked inhibition of sphere formation during long-term culture (Supplementary Figure S2C). Importantly, the reduction in cell proliferation appears to be specific for transformed cells, as TRIB2

knockdown in normal lung fibroblast and epithelial cells did not inhibit proliferation (Supplementary Figure S2D–E). Together, these data suggest that TRIB2 specifically affects the proliferative capacity of lung cancer cells, and may have a role in cancer stem cell self-renewal.

To more closely mimic the tumor microenvironment, the tumorigenic capacity of cells in which TRIB2 expression had been depleted was assayed *in vivo* by injection into NOD/SCID mice. H226 or H1650 cells with confirmed knockdown phenotypes were embedded in collagen or matrigel, and injected subcutaneously. Mice injected with mock-infected cells quickly developed large tumors, as did cells expressing non-targeting shRNA after a lag period. In contrast, mice injected with cells in which TRIB2 had been knocked down remained tumor free for up to 240 days after transplant (Figure 2c), at which time the experiment was terminated.

To confirm that inhibition of cell proliferation was indeed due to loss of TRIB2 expression, we performed rescue experiments in which shRNA-resistant TRIB2 cDNAs were overexpressed. Constructs were generated for TRIB2 that either lack the 3' non-coding region (Trib2s) and thus are not targeted for degradation by sh1, or are mutated such that they are no longer targeted for degradation by sh2 (Trib2m). The *in vitro* overexpression of Trib2m protein in lung cancer cell lines stably expressing sh2 was sufficient to rescue inhibition of cell proliferation (Figure 3a, Supplementary Figure S3). Similar results were obtained upon rescue of sh1-mediated TRIB2 knockdown with Trib2s (data not shown). We then examined whether introduction of Trib2s and Trib2m *in vivo* could overcome the antitumorigenic effect of TRIB2 knockdown. H226 or H1650 cells stably expressing sh1 or sh2 were infected with Trib2s or Trib2m, respectively, imbedded in collagen or matrigel and injected into NOD/SCID mice. Overexpression of Trib2m or Trib2s in the presence of shRNA directed against TRIB2 resulted in rapid tumorigenesis (Figure 3b and data not shown). Thus, both *in vitro* and *in vivo* inhibition of lung cancer tumor cell proliferation were specifically caused by inhibition of TRIB2 expression.

TRIB2 knockdown correlates with increased expression and activity of C/EBP α

Previously published work has suggested the existence of an interaction between C/EBP α and TRIB2 (Keeshan *et al.*, 2006; Dedhia *et al.*, 2010), and has shown that C/EBP α is expressed in lung cells during development (Flodby *et al.*, 1996). When TRIB2 expression levels are decreased, several lung cancer cell lines exhibit dramatic phenotypic changes (Figure 4a). As TRIB2 has been shown to be responsible for both downregulation of C/EBP α and inhibition of cell differentiation (Keeshan *et al.*, 2006; Naiki *et al.*, 2007; Dedhia *et al.*, 2010), we investigated whether the morphological changes observed upon TRIB2 knockdown in lung cancer cell lines could be due to increased levels of C/EBP α . Indeed, overexpression of C/EBP α in H226 cells resulted in morphological changes similar to those seen upon TRIB2 knockdown (Figure 4a). To further characterize these changes, immunofluorescence was performed using various lung lineage markers (Kim *et al.*, 2005). Parental H226 and H226shNT cells retained multiple lineage markers indicative of a more primitive cell type, whereas cells that had been infected with sh1 or sh2 to knockdown TRIB2 expression became restricted to either the alveolar type I or II lineages (Supplementary Figure S4A). This result is consistent with the reported role of C/EBP α as a necessary factor for differentiation during development (Flodby *et al.*, 1996), and suggests that upon TRIB2 knockdown, C/EBP α levels increase and drive lung cancer cell differentiation.

As the phenotypic changes observed upon TRIB2 knockdown were similar to the phenotypic changes induced by C/EBP α overexpression in lung cancer cell lines (Figure 4a), C/EBP α levels were also examined in primary lung tumor samples. Pearson product-

moment correlation analysis of microarray data of the same 68 primary samples detailed in Figure 1 indicates a negative correlation between TRIB2 and C/EBP α expression (-0.306 , $P < 0.02$; Supplementary Figure S4B). To confirm this correlation, qRT-PCR was performed on an independent cohort of primary lung tumor cDNAs, and a significant inverse correlation between TRIB2 and C/EBP α expression levels was again observed (Figure 4b). This indicates that, similar to mouse acute myelogenous leukemia studies (Keeshan *et al.*, 2006; Dedhia *et al.*, 2010), TRIB2 expression might affect the level of C/EBP α expression in human lung cancer.

To determine whether TRIB2 also affects the transactivation capabilities of C/EBP α , a transient transfection system in which forced expression of C/EBP α leads to luciferase reporter activation was employed. Specifically, a stable 293T cell line that expresses a luciferase reporter behind a $4 \times$ C/EBP-binding site was generated, and transient transfections of TRIB2, C/EBP α or both species simultaneously were performed. Transfection of the reporter with C/EBP α alone led to reporter activation approximately 10-fold greater than background levels (Figure 4c). In contrast, cotransfection of both C/EBP α and TRIB2 reproducibly resulted in a three-fold decrease in reporter activation (Figure 4c). Although there was a reduction in reporter activity, C/EBP α protein levels were not affected (Figure 4c). Thus, although coexpression of TRIB2 and C/EBP α leads to a decrease in C/EBP α transactivation activity, it is not due to degradation of C/EBP α .

To further validate the observation that TRIB2 expression affects C/EBP α function, the expression levels of C/EBP α target genes were examined after modulation of TRIB2 levels. After knockdown of expression of TRIB2 using shRNA constructs was confirmed using qRT-PCR and upregulation of C/EBP α was confirmed by western blotting (Figure 4d and Supplementary Figure S4C, respectively), expression of the C/EBP α target genes *GCSFR* (*CSF3*; Smith *et al.*, 1996) and *NE2* (Oelgeschlager *et al.*, 1996) were examined (Figure 4d). The expression levels of both C/EBP α target genes were increased as much as 5- to 10-fold when TRIB2 expression levels were reduced. This finding further supports the hypothesis that expression of TRIB2 inhibits C/EBP α activity.

TRIB2 interacts with TRIM21 and leads to degradation of C/EBP α

The ability of TRIB2 to affect C/EBP α activity could occur through at least two mechanisms. TRIB2 might bind C/EBP α directly and prevent its transactivation capabilities. Alternatively, the ability of TRIB2 to modulate expression of C/EBP α has been hypothesized to occur *via* targeting of the transcription factor for proteasomal degradation (Keeshan *et al.*, 2006; Dedhia *et al.*, 2010), although the exact mechanism remains unknown. To distinguish between these two possibilities and identify protein interactors of TRIB2, immunoprecipitation– mass spectrometry was performed. H1650 cells were retrovirally transduced with a C-terminal FLAG-6xHis dual-affinity-tagged TRIB2 or with a TRIB2 construct devoid of the C-terminal dual-affinity tag as a negative control. Clarified lysates were subjected to dual-affinity purification, and bound proteins were identified by mass spectrometry. The E3 ubiquitin ligase TRIM21 (also called Ro52; (Wada and Kamitani 2006; Wada *et al.*, 2009)) was clearly identified in the affinity-tagged construct sample but not observed in the negative control experiment (Supplementary Table S3). Coprecipitation of TRIB2 and TRIM21 in TRIB2-overexpressing H226 cells confirmed the TRIB2/TRIM21 protein interaction (Figure 5a). This, in combination with our results and previously published results detailing the regulation of C/EBP α by TRIB2 (Keeshan *et al.*, 2006; Dedhia *et al.*, 2010), suggests that TRIM21 might be an E3 ubiquitin ligase responsible for targeting C/EBP α for degradation in lung cancer, and that interaction between TRIM21 and TRIB2 is important to this process.

To test the hypothesis that TRIM21 has a role in regulation of C/EBP α levels, a 293T transient transfection system was utilized. Cotransfection of C/EBP α and TRIB2 in the absence of TRIM21 resulted in very little or no degradation of C/EBP α (Figure 5b, lane 1). Likewise, cotransfection of TRIM21 and C/EBP α resulted in little or no change in C/EBP α levels (Figure 5b, lane 3). However, cotransfection of all three species led to a significant decrease in C/EBP α levels (Figure 5b, lane 2). The apparent reduction in TRIM21 expression may be due to TRIM21 autoubiquitination and subsequent degradation (Sabile *et al.*, 2006). Taken together, these results suggest that both TRIB2 and TRIM21 are necessary for modulation of C/EBP α levels.

Discussion

The transcription factor C/EBP α has been shown to be a major player in lung differentiation, and recent reports have suggested that loss of C/EBP α expression may be instrumental in driving the formation of lung adenomas (Koschmieder *et al.*, 2009). Although loss of the chromosomal region surrounding C/EBP α is a common feature of up to 50% of NSCLCs, sequencing studies by several groups failed to identify mutations within the coding region of the gene itself, suggesting an alternative mechanism for C/EBP α downregulation (Koschmieder *et al.*, 2009). One possible mechanism may involve proteasomal degradation of the transcription factor driven by expression of TRIB2 (Grosshans and Wieschus 2000; Mata *et al.*, 2000; Seher and Leptin 2000; Naiki *et al.*, 2007; Dedhia *et al.*, 2010; Keeshan *et al.*, 2006, 2010). The results presented here support this hypothesis, and further suggest that TRIM21 may be the E3 ubiquitin ligase necessary for C/EBP α degradation. Additionally, the juxtaposition of the reporter data (Figure 4c) and the C/EBP α degradation data (Figure 5b) suggest that although TRIB2 alone is sufficient to block the transcriptional activity of C/EBP α , TRIM21 expression is necessary for downregulation of C/EBP α levels.

Formation of the TRIB2/TRIM21 complex and subsequent downregulation of C/EBP α suggest that TRIB2 may function as a molecular adapter, facilitating assembly of a TRIM21 E3 ligase complex and aiding in the subsequent ubiquitylation and degradation of its substrates, such as C/EBP α . A similar property for the protein kinase dual specificity tyrosine phosphorylation regulated kinase 2 (DYRK2) has been identified, in which DYRK2 functions as a scaffold that mediates assembly of the EDVP E3 ligase complex (Maddika and Chen, 2009). Depletion of DYRK2 by small interfering RNA disrupts formation of the EDVP complex. Additionally, the kinase activity of DYRK2 is necessary for substrate ubiquitylation and degradation by the EDVP complex. TRIB2 may perform a scaffolding function similar to DYRK2, although it may not possess the phosphorylation capabilities, as TRIB2 has been proposed to be a pseudokinase (Hegedus *et al.*, 2007).

An interaction between TRIB2 and the E3 ligase COP1 has previously been reported (Dedhia *et al.*, 2010; Keeshan *et al.*, 2010). The apparent ability of TRIB2 to bind to multiple E3 ligase proteins also supports the hypothesis that TRIB2 functions as a molecular scaffold, and is involved in many processes including C/EBP α degradation *via* the proteasome. Using an unbiased immunoprecipitation–mass spectrometry approach, we did not observe interaction between TRIB2 and COP1 in lung cancer cell lines, even though COP1 was expressed in those cell lines (data not shown). Perhaps the interaction of TRIB2 with different E3 ligases is context dependent, with the TRIB2/COP1 interaction involved in hematopoietic tumor generation, whereas TRIB2/TRIM21 is involved in solid tumor formation such as lung tumors.

Based on our results and those of others (Keeshan *et al.*, 2006; Dedhia *et al.*, 2010), we propose the following hypothesis for TRIB2 protein interactions and subsequent activities:

C/EBP α functions as a transcription factor in the absence of TRIB2 (Figure 5ci). When TRIB2 is present and bound to C/EBP α , transcriptional activity is lost, possibly because of its inability to bind DNA (Figure 5cii). Upon recruitment of TRIM21 to the TRIB2:C/EBP α complex, C/EBP α is degraded in a proteasome-dependent manner (Figure 5ciii). Thus, when TRIM21 is present, increased TRIB2 expression will result in a decrease in CEBP α levels. Moreover, as C/EBP α has been shown to undergo autoregulation (Timchenko *et al.*, 1995), decreased levels of the protein will lead to continued downregulation at the mRNA level.

As TRIM21 appears to mediate the effects of TRIB2, modulation of TRIM21 should phenocopy TRIB2 modulation. However, it has recently been shown that loss of TRIM21 results in the induction of a massive cytokine response, leading to cell death (Yoshimi *et al.*, 2009). Thus, we were unable to confirm the expected phenocopy (data not shown).

Here, we described the role of TRIB2 as a potential oncogene in human lung cancers. We found that the gene is enriched in cells grown as spheroids and, importantly, is overexpressed in a significant percentage of primary lung tumors. Further, we found that this overexpression could be due to genomic amplification of the gene. This finding could enable both better diagnostics and screening for lung cancer as well as improved predictions of lung cancer outcomes. Additionally, inhibition of overexpressed and/or amplified TRIB2 in lung cancers could provide a possible means of treatment, as our results indicate that reducing levels of TRIB2 can efficiently inhibit tumor growth. Together, our findings indicate that overexpression of TRIB2 inhibits the expression of C/EBP α through multiple mechanisms, including transcriptional inactivation and protein degradation, and could consequently lock cells in an undifferentiated, tumor-initiating cell state.

Materials and methods

Primary lung tumor samples, cell proliferation and tumorigenicity

The human primary tumor samples utilized in this study consisted of matched pairs including a bulk tumor sample and adjacent non-tumorous lung tissue from the same patient. They were obtained from Asterand (Detroit, MI, USA; Supplementary Table S1), with approval from the appropriate institutional review boards. Total RNA, complementary DNA (cDNA) or genomic DNA were either obtained directly from Asterand (Figure 1b, Supplementary Figure S1C and S4B) and Origene (Rockville, MD, USA; Figure 4b) or were extracted from bulk tissue samples and used to generate DNA (Qiagen, Valencia, CA, USA; Figure 1d). To assess cell proliferation, cells were seeded at 5000 cells/well in 96-well tissue culture plates and analyzed using CellTiterGlo (Promega, Madison, WI, USA). Tumor growth studies in NOD/SCID mice were performed according to the National Institutes of Health guidelines with protocols approved by the institutional review board of the Genomics Institute of the Novartis Research Foundation.

Constructs and lentiviruses

A 1032-bp fragment encoding the entire human *TRIB2* cDNA was subcloned into pcDNA3.1/V5/HIS (Invitrogen, Carlsbad, CA, USA) and pHAGE/CMV/MCS/IRES/ZSgreen vectors (Pan *et al.*, 2008). To generate the Trib2m mutant, silent mutations of the *TRIB2* sh2-binding region were introduced by site-directed mutagenesis. Lentiviral vectors expressing shRNA sequences directed against human *TRIB2* (sh1–sh4) as well as a non-targeting construct (shNT) were obtained from Sigma-Aldrich (St Louis, MO, USA). The C/EBP α luciferase reporter vector has previously been described (Behre *et al.*, 2002).

Other experimental methods

Additional details and methodologies regarding cell culture, antibodies, microarray procedures, constructs, lentiviruses, immunohistochemistry, immunofluorescence, expression analysis, immunoprecipitations, *in vivo* tumorigenicity, gene copy number analysis and mass spectrometry can be found in the Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

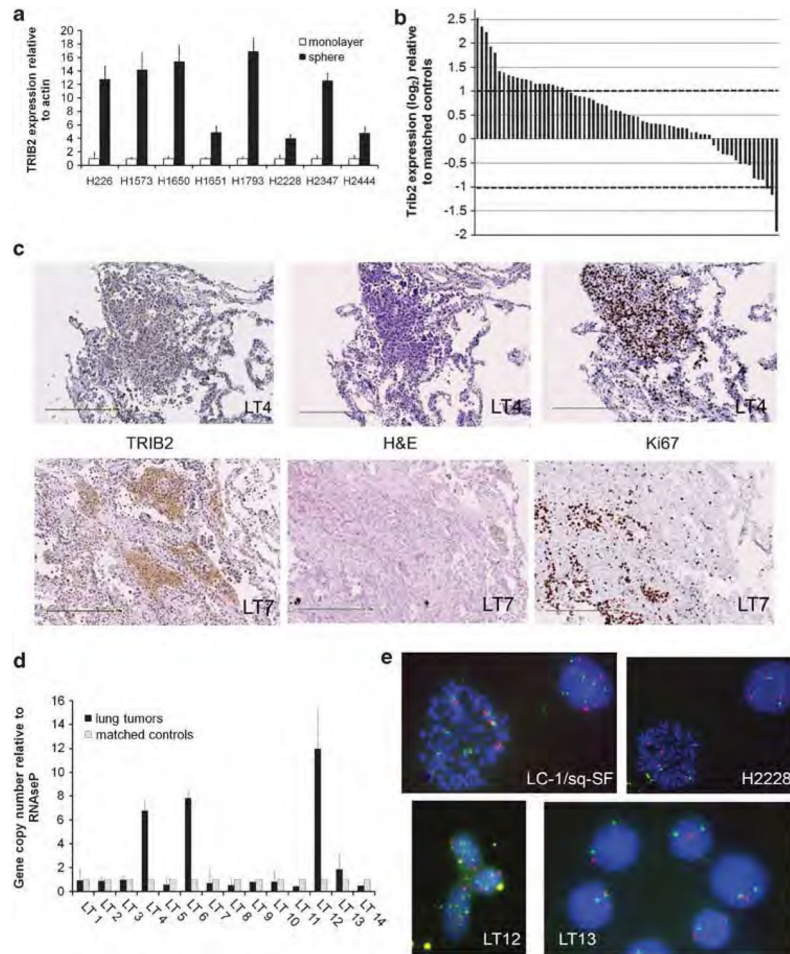
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**Figure 1.**

TRIB2 is overexpressed and amplified in a subset of human lung tumors. **(a)** Expression of TRIB2 in eight NSCLC cell lines grown as monolayers (white bars) or under low-adherence conditions as spheres (black bars). Expression levels in spheres were normalized to actin and expressed relative to the values in monolayer cultures ($P < 0.01$). **(b)** Microarray expression analysis of TRIB2 in 68 primary lung tumor samples. Data were normalized to the corresponding value from matched non-tumor samples and expressed as \log_2 . Changes greater than two-fold were considered significant, indicated by the dashed lines. **(c)** Histology of human lung tumors stained for TRIB2 and Ki67 expression (in brown). Human lung tumor sample LT4 (upper panels) and LT7 (lower panels) stained with TRIB2, hematoxylin and eosin (H&E) and Ki67 to identify dividing cells. Scale bar is 250 μm . **(d)** Quantitative PCR of *TRIB2* gene copy number based on a custom Taqman probe to the second intron. Results are normalized to *RNaseP* and expressed relative to matched non-tumor samples. Black bars represent gene copy numbers in tumor samples, gray bars represent the matched control tissues from each patient. **(e)** Fluorescence *in situ* hybridization analysis from lung cancer cell lines LC-1/sq-SF and H2228, and dissociated cells from human lung tumor samples LT12 and LT13. Probes for *TRIB2* gene (green) and centromere of chromosome 2 (red) were used. Yellow spots represent non-specific background.

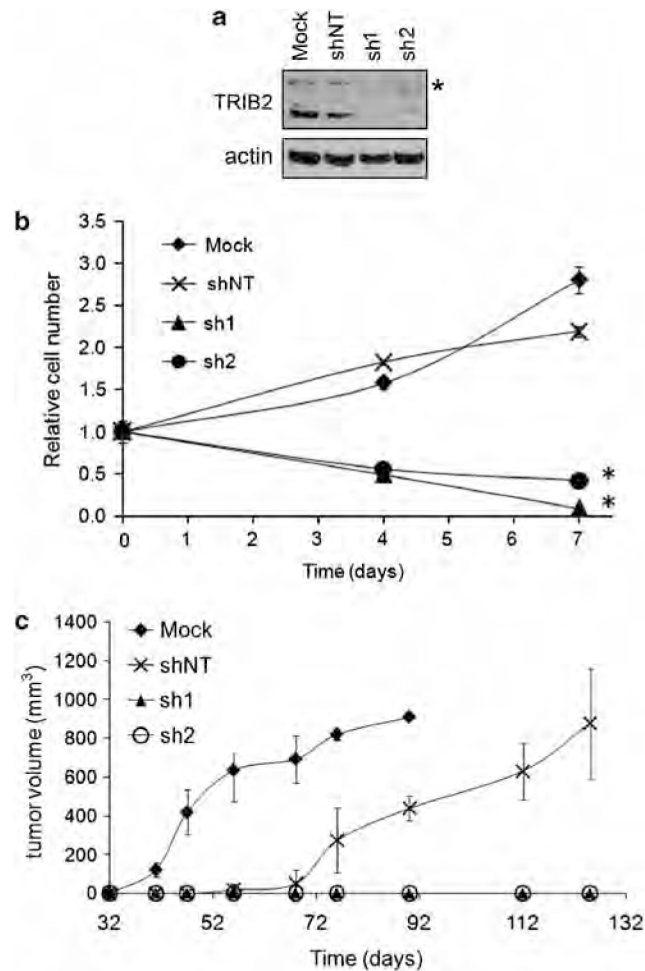


Figure 2. Knockdown of TRIB2 by shRNA inhibits cell proliferation and abrogates tumor growth. **(a)** Western blot of TRIB2 expression in U937 cells infected with sh1 or sh2. Actin is shown as a loading control. *Bands likely represent an alternate isoform of TRIB2. **(b)** Relative cell proliferation in H226 cells infected with sh1 or sh2. Values are expressed relative to cell number on day 0. * $P < 0.01$ by Student's t -test relative to non-targeting controls. **(c)** Tumor growth in NOD/SCID mice injected with 50 000 H226 cells infected (or mock infected) with the indicated shRNA construct directed against TRIB2. Animals were killed once tumor volume reached 1000mm^3 . Shown is a representative experiment ($n=8$). Error bars represent standard deviation.

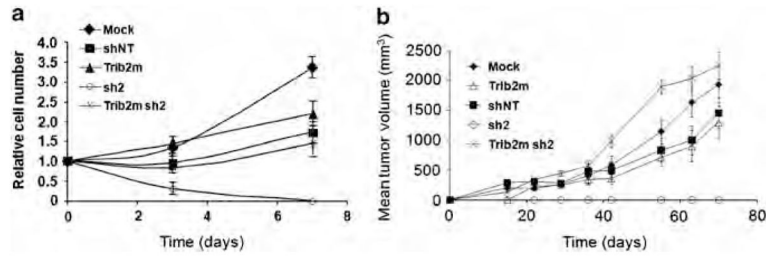
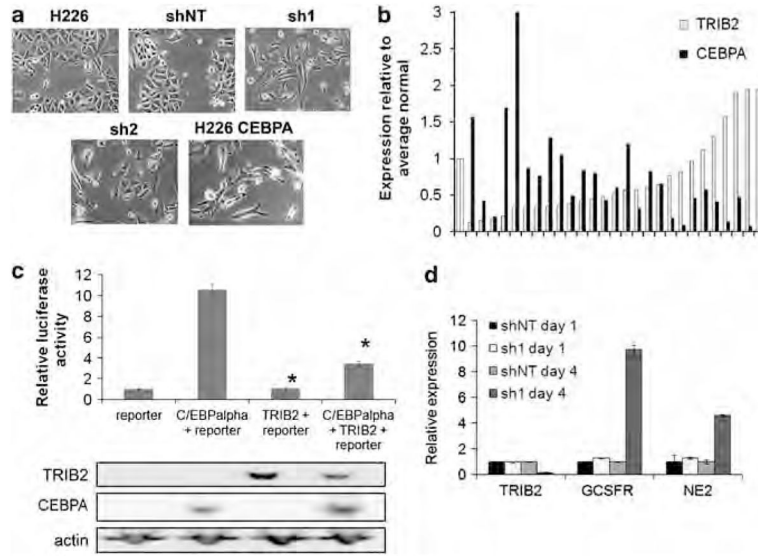


Figure 3.

Overexpression of TRIB2 rescues antitumorigenic phenotype of TRIB2 knockdown. **(a)** Relative cell proliferation of H226 cells infected (or mock infected) with shNT or sh2, followed by infection with Trib2m. **(b)** Tumor growth in NOD/SCID mice after injection of 100 000 H1650 cells infected (or mock infected) with shNT or sh2, followed by infection with Trib2m. All panels show representative experiments ($n=6$). Error bars represent standard deviation.

**Figure 4.**

TRIB2 inhibits C/EBP α function. **(a)** Phase-contrast images of H226 cells infected with shNT, sh1, sh2 or overexpressing C/EBP α . **(b)** Expression levels of TRIB2 and C/EBP α in the Origene lung cancer panel as measured using qRT-PCR. Values are normalized to actin and expressed relative to the average of the normal samples. Pearson correlation coefficient -0.38 , $P < 0.05$. **(c)** 293T cells were cotransfected with the indicated constructs as well as a reporter vector carrying a $4 \times$ C/EBP α -binding site. Top panel: luciferase activity of the transfected cells. Activity was measured at 24 h after transfection. Shown is a representative experiment ($n=6$). Error bars are standard error of the mean. *, $P < 0.05$, relative to reporter + CEBPA. Lower panel: western blot of TRIB2 and C/EBP α levels at 24 h after transfection with the indicated constructs. **(d)** Relative mRNA expression of TRIB2 and C/EBP α target genes normalized to actin. U937 cells were infected with sh1 or shNT on day 0. The next day, a subset of cells was collected (day 1 expression levels), and all-trans retinoic acid (ATRA, for granulocyte colony-stimulating factor receptor) or granulocyte colony-stimulating factor (for neutrophil elastase) was added to the culture media. Cells surviving puromycin selection were collected on day 4. Error bars represent standard deviation.

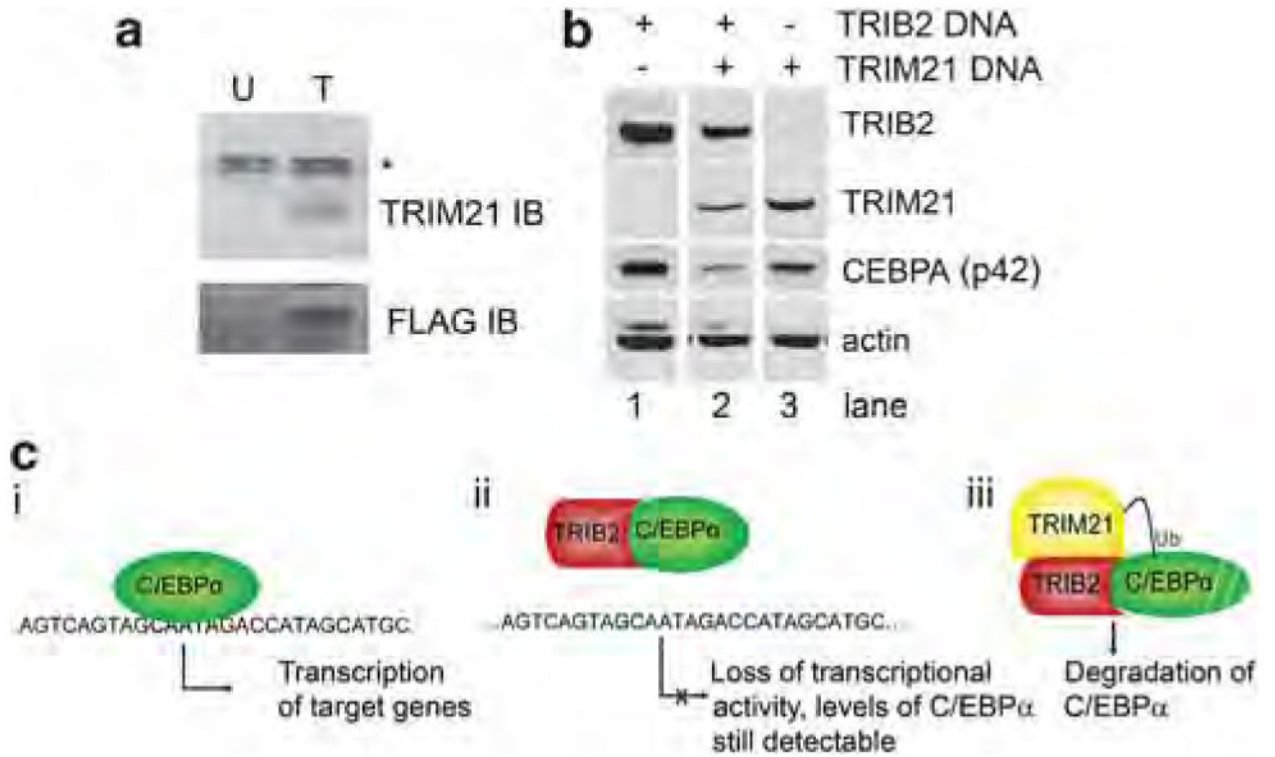


Figure 5.

TRIB2 interacts with TRIM21 and regulates C/EBP α . (a) H226 cells infected with a C-terminal FLAG-6xHis dual-affinity-tagged TRIB2 (T) or with a TRIB2 construct devoid of the C-terminal dual-affinity tag (U) as a negative control were subjected to nickel resin precipitation and immunoblotted (IB) with the indicated antibodies. *Non-specific band. (b) 293T cells were transiently transfected with CEBPA in the presence or absence of TRIB2 and TRIM21 transfection, then immunoblotted with the indicated antibodies. All lanes were derived from the same blot with the same exposure, but were non-adjacent in the original image. (c) Depiction of hypothesized protein interactions of TRIB2 and observed consequences. (i) C/EBP α acts as a transcription factor in the absence of TRIB2 and is able to activate transcription. (ii) In the presence of TRIB2, C/EBP α is no longer able to activate transcription, most likely because of an inability to bind DNA. (iii) Upon recruitment of TRIM21 to the TRIB2/C/EBP α complex, degradation of C/EBP α occurs, possibly through ubiquitylation and targeting to the proteasome.