A splicing isoform of TEAD4 attenuates the Hippo–YAP signalling to inhibit tumour proliferation

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Aberrant splicing is frequently found in cancer, yet the biological consequences of such alterations are mostly undefined. Here we report that the Hippo–YAP signalling, a key pathway that regulates cell proliferation and organ size, is under control of a splicing switch. We show that TEAD4, the transcription factor that mediates Hippo–YAP signalling, undergoes alternative splicing facilitated by the tumour suppressor RBM4, producing a truncated isoform, TEAD4-S, which lacks an N-terminal DNA-binding domain, but maintains YAP interaction domain. TEAD4-S is located in both the nucleus and cytoplasm, acting as a dominant negative isoform to YAP activity. Consistently, TEAD4-S is reduced in cancer cells, and its re-expression suppresses cancer cell proliferation and migration, inhibiting tumour growth in xenograft mouse models. Furthermore, TEAD4-S is reduced in human cancers, and patients with elevated TEAD4-S levels have improved survival. Altogether, these data reveal a splicing switch that serves to fine tune the Hippo–YAP pathway.
Hippo–YAP signalling is a key pathway that regulates cell proliferation, cell contact inhibition and organ size. The transcriptional output of this pathway is mediated by TEAD proteins that partner with YAP to activate genes that stimulate cell proliferation. As a key effector of the Hippo pathway, YAP lacks DNA-binding motif, and thus recognizes its targets through interacting with TEAD proteins. Under normal condition, YAP is translocated into the nucleus to promote cell growth; however, the activation of Hippo causes YAP phosphorylation, leading to cytoplasmic retention and degradation of YAP. Thus, defects of the Hippo signalling cause overgrowth phenotypes due to deregulation of proliferation and apoptotic defects. Hippo–YAP pathway is directly involved in cancer development, and inhibition of the YAP activity provides a valuable route for cancer prevention and treatment. In current model, Hippo signalling is mainly transcription co-activator YAP to promote transcription of target genes. The transcriptional output of this pathway is mediated by the Hippo–YAP signalling. The transcription factor-mediating YAP-dependent gene expression, TEAD4, contains two functional domains: an N-terminal domain specifically recognizing the enhancer of targeted genes through direct binding of DNA and a C-terminal motif that interacts with transcription co-activator YAP to promote transcription of target genes. On the basis of the annotation of the RNA sequencing data, we discovered that the exon 3 of human TEAD4 can be skipped to generate a new isoform, producing a truncated TEAD4 protein with an alternative start site at exon 6. This short isoform of TEAD4 (TEAD4-S) lacks an N-terminal DNA-binding domain, but still contains an intact C-terminal YAP-binding motif.

To validate the presence of this TEAD4-S isoform, we measured the levels of TEAD4-S mRNA and protein in different human tissues. While the full-length TEAD4 (TEAD4-FL) represents the major form in all tested tissues, the TEAD4-S mRNA is ubiquitously spliced with small tissue-specific variations. Consistently, using an antibody against the common sequence of both TEAD4 isoforms, we detected TEAD4-S protein in all tissues with abundance comparable to a canonical isoform in some tissues (for example, spleen and skeleton muscle; Supplementary Fig. 1d). These two TEAD4 isoforms have distinct subcellular localizations: while canonical TEAD4-FL is primarily localized in the nucleus, TEAD4-S is found in both the nucleus and cytoplasm with no obvious preference (Fig. 1b). Importantly, both TEAD4 isoforms were found to bind YAP protein in a co-immunoprecipitation assay (Fig. 1c), supporting the finding that TEAD4-S still contains an intact C-terminal YAP-binding motif.

Splicing of TEAD4-S is regulated by RBM4. To identify the possible splicing factors that control the AS of TEAD4, we analysed the sequence of exon 3 in TEAD4 and discovered a putative RNA motif resembling a known binding site of RBM4 (CGGCCGGG). RBM4 is a general splicing factor that functions as a tumour suppressor in a number of human cancers. This observation raises the possibility that RBM4 may directly control TEAD4 splicing. To test this possibility, we generated 293 cells that stably express RBM4 on tetracycline induction, and found that RBM4 promotes the splicing of TEAD4-S in a time-dependent manner (Fig. 1d; Supplementary Fig. 1e). Such regulation is consistent across various cell types, as overexpression of RBM4 increased the TEAD4-S mRNA and protein levels in various cultured cancer cells from pancreatic, lung, liver and breast cancers (Fig. 1e; Supplementary Fig. 1f).

Next, we generated a minigene reporter containing the exon 2–4 of TEAD4 (Fig. 1f, upper panel). Consistent with the previous results in Fig. 1d,e, RBM4 expression indeed caused skipping of exon 3 in this artificial reporter (Fig. 1f, lower-left panel). Remarkably, a mutant splicing reporter destroying the putative RBM4-binding site (mut 1, CTTATA) abolished the splicing regulation of TEAD4 by RBM4, whereas another mutant reporter with a replaced RBM4-binding motif (mut 2, GTAACG) restored the RBM4 regulation (Fig. 1f, lower-right panel). We further confirmed that RBM4 directly bound to TEAD4 pre-mRNA using RNA-IP (Fig. 1g). Consistently, the mutant RNA with the destroyed RBM4-binding site (mut 1) failed to interact with RBM4, while the replacement of this site with a different RBM4-binding sequence (mut 2) reinstated the RNA–RBM4 interaction (Fig. 1h). Furthermore, we used an antisense oligonucleotide (ASO3, TAATGGTGCCGGCGTG GCC) to block the RBM4-binding site in exon 3 of TEAD4. As expected, RBM4 could no longer regulate TEAD4 splicing in the presence of antisense oligos (Supplementary Fig. 1g). Collectively, these data suggest that RBM4 indeed recognizes this putative binding site to control TEAD4 splicing.
canonical TEAD4 with its YAP-binding domain. We directly test this hypothesis with a reporter system containing a luciferase gene driven by a TEAD-dependent promoter. We found that expression of YAP alone or co-expression of YAP/TEAD4-FL significantly increased luciferase activity, whereas co-expression of TEAD4-S/YAP suppressed the gene activation in a dose-dependent manner, suggesting that TEAD4-S has an opposite activity to canonical TEAD4. Importantly, when increasing amount of TEAD4-S was co-expressed with TEAD4-FL, TEAD4-S can inhibit the transcriptional activity of TEAD4-FL in a dose-dependent manner (Fig. 2a; Supplementary Fig. 2a). We further demonstrated that TEAD4-S can directly disrupt the binding of canonical TEAD4-FL to YAP as judged by reciprocal co-immunoprecipitation assays (Fig. 2b,c; Supplementary Fig. 2b), suggesting that TEAD4-S functions as a dominant negative isoform via competing with TEAD4-FL. In addition, while...
TEAD4-FL is found to bind the promoter of its endogenous target CTGF, TEAD4-S cannot bind the same DNA as judged by chromatin immunoprecipitation, presumably due to lack of the N-terminal DNA-binding domain (Fig. 2d; Supplementary Fig. 2c). Together, these data suggest that TEAD4-S functions as a dominant negative isoform.

In line with the finding that RBM4 promotes the splicing of TEAD4-S, co-expression of RBM4 with YAP also reduced TEAD4-mediated gene activation, whereas expression of RBM4 alone had no effect (Fig. 2a). This observation was further supported by measuring the endogenous targets of YAP/TEAD4, as co-expression of YAP/TEAD4 diminished the YAP-dependent activation of CTGF and ITGB in two different cell lines (Fig. 2e,f; Supplementary Fig. 2d). Consistently, co-expression of RBM4 with YAP can also reduced the activation of these two genes by YAP (Fig. 2e,f), while expression of RBM4 alone had no effect (Supplementary Fig. 2e,f), further confirming that RBM4-mediated new TEAD4 splicing switch to control the YAP signalling pathway.

To further assess the impact of the RBM4-mediated TEAD4 splicing switch on YAP signalling and cellular functions, particularly cell proliferation, we conducted transcriptome profiling by RNA-seq in cells transfected with YAP alone, YAP/TEAD4-FL, YAP/TEAD4-S and YAP/RBM4 (Supplementary Fig. 2f; Supplementary Data 1). As expected, cells with co-expression of YAP with TEAD4-FL or TEAD4-S displayed limited overlapping between significantly affected genes (Supplementary Fig. 2g). Consistent with the finding that RBM4 increases splicing of TEAD-S, the genes that were up- or downregulated on YAP/TEAD4-S expression significantly overlap with those altered in the same directions by YAP/RBM4 expression, whereas the genes altered in different directions are mutually exclusive (Supplementary Fig. 2h; \( P < 2.2 \times 10^{-16} \) by \( \chi^2 \)-test).

By comparing all samples with the vector-transfected control, we identified a set of 429 YAP-activated genes that were enhanced by co-expression of TEAD4-FL, but were repressed by co-expression of TEAD4-S or RBM4 (Fig. 2g; Supplementary Data 2). Amazingly, these genes form a densely connected network as evaluated by the analysis of STRING database of gene interaction (Fig. 2h). The largest cluster of genes contains many key regulators of cell cycle, including transcription factor (for example, E2F1 and Myc), some cyclins (for example, CCNB1 and CCNE2) and genes required for chromatin alignment and segregation (for example, NDC80, CDC2A8, SPDL1 and CENPF). In addition, the genes differentially regulated by two TEAD4 isoforms also include components in key signalling pathways that affect cell proliferation (for example, CSNK2A1, PCNA and other components of the Hippo pathway), as well as genes involved in RNA splicing (for example, SMN2, SP3A2 and many splicing factors) and translation (for example, elf5C, elf1B and ribosomal proteins).

In addition, gene ontology analysis also suggested that the cell cycle, cell proliferation and RNA processing are main functional pathways that are activated by YAP YAP TEAD4, but diminished by the non-canonical TEAD4 isoform (Supplementary Fig. 2i). Taken together, these data indicate that the short isoform of TEAD4 harbours functions to antagonize conventional TEAD4, thus attenuating the Hippo–YAP–TEAD signalling cascade.

**Figure 1 | Identification of an alternative splicing isoform of TEAD4 regulated by RBM4.** (a) Schematics of human TEAD4 pre-mRNA and protein. The full-length TEAD4 contains 1-12 exons, including the DNA-binding domain in the N terminal, whereas exon 3 of TEAD4 can be skipped to generate a new isoform whose translation is started at exon 6 to produce a truncated TEAD4-S protein. TEAD4-S lacks an N-terminal DNA-binding domain, but still contains an intact C-terminal YAP-binding motif. (b) The localizations of TEAD4-FL and TEAD4-S were determined by immunofluorescence assay. Flag-tagged TEAD4-FL or TEAD4-S was transfected into cells. The immunofluorescence assay was performed with anti-Flag antibody to show the localizations of Flag-TEAD4. Scale bar, 25 \( \mu \)m. (c) Interaction of two TEAD4 isoforms with YAP protein as judged by co-IP experiment. The Flag-tagged TEAD4-FL or TEAD4-S was co-transfected with HA-tagged YAP into 293T cells, and the binding of YAP was detected. (d) 293 cells expressing RBM4 on tetracycline induction were collected at different time points after induction to determine the expression levels of TEAD4-FL and TEAD4-S. (e) RBM4 regulates the splicing of TEAD4 in various cancer cells, including Panc1, HT1080, MDA-MB-231 and HepG2. The cells were stably transfected with RBM4 or vector control, and the splicing of TEAD4 was examined by RT-PCR. The representative gels were shown. (f) The schematic of TEAD4 pre-mRNA where the potential RBM4-binding site (CGGGCCGG) in red. TEAD4 splicing reporters with the indicated mutations (mut1: CTTTATA and mut2: GTAACG) were generated (upper panel). TEAD4 splicing reporters containing wild-type RBM4-binding site (lower-left panel) or mutations (lower-right panel) were co-expressed with RBM4 or vector control in 293T cells to assay for the splicing change of TEAD4. Representative gels from triplicate experiments were shown, with the mean ± s.d. of TEAD4-S% plotted below representative gels. (g) Binding of TEAD4-FL to RBM4 was examined by RNA-immunoprecipitation assay in cells exogenously expression FLAG-RBM4 or vector control. (h) 293 cells were co-transfected with Flag-RBM4 or vector control and the indicated mutant or wild-type (WT) TEAD4 reporters, and then immunoprecipitated with anti-Flag antibody. The co-precipitated RNAs were detected by RT-PCR.
To further examine the functional role of TEAD4 splicing switch, we applied a recently developed approach, engineered splicing factors (ESFs)\textsuperscript{39–42}, to specifically manipulate TEAD4 splicing and to test whether the splicing changes of TEAD4 can directly affect YAP-mediated EMT and tumour proliferation. We designed and generated a PUF domain that can specifically bind YAP, TEAD4, or RBM4.

### References

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to an 8-nit RNA sequence (GAGCCTTG) in exon 3 of TEAD4, and fused the designer PUF domain with a glycine-rich motif that can inhibit exon inclusion on binding to pre-mRNA. The resulting ESF, ESF-TEAD4, is designed to bind and inhibit the inclusion of exon 3 in TEAD4, thus promoting the TEAD4-S splicing (Fig. 4a). When transfecting ESF-TEAD4 in YAP stably expressed lung cancer cells, H157 and A549, we found that the ESF-TEAD4 specifically shifted TEAD4 splicing towards the short isoform as compared with control ESF (Fig. 4b). As expected, the splicing switch to TEAD4-S can indeed suppress the YAP-mediated EMT (Fig. 4c) and the tumour cell proliferation (Fig. 4d) in both lung cancer cell lines tested.

TEAD4-S inhibits tumour growth in vivo. To further assess whether TEAD4-S affects cancer growth in vivo, we determined whether expression of TEAD4-S could suppress tumour growth in a xenograft mouse model. We generated H157 cells with stable expression of YAP, YAP/TEAD4-FL, YAP/TEAD4-S or YAP/RBM4, and injected these cells subcutaneously into the flanks of nude mice to measure tumour growth. Consistent with our in vitro results, cells expressing TEAD4-S developed smaller tumours as compared with cells with YAP alone, YAP/TEAD4-FL or even the vector control (Fig. 5a), suggesting that TEAD4-S functions as an inhibitor of tumour development. In addition, the xenograft tumours with TEAD4-S developed much slower than cells with YAP, YAP/TEAD4-FL or even vector control (Fig. 5b), further supporting that TEAD4-S inhibits cancer progression in vivo. As a positive control, RBM4 also inhibited tumour development in vivo, consistent with its role as antitumour splicing factor to promote TEAD-S.

Next, we surgically collected paired non-small cell lung cancer (NSCLC) samples and adjacent normal tissues from seven patients, and measured the relative levels of two TEAD4 isoforms. Compared with the paired normal tissues, the relative mRNA levels of TEAD4-S were significantly reduced in six out of seven primary NSCLC specimens (Fig. 5c), and the TEAD4-S protein was substantially decreased in five out of seven specimens (Fig. 5d), suggesting a general reduction of TEAD4-S expression despite the obvious heterogeneity in different tumour specimens. Intriguingly, in three of the NSCLC specimens (samples 4, 5 and 7), the truncated TEAD4-S protein was the predominant isoform in normal tissues, but was almost completely eliminated in tumours, implying that the AS switch in TEAD4 could play a major role in the tumorigenesis of these patients.

To further evaluate the clinical relevance of TEAD4 splicing in all cancers, we analysed the databases from TCGA consortium that contains various large-scale RNA-seq results from thousands of patients with various tumours43 (Supplementary Fig. 4a). Strikingly, TEAD4-S levels were significantly altered in 7 out of 11 tumour types analysed with reduction in the majority of tumours (6 out of 7; Fig. 5e), consistent with our results in the anti-tumour activity of TEAD4-S. To further investigate the clinical significance of TEAD4 splicing in cancers, we used a survival analysis tool, Kaplan–Meier plotter, to analyse TCGA data sets for the overall survival of various cancer patients with different TEAD4-S levels. Strikingly, a higher level of TEAD4-S was significantly associated with the improved overall survival in patients with lung and colon cancers (Fig. 5f), and to a lesser extent, in patients with liver and breast cancers (Supplementary Fig. 4b, positive but non-significant association). These observations indicate that TEAD4-S could be recognized as an independent prognostic factor for the survival of cancer patients. Collectively, this finding validated the mechanistic link between the TEAD4 isoform and cancer progression, highlighting the importance of this splicing switch of the Hippo–YAP–TEAD pathway in regulating human cancer progression and patient survival.

Discussion

Extensive splicing misregulation is one of molecular hallmarks of cancer14–17; however, the functional implication is far from clear. Here we report a model in which the AS plays a key regulatory role in mediating the activation of Hippo–YAP signals. In the current model, activation of the Hippo pathway by multiple extracellular cues is converged to its main effector YAP, whose phosphorylation leads to cytoplasmic retention and protein degradation. When unphosphorylated, YAP translocates into the nucleus and interacts with transcription factors TEAD1–4 to activate gene expression and promote cell proliferation10. We demonstrated that the AS imposes a new layer of regulation: skipping of exon 3 in TEAD4 produces a short isoform that interacts and neutralizes YAP in both the nucleus and cytoplasm, leading to the attenuation of YAP signalling (Fig. 4g). The splicing of TEAD4-S is controlled by RBM4 through direct binding to its pre-mRNA. Consistently, RBM4 and TEAD4-S inhibit tumour progression in cultured cells and xenograft tumours. Altogether, these data represent a new mechanism on how the AS affects tumorigenesis through mediating the key signalling cascades such as the Hippo–YAP pathway.

Genomic analyses of TCGA data sets indicate that splicing of TEAD4 is commonly altered in cancer patients to reduce TEAD4-S. Strikingly, re-expression of TEAD4-S significantly reduced tumour development in cultured cells and mouse model. Since the Hippo–YAP pathway has a broad impact on
Figure 3 | TEAD4-S reduces cancer cell proliferation and EMT. (a) RNA levels (upper panel) and protein levels (lower panel) of TEAD4-S in various NSCLC lines and normal bronchial cells were measured by semi-quantitative RT–PCR and western blot. The mean ± s.d. for the percentage of TEAD4-S mRNA was plotted from three experiments. Each cancer cell line was compared with the two normal cell lines to calculate P values using Student’s t-test. (b) H157 or A549 cells stably expressing YAP, YAP/TEAD4-FL, YAP/TEAD4-S, YAP/RBM4 or vector control were analysed by colony formation assay. All experiments were performed in triplicates, with mean ± s.d. of relative colony numbers plotted (P values from t-test). Images of the whole plate were shown. (c) H157 or A549 cells stably expressing YAP, YAP/TEAD4-FL, YAP/TEAD4-S, YAP/RBM4 or vector control were analysed by soft agar assay. All experiments were performed in triplicates, with mean ± s.d. of relative colony numbers plotted (P values from t-test). (d) Western blot of epithelial and mesenchymal markers was performed using lysates from indicated H157 and A549 stable cells. (e) The subcellular localizations of TEAD4-FL or TEAD4-S with YAP. Cells were co-transfected with Flag-tagged YAP (red) and GFP-fused TEAD4 (green), and visualized with immunofluorescence assay. Scale bar, 25μm. The localization of TEAD4-FL and TAD4-S was quantified in transfected cells, and the per cent of cells with nuclear or cytoplasmic TEAD4 were plotted (>50 cells were captured and quantified in both samples).
tumorigenesis and cancer development, modulating TEAD splicing may provide a new approach for potential therapeutic interventions of cancer. In particular, TEAD4-S is controlled by RBM4, a master regulator of many cancer-related splicing events. Activation of RBM4 increases the production of TEAD4-S, which in turn may potentially inhibit tumorigenesis through multiple oncogenic pathways.

We also noticed that the mRNA and protein levels of two TEAD4 isoforms are not always consistent across different cell lines and tissues (Figs 3a; and 5c,d), suggesting that the two isoforms may be differentially controlled in the levels of protein translation and/or degradation. This observation adds additional layer of complexity in controlling TEAD4 isoforms in addition to splicing regulation at RNA level. Adding to the complexity is the possibility that TEAD4-S functions as a dominant negative isoform to sequester/neutralize YAP imply that it may also antagonize all other TEAD paralogues. It is possible that this finding presents a general regulatory switch of all TEAD proteins, and future studies are warranted to fully address the role of all noncanonical TEAD isoforms.

Figure 4 | The splicing switch to TEAD4-S inhibits EMT and cancer cell proliferation. (a) Schematics of the engineered splicing factor (ESF-TEAD4) that regulates the splicing of TEAD4. PUF domain was engineered to recognize an 8-nt RNA sequence (GAGCCCTTG) in exon 3 of TEAD4, and fused with a glycine-rich motif of hnRNP A1 that can inhibit exon inclusion on binding to pre-mRNA. The resulting ESF, ESF-TEAD4, was designed to bind and inhibit the inclusion of exon 3 in TEAD4, thus inducing splicing switch towards TEAD4-S. (b) ESF-TEAD4 or control ESF was transfected into YAP-expressing H157 and A549 cells. RNAs were extracted from the transfected cells, and the splicing of TEAD4 was examined with RT–PCR. The representative gel was shown. The mean ± s.d. for the percentage of TEAD4-S mRNA was plotted from three experiments. (c) Total proteins were purified from the same set of transfected cells as in b, and the epithelial and mesenchymal markers were detected with western blots. (d) ESF-TEAD4 or control ESF was transfected into YAP-expressing H157 or A549 cells. Colony formation assay was carried out to examine tumour cell proliferation. All experiments were performed in triplicates, with mean ± s.d. of relative colony numbers plotted (P values from t-test). Images of the whole plate were shown.
controlled by a phosphorylation cascade that elicits rapid regulation, the new control mode through splicing may present a slower but more stable regulatory dynamics that is important to cancer cell reprogramming. We speculate that splicing misregulations of other components in the Hippo–YAP pathway also play critical roles in cancer development, and thus should be explored as a new route of potential cancer therapy.

Figure 5 | TEAD4-S can inhibit tumour growth in vivo. (a) H157 cells stably expressing YAP, YAP/TEAD4-FL, YAP/TEAD4-S, YAP/RBM4 or vector control were subcutaneously injected into the flank of nude mice. Each group contained nine mice. Pictures of the tumours removed after 17 days were shown. Scale bar, 1 cm. (b) The growth of xenograft tumours was monitored and the average sizes of xenograft tumours were measured every 2 days (n = 9, error bars indicate ± s.d., P < 0.05 by t-test). (c) Total RNA isolated from seven paired NSCLC tumours and adjacent normal tissues were assayed by semi-quantitative RT–PCR. The mean ± s.d. from three experiments was plotted. (d) TEAD4 levels from seven paired NSCLC tumours and adjacent normal tissues were analysed by western blot. (e) The splicing alteration of TEAD4 was examined in various cancers by analysing the TCGA consortium containing RNA-seq data sets from thousands of patients. The PSI of TEAD4 in the cancer or normal samples are presented as standard box plot, with the boxes presenting the first and third quartiles and the whiskers representing the 2nd and 98th percentiles. ***P < 0.0005; **P < 0.005; *P < 0.05; and NS, not significant. (f) The overall survivals of lung cancer and colon cancer patients with different ratios of TEAD4 splicing isoforms were analysed. (g) The model of how the Hippo–YAP pathway is regulated through a TEAD4 splicing switch.
**Methods**

**Splicing reporter constructs.** To construct the TEAD4 reporter, we used PCR reactions to amplify a fragment containing exon 2, part of intron 2, exon 3, part of intron 3 and exon 4 of TEAD4, and ligated this fragment to the pCDNA3-FLAG vector digested with Nhel/NotI. To generate TEAD4 reporters with mutated RBM4-binding sites, Quikchange approach was applied with different paired primers. All primers used in this study were listed in Supplementary Table 1.

**ESF expression constructs.** To express ESFs in cultured cells, we generated expression constructs using the pCI-neo vector (Promega). We started with an expression vector that encodes from N- to C-terminal, FLAN domain, Gly-rich domain of hnRNP A1 (residues 195–320 of NP_002127), and the MS2 coat protein (gift of Dr R. Brentnach form Institut de Biologie-CHR 1). The fragment encoding the MS2 coat protein fragment was removed using BamHI/SalI digestion and replaced with a fragment encoding a NLS (PPPKKRRK) and the PUromycin domain of RBM4 on tetracycline induction, we used To generate stable cell line expressing RBM4 on tetracycline induction, we used the pCDNA5-FRT/TO vector and 293 FlpIn/T-Rex cells (Invitrogen). The FLAG-microbiological culture method, and all the lines were free of mycoplasma.

**Human tissue total protein and cDNA panels.** Human Tissue total protein and complementary DNA (cDNA) panels were purchased from Ambio Company. Each panel contained 10 different human tissues, including the brain, colon, liver, kidney, heart, lung, skeletal muscles, pancreas, spleen and stomach.

**Cell culture and transfection.** 293 FlpIn/T-Rex cells were purchased from Invitrogen. All other cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). The HEK293 human embryonic kidney, Hela cervical cancer, 293 FlpIn/T-Rex and PANC-1 human pancreatic carcinoma cell lines were cultured in DMEM (high glucose) medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S). The A549 human lung carcinoma cell line was cultured in F-12 K medium containing 10% FBS and 1% P/S. The H157 human lung carcinoma cell line was cultured in RPMI-1640 medium containing 10% FBS and 1% P/S. The HT-1080 human fibrosarcoma and HepG2 human hepatocellular carcinoma cell lines were cultured in Eagle’s Minimum Essential Medium containing 10% FBS and 1% P/S. The MDA-MB-231 human breast cancer cell line was cultured in L-15 medium containing 10% FBS and 1% P/S. The cell lines were tested for mycoplasma contamination using the microbiological culture method, and all the lines were free of mycoplasma.

**To generate stable cell line expressing RBM4 on tetracycline induction, we used** pCDNA5-FRT/TO vector and 293 FlpIn/T-Rex cells (Invitrogen). The FLAG-tagged full-length RBM4 was cloned into the vector, and transfected with pOG44 in 1:9 ratio. The stably integrated cells were selected with 100 µg/ml of hygromycin at 2 days after transfection for ~2 weeks to obtain individual colonies. One day before the induction, the cells were transfected to hygromycin-free medium. The inducible constructs were inactivated reserve transfection in a final concentration of 2 µg/ml. The induced cells were collected at several time points after induction to extract RNA and perform further analysis.

**To determine the localization of TEAD4-FL or TEAD4-S, Hela cells were plated onto a coverslip in six-well plates 1 day before transfection. An amount of 1 µg of GFP-tagged TEAD4-FL or TEAD4-S vectors was transfected using lipofectamine 2000 according to the manufacturer’s protocols.** The supernatant media containing virus was collected by centrifugation to remove any cellular contaminant. The resulting viral particles were used to infect H157 cells, and stably integrated cells were selected with 100 µg/ml of puromycin for 1 week. The expression transgene was confirmed by Western blots before further analysis.

**To determine the effects of overexpression of RBM4 on TEAD4 splicing changes, 0.2 µg of TEAD4 mini-gene reporters was co-transfected with 0.4 µg of RBM4, using lipofectamine 2000 according to the manufacturer. After 48 h, cells were fixed for further immunofluorescence analysis.**

To stably express RBM4 (or other proteins) in PANCl cells (or other cells), we used lentiviral vectors. We transfected 293 cells with pCDH-flag-RBM4 or pCDH-flag-empty vectors as per the manufacturer’s protocols. The supernatant media containing virus was collected by centrifugation to remove any cellular contaminant. The resulting viral particles were used to infect H157 cells, and stably integrated cells were selected with 100 µg/ml of puromycin for 1 week. The expression transgene was confirmed by Western blots before further analysis.

**To determine the effects of overexpression of RBM4 on TEAD4 splicing changes, 0.2 µg of TEAD4 mini-gene reporters was co-transfected with 0.4 µg of RBM4, using lipofectamine 2000 according to the manufacturer. After 48 h, cells were collected for further analysis of RNA and protein levels.**

**Assay of splicing with semi-quantitative RT-PCR.** The total RNA was isolated from transfected cells with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions, followed by 1-h DNase I (Invitrogen) treatment at 37°C. Then, we heat inactivation of DNase I. Total RNA (2 µg) was then reverse-transcribed with SuperScript III (Invitrogen) using poly T primer, and one-tenth of the room temperature product was used as the template for PCR amplification (25 cycles of amplification, with trace amount of Cys-DCTP in addition to non-fluorescent rNTPs). Reverse transcription–PCR (RT–PCR) products were separated on 10% polyacrylamide gel electrophoresis (PAGE) gels, stained using a Typhoon 9400 scanner (Amersham Biosciences). The amount of each splicing isoform was measured using ImageQuant 5.2.

**Western blot.** Cells were lysed in lysis buffer containing 50 mM HEPES, 150 mM NaCl (4.38 g), 1 mM EDTA, 1% (w/v) CHAPS and Sigma protease inhibitor cocktail. Subsequently, the cell lysates were boiled in 2 × SDS–PAGE loading buffer for 10 min, and then resolved by 10% SDS–PAGE and transferred to the nitrocellulose membrane. All primary antibodies were diluted 1,000 times for western blotting if not specified. The following antibodies were used in this study: TEAD4 (αab58310) antibody, anti-Myc tag antibody (αab9106) and α-HA tag antibody (αab9110) were purchased from Abcam; N-cadherin (αab10921) antibody was purchased from BD; Vimentin (α5741) and YAP (α12395) antibodies were purchased from Cell Signaling Technology; and alpha-tubulin (αT5168, 1:5000 dilution) and FLAG M2 (αF1804) were purchased from Sigma-Aldrich. RBM4 antibody (#16141-1-AP) was purchased from Proteintech. Bound antibodies were visualized using the ECL kit (GE Healthcare).

**Clinical tissues samples collection.** Fresh lung cancer tissues and normal adjacent tissues were collected from patients with pathologically and clinically confirmed lung carcinomas. All human tumour tissues were obtained with written informed consent from patients or their guardians before participation in the study. The Institutional Review Board of the Dalian Medical University approved use of the tumour specimens in this study. All of tissue specimens were kept in liquid nitrogen and sectioned for protein or mRNA extraction.

**High-throughput mrNA-sequence and data analysis.** RNAs from H157 cells stably expressing YAP, YAP/TEAD4-FL, YAP/TEAD4-S, YAP/RBM4 and control vectors were purified using Trizol method, and subsequently cleaned using the RNAeasy Kit (Qiagen). The RNAs were digested in column with RNase-free DNase as per the manufacturer’s instruction. Total RNA not exceeding 3 µg was further used to purify polyadenylated RNA using the Illumina TruSeq Total RNA Sample Prep Kits. We used the Ribo-Zero Human to remove the cytoplasmic RNA. The mRNA purified was further analysed using the Bioanalyzer (Agilent Technologies) before generation of cDNA library with bar-coded ends. RNA-seq libraries were robotically prepared using the Illumina TruSeq Total RNA Sample Prep Kit according to the manufacturer’s protocol. The RNA-seq data set was deposited to the Gene Expression Omnibus with accession number GSE80372.

To estimate the gene expression levels, we used RSEM package and bowtie2 (refs 51,52) to align all reads to human reference genome (UCSC hg19 version). Then, we provided a fragment length distribution with options of “-frag-length-mean 75 ’ and ‘–frag-length-sd 10’ to calculate transcript expression levels. Subsequently, we used ESeq tool53 to examine differential expression genes of pairwise-comparison based on empirical Bayesian methods.

**Heat map.** We included genes that met the following criteria: (i) FPKM (fragments per kilobase of transcript per million mapped reads) values of a given gene are not equal in all samples; (ii) at least one of the FPKM values in all samples is ≥3; and (iii) the ratio of the maximum FPKM value and the minimum FPKM value in all samples is ≥2. Then, we used the log2 ratio of FPKM values of the included genes, normalized by the FPKM value of control sample, as input of Cluster 3.0 (ref. 34). We clustered the dataset using the hierarchical clustering method based on Pearson correlation with average linkage, and further viewed the results using Java TreeView.

We selected the cluster in this pattern—upregulated by YAP–TEAD434, but downregulated by YAP–TEAD305 and YAP–RBM4—as our target data set. This data set includes the genes that are differentially regulated by TEAD4 isoforms. The heat map shown is ordered by the FPKM value of YAP.

**The gene ontology analysis was performed using DAVID gene ontology analysis software to search for enriched pathways.** The functional association of TEAD4 targets were analysed using the protein interaction data from STRING database, generating a set of functional interaction networks. The sub-network containing more than five nodes was demonstrated.

**Soft agar assay.** Equal volumes of 1.2% agar and 2 × DMEM (or RPMI-1640) mediums were mixed and placed onto six-well dishes to generate 0.6% base agar. A549 cells (or H157 cells) expressing YAP, YAP/TEAD4-FL, YAP/TEAD4-S, YAP/RBM4 and control vectors (5,000 cells per dish) were seeded in the 10-cm dishes and incubated at 37°C in humidified incubator for 2 weeks. Colonies were fixed and stained with crystal violet, and the number of colonies was counted.

**Colony formation assay.** A549 cells (or H157 cells) expressing YAP, YAP/TEAD4-FL, YAP/TEAD4-S, YAP/RBM4 and control vectors (5,000 cells per dish) were seeded in the 10-cm dishes and incubated at 37°C in humidified incubator for 2 weeks. Colonies were fixed and stained with crystal violet, and the number of colonies was counted.

**Xenograft assays.** Forty-five 4-week-old female BALB/c nude mice were purchased from Vital River Laboratories for in vivo tumorigenesis study. The Institutional Animal Care and Use Committee of the Dalian Medical University approved the use of animal models in this study. Mice were injected with...
subcutaneously with 1 × 10^3 H157 cells expressing YAP, YAP/TEAD4-FL, YAP/TEAD4-S, YAP/RBM4 and control. Nine mice were used for each group. Mice were raised in the following 3 weeks. The mice were then monitored for tumour volume and overall health. The size of the tumour was determined by caliper measurement of the subcutaneous tumour mass every 3 days. Tumour volume was calculated according to the formula (4/3)πr^2h, (r, h). Each experimental group contained nine mice. At the end of 17 days, all mice were killed, and tumours were removed for further analysis. For all data points, three independent measurements were performed and means were used for calculation.

**RNA immunoprecipitation.** 293T cells (1 × 10^6) expressing RBM4 or control vector are collected and washed twice with 10 ml of PBS, and then resuspended in 10 ml of PBS. Formaldehyde (37% stock) is added to the above solution to a final concentration of 1% and incubated at room temperature for 10 min with slow rotating. Crosslinking reactions are quenched by the addition of glycine solution (pH 7.0) to a final concentration of 0.25 M, followed by incubation at room temperature for 5 min. The cells are collected by centrifugation at 700 g for 4 min at 4°C, followed by two washes with ice-cold PBS. Fixed cells are resuspended in 2 ml of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-CL, pH 7.5, 1% NP-40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl) containing protease inhibitors. The cells are subsequently lysed by three rounds of sonication. Insoluble material is removed by microcentrifugation at 16,000g for 10 min at 4°C. An aliquot of solubilized cell lysate is mixed with protein A–Sepharose beads along with non-specific competitor RNA. This mixture is rotated for 1 h at 4°C, followed by microcentrifugation at 1200g for 5 min. The supernatant is removed, and the immunoprecipitates are collected and then incubated with the Flag antibody diluted in 1% bovine serum albumin for 2 h. Subsequently, slides were rinsed twice with 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 M NaCl, 1 M urea, 0.2 mM phenylmethyl sulphonyl fluoride) by 10-min rotation at room temperature. The beads containing the immunoprecipitated samples are collected and resuspended in 100 μl of 50 mM Tris-CL, pH 7.0, 5 mM EDTA, 10 mM dithiothreitol and 1% SDS. Samples (resuspended beads) are incubated at 70°C for 45 min to reverse the crosslinks. The RNA is extracted from these samples using Trizol according to the manufacturer’s protocol, and reverse-transcribed into cDNA for PCR detection.

**Immunofluorescence staining.** To determine the localization of TEAD4-FL, TEAD4-S and YAP, we performed immunofluorescence assay. In brief, cells were plated on coverslips in 24-well plates. Cells were co-transfected with 100 ng of pCMV promoter/firefly luciferase reporter plasmid and different amount of YAP (100 ng), TEAD4-FL (100 ng), TEAD4-S (20 or 100 ng), 100 ng TEAD4-FL with increasing amounts of TEAD4-S (20, 50 and 100 ng) and RBM4 (100 ng) plasmids and 5 ng of pRL-TK Renilla plasmids using lipofectamine 2000 (Invitrogen). After 48 h of transfection and used for immunoprecipitation incubation or in passive lysis buffer (Promega) for luciferase assay measured with the Dual-Luciferase Reporter Assay System (Promega), using the TD-20/20 Luminometer (Turner Designs). The relative luciferase activities were determined by calculating the ratio of firefly luciferase activities over Renilla luciferase activities.

**Assay of CTGF and ITGB expression with real-time PCR.** The real-time PCR was performed using the Maxima SYBR Green qPCR Master Mix (Thermo Scientific) and a 7500 real-time PCR system (Life Technologies) according to the manufacturer’s instructions. The expression level of CTGF and ITGB was normalized to the endogenous expression of GAPDH.

**Statistics.** Statistical analyses of colony formation, soft agar and splicing changes were performed using Student’s t-test.
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Author contributions
Y.W. and Z.W. designed the experiments, interpreted the results and wrote the manuscript. Y.Q., W.H., H.Q., J.Z., H.W. and W.Z. performed the experiments. Q.L. and S.M. help to interpret the data. J.Y., X.F. and Y.T. analysed the RNA-seq and TCGA data.

Additional information
Accession codes: RNA-seq data that support the findings of this study have been deposited in Gene Expression Omnibus of NCBI with the accession code GSE80372.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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