RhoBTB Proteins Regulate the Hippo Pathway by Antagonizing Ubiquitination of LKB1

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ABSTRACT The Hippo pathway regulates growth and apoptosis. We identify RhoBTB proteins as novel regulators of Hippo signaling. RhoBTB depletion in the Drosophila wing disc epithelium cooperated with Yki to drive hyperplasia into neoplasia. Depletion of RhoBTB2 caused elevated YAP activity in human cells. RhoBTB2 deficiency resulted in increased colony formation in assays for anchorage-independent growth. We provide evidence that RhoBTBs acts on Hippo signaling through regulation of the kinase LKB1. LKB1 protein levels were reduced upon RhoBTB2 depletion, which correlated with increased LKB1 ubiquitination. Restoring LKB1 levels rescued loss of RhoBTB in Drosophila. Our results suggest that RhoBTB-dependent LKB1 regulation may contribute to its tumor-suppressive function.

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KEYWORDS

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(Hamaguchi et al. 2002; Mao et al. 2009). Downregulation of RhoBTB2 was further observed in lung, bladder, bone and gastric cancer (Cho et al. 2009; Shi et al. 2008; Dong et al. 2012; Jin et al. 2013). Obtained in breast cancer cells, evidence suggests that RhoBTB2 exerts a tumor-suppressive function by inhibiting cancer cell proliferation, migration and invasiveness (Ling et al. 2014; Mao et al. 2011). Similar anti-tumorigenic properties were also found for the other RhoBTB isoforms. RhoBTB2 was found heterozygously deleted in head and neck tumors (Beder et al. 2005) and colon cancer (Xu et al. 2013). RhoBTB3 expression was greatly reduced in renal carcinoma and acts as a tumor suppressor through promoting ubiquitination and degradation of HIFs (Zhang et al. 2015).

Here, we provide evidence that RhoBTB proteins behave as tumor suppressors by regulating Hippo pathway activity in Drosophila and human cells. We show that RhoBTB2 acts via ubiquitination-dependent regulation of LKB1. Our work illustrates a novel aspect of the multifaceted molecular function of RhoBTB proteins.

**MATERIALS AND METHODS**

**Drosophila genetics and immunocytochemistry**

The use of *Drosophila* imaginal wing discs as a model for epithelial tumor formation was previously described (Herranz et al. 2012; Song et al. 2017; Herranz & Cohen 2017). RhoBTB was a validated candidate from a genome-wide screen identifying tumor suppressors whose knockdown promoted oncogenic activity of Yki as a tumor driver in *Drosophila* (Groth et al. 2019 preprint).

Briefly, male flies from the KK transgenic RNAi stock library of the Vienna *Drosophila* RNAi Center (VDRC, www.vdrc.at) carrying four different inducible USAs-RNAi constructs (P[TriP.HMC02368]attP40/CyO, P[TriP.HMC03199]attP40, P[TriP.HMS00411]attP, P[KK100815]VIE-260B, P[VSH330130]attP40) targeting RhoBTB were crossed to 10-15 virgins from the Yki driver stock used in the screen with the following genotype: w*, ap-Gal4, UAS-GFP/CyO; UAS-Yki, tub-Gal80*;TM6B. Crosses were carried out at 18° and flipped to new vials after 3 days. On day 11 post-mating, larvae-containing vials were moved to a 29° incubator to induce Yki expression. Crosses were left at 29° for another 9 days and larvae were scored for size and wing disc overgrowth phenotypes on the day 20 post-crossing (induction day 10). Other fly crosses were conducted using the same protocol. All other RNAi transgenic lines including P[VSH330167]attP40 (GD), P[KK108675]VIE-260B (attP40), P[TriP.GL00019]attP2, P[TriP.HMS01351]attP2 (targeting LKB1) and control lines were obtained from VDRC. The UAS-LKB1 (wild type) and UAS-LKB1 (KI) fly strains (Mohseni et al. 2014) were a kind gift of Jongkyeong Chung.

Imaginal wing discs were dissected and processed as described (Song et al. 2017). Antibodies were mouse anti-MMP-1 (1:10, DSHB, 3A6B4/5H7B11/3B8D12 were mixed in equal amounts), mouse anti-Dlg (1:200, DSHB, 4F3) and rat anti-DE-Cadherin (1:100, DSHB, DCAD2).

**Plasmids, siRNAs and shRNAs**

8xGTIIC-luciferase was a gift from Stefano Piccolo (Addgene plasmid #34615). The pRL-CMV (Renilla, #E2261) was purchased from Promega (Madison, WI, USA). Smart pool siRNAs against RhoBTB2 were obtained from Dharmacon. shRNAs against RhoBTB2 were expressed from the pSuper expression vector (Brunmelkamp 2002) with target sequences as listed in the Supplemental Table 1. RASG12V and LATS2 shRNA constructs were described previously (Voorhoeve et al. 2006).

**Quantitative reverse transcriptase PCR (qPCR)**

RNA was extracted using Triozl (Invitrogen) and cDNA was prepared using the iScript cDNA synthesis kit (Biorad) with random hexamers following the manufacturer’s instructions. Specific primers used for RT-qPCR are listed in Supplemental Table 1. qPCR was performed using the Solis BioDyne Firepol qPCR Master Mix and the Biorad SYBR Green Master Mix.

**Luciferase assay**

Luciferase assay to measure YAP/TAZ activity were performed using a dual luciferase kit (E1960, Promega) according to the manufacturer’s instructions and as previously described (Nguyen et al. 2017).

**Cell culture experiments**

All cell lines were purchased from the ATCC and cultured under standard conditions. All cell-based assays were performed as described (Nguyen et al. 2017; Nguyen et al. 2016). YAP localization was scored using mouse-anti-YAP (sc-101199, Santa Cruz) as previously described (Nguyen et al. 2017). Antibodies to phospho-YAP Ser127 (Cat #4911), YAP (#4912), TAZ (#2149), LATS2 (#5888), phospho-LATS1/2 (#8654), Myc-Tag (#2272), LKB1 (#3050), MARK1 (#3319) and p-MARK1-4 (#4386) were from Cell Signaling. YAP (sc-101199) antibody used for immunohistochemical staining was obtained from Santa Cruz Biotechnology. Anti-FLAG (M2, #F1365) and RhoBTB2 (SAB1407189) were from Sigma. Anti-actin (#MAB1501) was from Millipore.

**Data availability**

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, supplementary figures and tables. Supplementary figures were uploaded to figshare. Supplemental Table S1 contains primer and shRNA sequences. Supplementary figure S1 shows phenotypes caused by additional RNAi lines used in the study. Supplementary figure S2 illustrates how YAP nuclear localization was scored. Supplementary figure S3 shows results of colony formation assays using cancer cell lines. Supplemental material available at figshare: https://doi.org/10.25387/g3.11894592.

**RESULTS AND DISCUSSION**

**Depletion of RhoBTB cooperates with Yki in a Drosophila epithelial transformation model**

RhoBTB proteins perform a variety of molecular functions and can target proteins for proteasomal degradation (Choi et al. 2016). To study whether RhoBTB activity can regulate the Hippo pathway, we used a *Drosophila* epithelial tumor model (Song et al. 2017) which allows the spatio-temporally controlled expression of transgenes. In this system, conditional expression of Yki leads to mild hyperplasia in the imaginal wing disc epithelium (Figure 1A). RNAi-mediated RhoBTB depletion had little or no effect on its own (Figure 1A). However, co-expression of Yki with several independent RhoBTB-targeting RNAi transgenes caused formation of massively overgrown tumors in a subset of larvae (Figure 1A and Suppl. Fig. S1). On average 3-4 phenotypically noticeable larvae were observed per vial. These larvae were developmentally delayed but did not display the characteristic ‘giant larve’ phenotype often associated with tumorigenesis in *Drosophila*. Tumor formation did not occur at high frequency, but was consistently observed in replicate experiments, independent crosses and with different RNAi lines targeting *RhoBTB*, arguing against off-target effects. The relatively low frequency of tumor-bearing larvae might be due to only partial efficiency of the
transgenes targeting RhoBTB as well as due to competition with sibling larvae with non-inducing genotypes under crowded conditions.

Tumors did neither exhibit evident loss of polarity as assessed by several markers (DE-Cadherin, Dlg and actin), nor upregulation of Matrix Metalloprotease-1 (MMP1) (Figure 1A). Based on these observations, we decided to test whether interactions between RhoBTB activity and the Hippo pathway might be a conserved feature in human cells.

**YAP activity and YAP target expression are influenced by RhoBTB2 in human cells**

YAP and TAZ are the mammalian orthologs of *Drosophila* Yki (Huang et al. 2005). To test whether modulation of RhoBTB activity would impact on the Hippo pathway, we utilized a luciferase reporter containing octameric TEAD binding sites in HEK293T cells. In these cells, the reporter responds sensitively to perturbations of YAP and TAZ activity (Dupont et al. 2011; Nguyen et al. 2016). HEK293T cells express high levels of RhoBTB2. Of note, HEK293T and the other cell lines used in this study expressed very low levels of RhoBTB1 mRNA as compared to RhoBTB2 and undetectable levels of RhoBTB3 (data not shown). Three independent RhoBTB2 shRNAs, which strongly reduced RhoBTB2 expression but had little or no effect on RhoBTB1 (Figure 1B), strongly induced reporter activity (Figure 1C). Consistently, several endogenous transcriptional YAP targets (CTGF, AREG an CYR61) were upregulated in RhoBTB2 shRNA-depleted cells compared to the control (ANOVA P < 0.001). Error bars represent mean +/- SD for three independent experiments.

**YAP is post-transcriptionally regulated by RhoBTB2**

Upon phosphorylation by the Hippo pathway core cassette components LATS1 and LATS2, YAP is retained in the cytoplasm and

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**Figure 1** RhoBTB regulates the Hippo pathway. (A) Representative images showing the synergistic effect between Yki overexpression and RhoBTB depletion in a *Drosophila* transformation model. Genotypes are indicated in the figure. DAPI (blue), GFP (green), DE-Cadherin (red). MMP1 staining is shown as a separate panel (white). The rightmost image shows a representative Y-Z projection illustrating the epithelial polarity of the adjacent tumor (DE-Cadherin, red). (B) qPCR quantification of RhoBTB2, YAP, and YAP-targets CTGF, AREG and CYR61 in HEK293T cells treated with a control shRNA or three independent shRNAs targeting RhoBTB2. Changes in CTGF, AREG and CYR61 were significant in RhoBTB2 shRNA-depleted cells compared to the control (ANOVA P < 0.001). Error bars represent mean +/- SD for three independent experiments. (C) Luciferase reporter assays performed in HEK293T cells treated with a control shRNA or three independent shRNAs targeting RhoBTB2. Changes in luciferase activity were significant in all shRNAs vs. control (ANOVA P < 0.0001). Error bars represent mean +/- SD for three independent experiments. (D) Scoring of nuclear YAP localization in BJ cells treated with a scrambled control siRNA (black bars) or a siRNA pool targeting RhoBTB2 (gray bars). Changes in number of cells exhibiting high and low nuclear YAP were significant in RhoBTB2 shRNA expressing vs. control cells (ANOVA P < 0.01). (E) Western blots of cellular extracts from cells treated with a control shRNA or two different shRNAs targeting RhoBTB2. Blots were probed with antibodies specific for phospho-LATS1/2 (T1079), LATS 2, phospho-YAP (S127), YAP, TAZ. ACTIN was used as a loading control. Grayscale values of inverted bands measured with ImageJ and normalized against ACTIN are shown above the corresponding bands to indicate the band intensities.
ultimately targeted for degradation (Zhao et al. 2010). In contrast, non-phosphorylated YAP translocates to the nucleus to activate target gene expression. Since knockdown of RhoBTB2 led to an increase of YAP/TAZ activity, we asked whether RhoBTB2 inhibition would affect the subcellular localization of YAP. We utilized a previously established fibroblast cell model expressing human TERT, p53 shRNA, p16 shRNA and RASG12V (BJhTert/p53kd/p16kd/RASG12V) that permits detecting YAP translocation by immunochemical staining of YAP upon inhibition of the Hippo pathway (Nguyen et al. 2017). In comparison to control cells, RhoBTB2 depletion caused a statistically significant shift toward increased nuclear YAP levels (Figure 1D and Suppl. Fig. S2). We next assessed the expression and phosphorylation of Hippo proteins in the context of RhoBTB2 depletion in these cells by Western blot analysis. Knockdown of RhoBTB2 using two different shRNAs led to an increase in the protein level of YAP and TAZ (≥ 1.5-fold increase in protein levels, which correlated with reduced phosphorylation of YAP (p-Ser127: reduced by more than 0.5 fold) (Figure 1E). LATS2 protein levels did not change or increased slightly (~1.2-fold). In contrast, we observed a more than twofold reduction of LATS phosphorylation, consistent with reduced LATS activity.

Taken together, these data are consistent with the idea that RhoBTB2 deficiency prevents the Hippo core cassette from phosphorylating YAP, which would consequently promote transcription of target genes in the nucleus. Therefore, our results suggest that RhoBTB2 regulates YAP at the post-transcriptional level through the Hippo core cassette.

**RhoBTB2 inhibits cell growth by regulating Hippo pathway activity**

Active YAP can promote cell proliferation and growth via transcriptional regulation of numerous target genes (Zhao et al. 2007; Ehmer & Sage 2016). We therefore asked whether depletion of RhoBTB2 would cause phenotypes associated with increased YAP activity. To address this question, we studied the effect of RhoBTB2 suppression on colony formation in soft agar in two partially transformed human fibroblast cell models expressing human TERT, p53 shRNA, p16 shRNA and small T (BJhTert/p53kd/p16kd/small T) or RASG12V (BJhTert/p53kd/p16kd/RASG12V). These genetically defined cells were previously shown to be sensitive to perturbations of YAP activity, which is coordinately regulated by the Hippo and RAS pathways (Nguyen et al. 2014; Hong et al. 2014). As expected, expression of active RAS (Figure 2A) or depletion of LATS2 (Figure 2B), which induces YAP activation, caused enhanced transformation phenotypes upon inhibition of Hippo activity. Depletion of RhoBTB2 in these cell lines increased anchorage-independent growth in soft agar assays (Figure 2A and 2B). These data are consistent with the
idea that RhoBTB2 controls cell growth and proliferation by modulating Hippo pathway activity. We observed a similar growth-suppressive behavior of RhoBTB2 in several cancer cell lines. Depletion of RhoBTB2 led to an increase in colony formation in the cell lines HT-15, MDA-MB-468, HeLa, HCT116 and HT-29 (Suppl. Fig. S3). The cell lines MDA-MB-231, DU-145, A549 and H1299 did not display a change in colony numbers. Surprisingly, MCF-7 cells showed reduced cell growth when RhoBTB2 was depleted (data not shown). The role of RhoBTB2 in these cell lines remains unclear. Taken together, however, these results suggest that RhoBTB2 could be part of the machinery that limits proliferation of cancer cells, depending on the specific cellular context.

Figure 3 RhoBTB2 depletion affects the Hippo pathway through the LKB1/MARK axis. (A) Western blots of HEK293T cells transfected with a control shRNA or two independent shRNAs targeting RhoBTB2. Blots were probed for LKB1, phospho-MARK1-4 and MARK1 antibodies. ACTIN was used as a loading control. Grayscale values of inverted bands measured with ImageJ and normalized against ACTIN are shown above the corresponding bands to indicate the band intensities. (B) Ubiquitination assay of cells transfected with myc-Ubiquitin, FLAG-LKB1 and either a scrambled siRNA pool, or with a siRNA pool targeting RhoBTB2. Blots were probed with anti-myc and anti-FLAG.

Figure 4 LKB1 resupply suppresses tumor formation induced by depletion of RhoBTB2. (A) Confocal images of wing discs showing the effect of LKB1 depletion. GFP (green), DAPI (blue) and Dlg (red). Genotypes are indicated in the figure. All images correspond to the same scale. (B) Representative images of wing discs showing the influence of wildtype LKB1 overexpression respectively kinase-inactive LKB1 expression on the RhoBTB2 depletion phenotype. Discs were stained for actin. Genotypes are indicated. All images were taken at the same magnification. (C) Graphs showing the effect of wildtype LKB1 (LKB1WT) overexpression respectively kinase-inactive LKB1 (LKB1KI) expression on the frequency of wing disc tumor formation. Data are presented as the number of tumor-bearing larvae scored per day ± SD p-values were determined using a non-parametric Mann-Whitney test.
RhoBTB2 acts on the Hippo pathway via regulation of LKB1

To elucidate how RhoBTB2 regulates Hippo signaling, we searched the BioGRID database (Oughtred et al. 2018) for potential interaction partners. This approach identified the kinase LKB1 (also known as STK11) as a candidate RhoBTB2 interactor. LKB1 acts on the Hippo pathway via phosphorylation of MARK kinases and thereby modulates YAP activity to regulate organ growth and proliferation (Mohseni et al. 2014). We observed reduced LKB1 expression (≥ threefold) and MARK phosphorylation (40% reduction) in RhoBTB2-depleted cells (Figure 3A). Since LKB1 is a known substrate of the Skp2-SCF ubiquitination complex that targets several Hippo components (Lee et al. 2015), we asked if RhoBTB2 depletion would affect LKB1 ubiquitination. Consistent with the reduced LKB1 levels, LKB1 was more extensively ubiquitinated in RhoBTB2-depleted cells than in the control (Figure 3B). These data are consistent with the idea that RhoBTB2 impacts on Hippo pathway activity through the LKB1/MARK axis.

Resupply of LKB1 rescues tumor formation induced by RhoBTB2 depletion

Depletion of RhoBTB drives Yki-mediated hyperplasia into tumor formation (Figure 1A). To test whether a decrease in LKB1 levels produces a similar effect, we depleted LKB1 in the Drosophila wing disk epithelium using several independent RNAi lines. Concomitant with Yki overexpression, but not on its own, LKB1 depletion led to the formation of larvae bearing large tumors (Figure 4A). We did neither detect a loss of epithelial polarity nor significant changes in MMP-1 levels or occurrence of giant larvae (not shown). The phenotypical similarities between individuals depleted for RhoBTB and LKB1 levels or occurrence of giant larvae (not shown). The phenotypical similarities between individuals depleted for Yki/YAP/TAZ.

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