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# TRB3 is elevated in psoriasis vulgaris lesions and mediates HaCaT cells proliferation in vitro

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## ABSTRACT

Psoriasis is a chronic skin disease characterized by abnormal keratinocyte proliferation and differentiation, inflammation, and angiogenesis. Overexpression of tribbles homolog3 (TRB3), which belongs to the tribbles family of pseudokinases, has been found in several human tumors and metabolic diseases, but its role in psoriasis has not been fully clarified. The aim of this study is to investigate the expression of TRB3 in psoriasis and explore its roles in the proliferation of keratinocytes. Twenty-four patients with psoriasis vulgaris were recruited for the study. Diagnosis of psoriasis was based on clinical and histologic examinations. Immunohistochemistry and real-time reverse transcription PCR (RT-PCR) were performed to determine protein and messenger RNA (mRNA) expression of TRB3 in psoriasis lesions. 5-Bromo-2-deoxyUridine (BrdU) incorporation assay were performed for cell proliferation. Cell cycle distribution was assessed by flow cytometry analysis. The levels of TRB3 is elevated in psoriatic lesions compared with psoriatic non-lesions. The HaCat cells expressed the TRB3 gene. We found TRB3 silencing to significantly inhibit HaCat cell proliferation. Furthermore, the specific knockdown of TRB3 slowed down the cell cycle at the gap 0/first gap phase. In conclusion, our data suggest that TRB3 is overexpressed in lesions of patients with psoriasis and may be involved in the abnormal proliferation of keratinocytes. Therefore, TRB3 may be a potential therapeutic target for psoriasis.

## INTRODUCTION

Tribbles homolog3 (TRB3; also named TRIB3), which belongs to the tribbles family of pseudokinases, was first described in *Drosophila* sp as a negative regulator of cell division in early embryogenesis and has been proposed to interact with different targets including mitogen activated protein kinases and several transcription factors.<sup>1-3</sup> TRB3 is expressed in various tissues, including liver, thymus, adipose tissue, heart, prostate and skeletal muscle.<sup>4-6</sup> Emerging evidence suggests that TRB3 are crucial modulators of tumorigenesis.<sup>7,8</sup> In addition, TRB3 are also involved in a series of non-neoplastic disorders including metabolic and neurologic diseases.<sup>9-13</sup> Relatively little is known about the actions and role of TRB3 in skin.

Psoriasis is a common, chronic inflammatory and immune-mediated skin disease.<sup>14,15</sup>

It presents clinically as a sharply demarcated erythematous plaque with silvery white scales. Histologically, it is characterized by hyperkeratosis, parakeratosis, acanthosis of the epidermis, tortuous and dilated vessels, and an inflammatory infiltrate composed mostly of lymphocytes. The pathogenesis of psoriasis is complex and the exact mechanism remains elusive, but abnormal proliferation of keratinocytes is an important mechanism of psoriasis, resulting in epidermal hyperplasia and a morphologic characteristic of psoriasis.<sup>16</sup> Numerous well established antipsoriatic treatments, including phototherapy, oral retinoid and topical calcipotriol, improve the condition of patients with psoriasis via influencing keratinocytes proliferation.<sup>17</sup>

Therefore, aberrant keratinocyte biology may be a pathogenic driver in psoriasis.

In order to evaluate the function of TRB3 in cell growth, apoptosis and inflammation involved in psoriasis, we examined the expression of TRB3 in psoriasis lesions and the effects of RNA interference (RNAi)-mediated knock-down of TRB3 on the cell proliferation and cell cycle progression in HaCaT cells, a human immortalization keratinocyte cell line. Our results suggest that TRB3 may play a role in the pathogenesis of psoriasis and may be a novel therapeutic target for psoriasis.

## MATERIALS AND METHODS

### Subjects and skin specimens

The study was approved by the local ethical committee and carried out according to the Declaration of Helsinki principles. Informed consent was obtained from all subjects before the study. A total of 24 patients with psoriasis vulgaris was recruited for the study. Diagnosis of psoriasis was based on clinical and histologic examinations. The group consisted of 16 men and 8 women, with a mean age of 49.5 years and an age range of 18–81 years. Patients had not been treated with any systemic drugs and did not apply any topical drugs to the biopsy area for several weeks. Skin specimens comprised lesional skin together with non-lesional skin surgically excised from the extremities.

### Real-time RT-PCR

Total RNA from the epidermis was extracted using TRIzol reagent (Invitrogen, Carlsbad, California, USA). Total RNA (2 mg) was



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reversely transcribed and synthesized to complementary DNA (cDNA) in a 20- $\mu$ L reaction system using reverse transcriptase (Promega, Madison, Wisconsin, USA). cDNA of TRB3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified with LightCycler FastStart DNA Master Synergy Brands (SYBR) Green I (Roche Diagnostics, Tokyo, Japan). The following primers were used: TRB3 forward, 5'-TGCCCTACAGGCACTGAGTA-3'; TRB3 reverse, 5'-GTCCGAGTGAAAAAGGCGTA-3'. GAPDH forward, 5'-GTCAACGGATTTGGTCGTATTG-3'; GAPDH reverse: 5'-TGGAGGGATCTCGCTCTG GAAGAT-3'. The cycling conditions were as follows: denaturation for 10s at 95°C, annealing for 10s at 60°C, extension for 10s at 72°C, and for 30s at the specific melting temperature. The fluorescence emitted by the SYBR Green I was measured at the end of each cycle.

can you please upload the replacement figure in jpg/tif/png formats and try proofingThe amount of TRB3, GAPDH transcripts was calculated from these standard curves using the LightCycler software. Samples were tested in triplicate and the average values were used for quantification. For each sample, the ratio between the relative amounts of TRB3 and GAPDH was calculated to compensate for variations in quantity or quality of starting messenger RNA (mRNA) as well as for differences in reverse transcriptase efficiency.

### Immunohistochemistry

For immunohistochemical staining 4- $\mu$ m thick tissue sections were performed. The sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Following incubation with normal goat serum for 20 min, the sections were incubated for 4 hours at room temperature with anti-TRB3 antibodies (Santa Cruz, California, USA) diluted 1:200 in phosphate buffer saline (PBS). After the sections were washed, they were incubated with the secondary antibody conjugated with horseradish peroxidase at a 1:400 dilution for 1 hour at room temperature. As a control, normal human skin was obtained during surgery for an epidermal cyst. All analyses were performed in multiple randomly selected high-power microscopic fields (original magnification  $\times$  200) in each specimen.

### Cell culture

Human keratinocyte cell lines HaCat cells were cultured in dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin at 37°C in a 5% humidified CO<sub>2</sub> atmosphere.

### Plasmids and transfection

We silenced TRB3 with plasmid DNA by using Lipofectamine2000 (Invitrogen, USA) according to the manufacturer's recommendations. One day before transfection, the cells were cultured in the medium without antibiotics so that they would be 85%–90% confluent at the time of transfection. The recovered cells were mixed gently with plasmid DNA and Lipofectamine2000, and then incubated at 37°C in a CO<sub>2</sub> incubator, and then the culture medium was replaced after 5 hours.

### Cell proliferation assay

The transfected cells were seeded in 96-well plates at a density of  $2 \times 10^4$  cells/well. Ten microliters of BrdU labelling solution were added to each well, and the cells were incubated at 37°C. Cell proliferation was determined using a cell proliferation ELISA, BrdU (colorimetric) kit (Roche, Burgess Hill, UK), following the manufacturer's instructions.

### Cell cycle analysis

Cell cycle distribution was assessed by staining DNA content with propidium Iodide (PI) as previously described method. Briefly, the transfected cells were harvested by trypsinization, centrifuged at 800 rpm for 5 min, washed with PBS and fixed in 75% ethanol at 4°C for 12 hours. Cells were collected by centrifugation and resuspended in PBS containing 100  $\mu$ g/mL RNase A and 40  $\mu$ g/mL PI, and then incubated at 4°C for 30 min. Cells were analyzed by flow cytometry using an FACSCalibur flow cytometer (Becton-Dickinson, California, USA). The fractions of the cells in gap 0 (G0)/first gap (G1), synthesis (S), and second gap (G2)/mitosis (M) phases were analyzed using dedicated software (Becton-Dickinson, California, USA).

### Statistical analysis

Data are expressed as mean  $\pm$  SD. Student's t-test was performed. For analysis we used the SPSS V.11.0 statistical software. Differences with p values <0.05 were considered statistically significant.

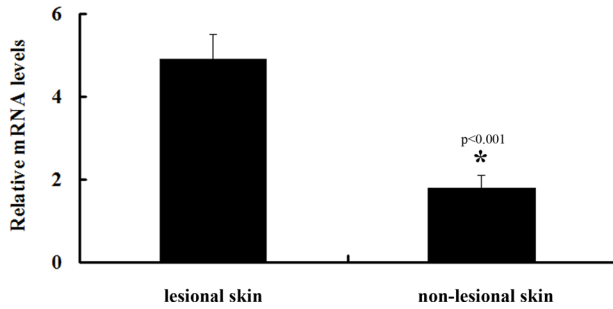
## RESULTS

### Expression of TRB3 mRNA in non-lesional and lesional skin of patients with psoriasis vulgaris and HaCat cell line

In order to investigate its potential role in psoriasis, we first measured the expression of TRB3 in the skin lesions of patients with psoriasis and HaCat cells by real-time RT-PCR. The real-time RT-PCR assays showed that mRNA of TRB3 is expressed in HaCaT cells. We next investigated the expression of TRB3 mRNA in paired lesional and non-lesional samples. The mean expression value of TRB3 mRNA (normalized by GAPDH gene expression) was significantly increased in lesional regions compared with the corresponding non-lesional regions ( $p < 0.001$ ; Student's t-test) (figure 1).

### Expression of TRB3 protein in lesional and non-lesional skin of patients with psoriasis vulgaris

To examine the expression and location of TRB3 in lesional and non-lesional psoriasis skin, immunohistochemistry was performed using the anti-TRB3 antibody. TRB3 staining was observed mainly in epidermal keratinocytes, whereas weaker and diffuse staining was detected in dermal fibroblasts. In lesional psoriasis skin, TRB3 was highly expressed throughout all layers of the epidermis and strong staining was observed in both nucleus and cytoplasm of keratinocytes, and about 96% ( $n = 23$  of 24) psoriatic lesions examined showed this pattern. However, in non-lesional psoriasis skin, the expression of TRB3 was weaker and predominantly distributed to the epidermal basal layer, and such pattern accounted for 83% ( $n = 20$  of 24) in all (figure 2).



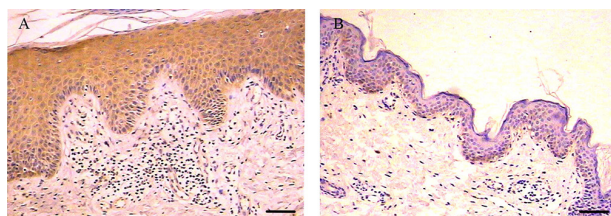
**Figure 1** Tribbles homolog 3 (TRB3) mRNA expression in lesional skin compared with non-lesional skin by real time RT-PCR. Quantitative real-time RT-PCR on 24 paired clinical samples showed that the mean expression value of TRB3 mRNA in lesional regions (normalized by GAPDH gene expression) was significantly higher than the value in the corresponding normal regions.

**Effect of TRB3 inhibition in HaCat cells growth**

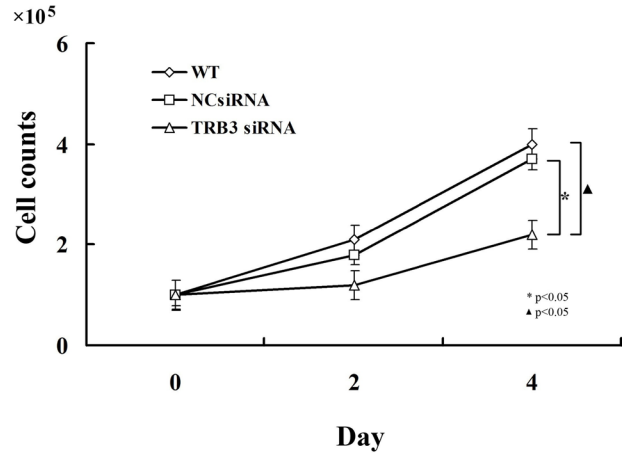
Excessive proliferation of keratinocytes is an important pathologic feature of psoriasis, and most of the treatments that improve the condition of psoriasis could suppress the proliferation of the keratinocytes. In order to illustrate the in vitro significance of TRB3 on the proliferation of keratinocytes, we applied small interfering RNA (siRNA) targeting TRB3 to suppress TRB3 expression in HaCaT cells. Real-time RT-PCR assays confirmed endogenous TRB3 expression was significantly suppressed by siRNA in the examined HaCat cell lines. To further evaluate whether silencing the TRB3 gene in HaCat cells may inhibit cell proliferation, BrdU incorporation assay was performed. TRB3 siRNA decreased the growth of HaCat cells in a time-dependent manner (figure 3). The number of HaCaT cells significantly decreased in TRB3 siRNA compared with wild type or negative control ( $p<0.05$ ), but no statistically significant difference was observed between negative control and wild type.

**Effect of TRB3 downregulation on cell cycle progression in HaCat cells**

In order to illustrate the reasons for the reduction of cell growth by suppressing TRB3, we detected cell proliferation of HaCaT cells by flow cytometry analysis. As shown in figure 4, the knockdown of TRB3 induced an increase in the percentage of cells in the G0/G1 phase of the cell cycle, parallel to a decrease in the percentage of cells in the S phase. These results indicate that TRB3 knockdown can inhibit HaCat cell proliferation by the induction of G0/G1 arrest.



**Figure 2** Representative immunohistochemical staining pattern for tribbles homolog 3 (TRB3) in lesional (A) and non-lesional (B) psoriasis skin. In the keratinocytes of psoriasis lesions, strong staining for TRB3 was observed in both nucleus and cytoplasm. Bar=50  $\mu$ m (original magnification  $\times 20$ ).



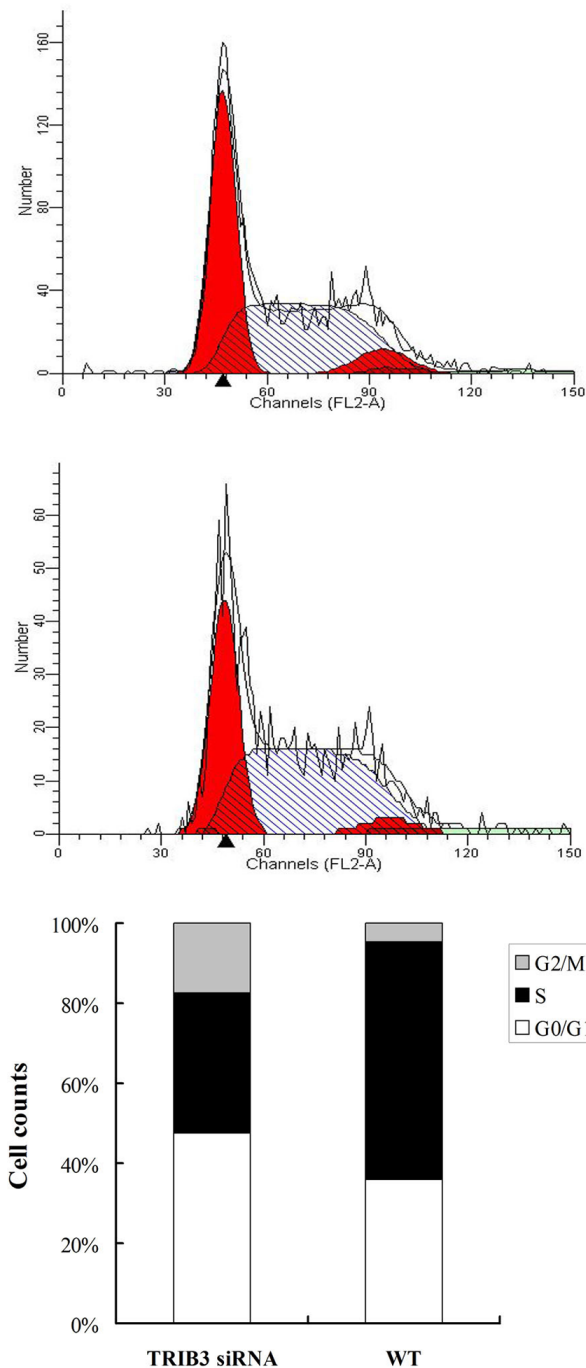
**Figure 3** Proliferation assay with siRNA inhibition in HaCat cell lines. Proliferation assay was performed in HaCat cells. There were significant differences between WT or NC, and TRB3 siRNA. Values are presented as means $\pm$ SD of three independent experiments. NC, negative control; TRB3, tribbles homolog 3; WT, wild type.

**DISCUSSIONS**

In mammals, there are three tribbles homologs—TRB1, TRB2 and TRB3. The tribbles family of proteins is characterized by their central pseudokinase domain that is well conserved during evolution. Among the members of the TRB family, TRB3 is the best studied member. Previous studies have shown that TRB3 mRNA levels are downregulated in different tumor types.<sup>18 19</sup> Likewise, high TRB3 protein levels are associated with good prognosis in patients with breast cancer.<sup>20</sup> Nevertheless, other studies have found that TRB3 mRNA levels are increased in certain types of human cancers and have proposed that TRB3 may play an oncogenic role.<sup>21 22</sup> In addition, previous observations by other laboratories indicate that TRB3 can enhance proliferation and invasiveness of cancer cells in vitro.<sup>23 24</sup> Therefore, inactivation or enhanced expression of TRB3 may produce a different outcome in a distinct genetic or cellular context.

In the current study, we investigated the role of TRB3 in the pathogenesis of psoriasis. Our research revealed for the first time that the expression of TRB3 is elevated in the lesion compared with the non-lesion of psoriasis patients, and is expressed in HaCaT cell lines. So, we performed an in vitro study with HaCaT cells to detect the role of TRB3 in keratinocytes proliferation. Our data showed that silencing of TRB3 inhibited HaCat cell proliferation. We also observed that cells were arrested in the G0/G1 phase of the cell cycle in HaCat cells transfected with siRNA against TRB3 by flow cytometry.

The cell cycle is a series of events that describe the growth and division of a cell.<sup>25</sup> In the G1 phase, there is a high rate of translation as components required for DNA synthesis are created. Cells can enter G0 during the G1 phase, if mitogenic stimulants are not present where the cell metabolic rate is low. After G1, cells enter the S phase, where the genome of the cell is replicated. Following this is the G2 phase, where protein synthesis and the metabolic rate are again high. Finally, in the M phase, the DNA chromatids and cell contents are split into two daughter cells.



**Figure 4** Effect of tribbles homolog 3 (TRB3) expression on cell cycle progression in HaCat cells. Cell cycle distribution was performed by flow cytometry analysis. Knockdown of TRB3 by RNAi in HaCat cells induced cell cycle arrest in the G0/G1 phase 2 days after transfection. Quantitative assessment of the percentage of cellular population associated with each phase of the cell cycle is illustrated between TRB3 siRNA and wild type (WT). Data represent the mean $\pm$ SD of three independent experiments.

Our research suggests that the suppression of cell growth may due to the induction of G0/G1 arrest. Our research found that silencing of TRB3 inhibited HaCat cell proliferation, and HaCat cells were arrested in the G0/G1 phase of the cell cycle with siRNA against TRB3. Together, the

present results suggest that the suppression of cell growth may due to the induction of G0/G1 arrest, and indicate that TRB3 may play a role in cellular proliferation of keratinocytes involved in psoriasis. However, further experimental studies are needed to clarify the mechanism for TRB3 silencing inducing cell cycle arrest in HaCat cells. TRB3 is expected to be a potential therapeutic target for the treatment of psoriasis.

**Contributors** X-JY designed and implemented the study, wrote the manuscript and approved the study. T-JS and L-WZ performed the experiments, and analyzed and interpreted the data. YS and K-YW assisted with manuscript preparation.

**Competing interests** None declared.

**Patient consent** Detail has been removed from this case description/these case descriptions to ensure anonymity. The editors and reviewers have seen the detailed information available and are satisfied that the information backs up the case the authors are making.

**Ethics approval** Ethics Committee of Shandong University Qilu Hospital.

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## REFERENCES

- Grosshans J, Wieschaus E. A genetic link between morphogenesis and cell division during formation of the ventral furrow in *Drosophila*. *Cell* 2000;101:523–31.
- Mata J, Curado S, Ephrussi A, *et al*. Tribbles coordinates mitosis and morphogenesis in *Drosophila* by regulating string/CDC25 proteolysis. *Cell* 2000;101:511–22.
- Seher TC, Leptin M. Tribbles, a cell-cycle Brake that coordinates proliferation and morphogenesis during *Drosophila* gastrulation. *Curr Biol* 2000;10:623–9.
- Hua F, Mu R, Liu J, *et al*. TRB3 interacts with SMAD3 promoting tumor cell migration and invasion. *J Cell Sci* 2011;124:3235–46.
- Zhang J, Wen HJ, Guo ZM, *et al*. TRB3 overexpression due to endoplasmic reticulum stress inhibits AKT kinase activation of tongue squamous cell carcinoma. *Oral Oncol* 2011;47:934–9.
- Izrailit J, Berman HK, Datti A, *et al*. High throughput kinase inhibitor screens reveal TRB3 and MAPK-ERK/TGF $\beta$  pathways as fundamental Notch regulators in breast cancer. *Proc Natl Acad Sci U S A* 2013;110:1714–9.
- Bowers AJ, Scully S, Boylan JF. SKIP3, a novel *Drosophila* tribbles ortholog, is overexpressed in human tumors and is regulated by hypoxia. *Oncogene* 2003;22:2823–35.
- Zhou Y, Li L, Liu Q, *et al*. E3 ubiquitin ligase SIAH1 mediates ubiquitination and degradation of TRB3. *Cell Signal* 2008;20:942–8.
- Du K, Herzig S, Kulkarni RN, *et al*. TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. *Science* 2003;300:1574–7.
- Koo SH, Satoh H, Herzig S, *et al*. PGC-1 promotes insulin resistance in liver through PPAR-alpha-dependent induction of TRB-3. *Nat Med* 2004;10:530–4.
- Qi L, Heredia JE, Altarejos JY, *et al*. TRB3 links the E3 ubiquitin ligase COP1 to lipid metabolism. *Science* 2006;312:1763–6.
- Avery J, Etzion S, DeBosch BJ, *et al*. TRB3 function in cardiac endoplasmic reticulum stress. *Circ Res* 2010;106:1516–23.
- Aimé P, Sun X, Zareen N, *et al*. Trib3 is Elevated in Parkinson's Disease and Mediates Death in Parkinson's Disease Models. *J Neurosci* 2015;35:10731–49.
- Rodríguez-Cerdeira C, Molares-Vila A, Sánchez-Blanco E, *et al*. Study on certain biomarkers of inflammation in Psoriasis through "OMICS" Platforms. *Open Biochem J* 2014;8:21–34.
- Lowes MA, Suárez-Fariñas M, Krueger JG. Immunology of psoriasis. *Annu Rev Immunol* 2014;32:227–55.
- Boehncke WH, Schön MP. Psoriasis. *Lancet* 2015;386:983–94.



- 17 Raut AS, Prabhu RH, Patravale VB. Psoriasis clinical implications and treatment: a review. *Crit Rev Ther Drug Carrier Syst* 2013;30:183–216.
- 18 Salazar M, Lorente M, García-Taboada E, et al. Loss of Tribbles pseudokinase-3 promotes Akt-driven tumorigenesis via FOXO inactivation. *Cell Death Differ* 2015;22:131–44.
- 19 Salazar M, Lorente M, García-Taboada E, et al. TRIB3 suppresses tumorigenesis by controlling mTORC2/AKT/FOXO signaling. *Mol Cell Oncol* 2015;2:e980134.
- 20 Wennemers M, Bussink J, Grebenchtchikov N, et al. TRIB3 protein denotes a good prognosis in breast cancer patients and is associated with hypoxia sensitivity. *Radiother Oncol* 2011;101:198–202.
- 21 Xu J, Lv S, Qin Y, et al. TRB3 interacts with CtlP and is overexpressed in certain cancers. *Biochim Biophys Acta* 2007;1770:273–8.
- 22 Wennemers M, Bussink J, Scheijen B, et al. Tribbles homolog 3 denotes a poor prognosis in breast cancer and is involved in hypoxia response. *Breast Cancer Res* 2011;13:R82.
- 23 Bowers AJ, Scully S, Boylan JF. SKIP3, a novel Drosophila tribbles ortholog, is overexpressed in human tumors and is regulated by hypoxia. *Oncogene* 2003;22:2823–35.
- 24 Hua F, Mu R, Liu J, et al. TRB3 interacts with SMAD3 promoting tumor cell migration and invasion. *J Cell Sci* 2011;124:3235–46.
- 25 van den Heuvel S. Cell-cycle regulation. *WormBook* 2005;21:1–16.