Alternative splicing reverses the cell-intrinsic and cell-extrinsic pro-oncogenic potentials of YAP1

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In addition to acting as a transcriptional co-activator, YAP1 directly mediates translocalization of the pro-oncogenic phosphatase SHP2 from the cytoplasm to nucleus. In the cytoplasm, SHP2 potentiates RAS–ERK signaling, which promotes cell proliferation and cell motility, whereas in the nucleus, it mediates gene regulation. As a result, elucidating the details of SHP2 trafficking is important for understanding its biological roles, including in cancer. YAP1 comprises multiple splicing isoforms defined in part by the presence (as in YAP1-2γ) or absence (as in YAP1-2α) of a γ-segment encoded by exon 6 that disrupts a critical leucine zipper. Although the disruptive segment is known to reduce co-activator function, it is unclear how this element impacts the physical and functional relationships between YAP1 and SHP2. To explore this question, we first demonstrated that YAP1-2γ cannot bind SHP2. Nevertheless, YAP1-2γ exhibits stronger mitogenic and motogenic activities than does YAP1-2α because the YAP1-2α–mediated delivery of SHP2 to the nucleus weakens cytoplasmic RAS–ERK signaling. However, YAP1-2γ confers less in vivo tumorigenicity than does YAP1-2α by recruiting tumor-inhibitory macrophages. Mechanistically, YAP1-2γ transactivates and the YAP1-2α–SHP2 complex transrepresses the monocyte/macrophage chemotaxtractant CCL2. Thus, cell-intrinsic and cell-extrinsic pro-oncogenic YAP1 activities are inversely regulated by alternative splicing of exon 6. Notably, oncogenic KRAS down-regulates the SRSF3 splicing factor that prevents exon 6 skipping, thereby creating a YAP1-2α–dominant situation that supports a “cold” immune microenvironment.

YAP1 (yes-associated protein 1, and its paralog TAZ) is a transcriptional co-activator that interacts with a number of sequence-specific transcription factors, most notably TEADs, and thereby transactivates downstream target genes, which stimulates cell proliferation while preventing cell death (1). Accordingly, YAP1 is considered to be a pro-oncogenic co-activator, deregulation of which promotes the development of cancer (2). YAP1 shuttles between the cytoplasm and nucleus in response to various signals including the Hippo signal, which plays an important role in restricting organ size by controlling cell proliferation and apoptosis (3). The Hippo pathway contains two core serine/threonine kinases: MST and LATS. When the Hippo signal is off, nonphosphorylated YAP1 enters the nucleus, where it acts as a transcriptional co-activator by binding with TEADs (4, 5). When the Hippo signal is on, however, MST-activated LATS phosphorylates cytoplasmic YAP1 to prevent its nuclear translocalization (6). Thus, impaired Hippo signaling also contributes to neoplastic transformation of cells by deregulating YAP1 (7). The YAP1 gene comprises nine exons and generates at least eight differentially spliced YAP1 isoforms (YAP1-1α, YAP1-1β, YAP1-1γ, YAP1-1δ, YAP1-2α, YAP1-2β, YAP1-2γ, and YAP1-2δ) (Fig. 1A) (8, 9). YAP1-1 and YAP1-2 isoforms are made by exclusion and inclusion, respectively, of exon 4, which encodes the second WW domain. Skipping of exon 6, which encodes the γ-segment comprising a 16-amino acid sequence, gives rise to α isoforms, which are characterized by the presence of a functional leucine zipper motif that can mediate protein–protein interaction. Conversely, inclusion of exon 6 results in the generation of γ isoforms, which do not possess a leucine zipper because of insertion of the γ segment. Additionally, an alternative splice donor site in intron 5 makes a variant exon 5 transcript encoding a 4-amino acid (VRPQ) extension, which also disrupts the leucine zipper heptad repeats. Thus, variant exon 5 generates β and δ isoforms in the absence and presence of exon 6, respectively (Fig. 1A) (8, 9). YAP isoforms containing two WW domains exhibit stronger co-activator activity than do those having a single WW domain (10). Conversely, YAP1 isoforms lacking the canonical leucine zipper exhibit a dramatically reduced co-activator function (11). Of these YAP1 isoforms, YAP1-2α and YAP1-2γ are two well-studied YAP1 splicing isoforms that correspond to the shorter and longer forms of Yap1 (mouse ortholog of human YAP1), respectively (10, 12). In contrast to YAP1, there has been no report showing the presence of multiple splicing isoforms of human TAZ.

SHP2, encoded by the PTPN11 gene, is a ubiquitously expressed non–receptor-type tyrosine phosphatase. Gain-of-function mutations in PTPN11 have been found in a variety of sporadic human malignancies (13). Deregulation of SHP2 by the Helicobacter pylori CagA oncoprotein also plays an important role in the development of gastric cancer (14). Thus, SHP2 is regarded as a pro-oncogenic phosphatase. In the cytoplasm, SHP2 potentiates the magnitude of RAS–ERK signaling (15, 16), and indeed, recent studies have shown that the growth of cancer cells carrying oncogenic KRAS mutations is still dependent on SHP2 activity (17). Like YAP1, SHP2 is specifically present in the cytoplasm at a high cell density but is abundantly distributed in the nucleus at a low cell density. The cell...
Figure 1. Leucine zipper–mediated YAP1-2α/TAZ-SHP2 interaction promotes SHP2 nuclear localization. A, structure of the alternatively spliced transcript isoforms of the human YAP1 and mouse Yap1 gene. The second WW domain encoded by exon 4 (yellow) presents in hYAP1-2 isoforms and mouse Yap isoforms. The extended transcript of exon 5 encodes additional four amino acids, termed β-segment (purple), and the exon 6 encodes 16 amino acids, termed γ-segment (green). The presence of these additional amino acids disrupts the leucine zipper motif (blue).

B, co-precipitation analysis of HEK293T cells expressing FLAG–SHP2 and a various splicing isoform of HA-YAP1. The cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody. The total cell lysates (TCL) and the anti-FLAG immunoprecipitates (IP) were subjected to immunoblot analysis with the indicated antibodies. The experiment was performed three times independently, and the results were totally reproducible.

C, co-precipitation analysis of HEK293T cells co-expressing FLAG–SHP2 and HA-YAP1-2α (WT) or HA-YAP1-2αLZM (LZM). D, co-precipitation analysis of HEK293T cells co-transfected with the indicated expression plasmids. E, co-precipitation analysis of HEK293T cells co-transfected with plasmids expressing FLAG-YAP1-2α or FLAG-YAP1-2αLZM and Myc-TAZ or Myc-TAZLZM. F, co-precipitation analysis of HEK293T cells co-transfected with plasmids expressing FLAG-YAP1-2α or FLAG-YAP1-2β and Myc-TAZ, G, co-precipitation analysis of HEK293T cells co-transfected with plasmids expressing FLAG-SHP2 and HA-YAP1-2α, TAZ knockdown was performed by transfecting TAZ-specific shRNA#1. H, upper panels, AGS (left panels) and NIH3T3 (right panels) cells transiently transfected with an HA-YAP1-2αS127A or HA-YAP1-2γS127A vector were cultured at high cell density and then stained with an anti-SHP2 antibody (green), anti-HA antibody (YAP, red) and 4',6-diamino-2-phenylindole (DAPI, nuclei, blue). The arrows indicate cells showing nuclear distribution of HA-YAP1-2α or HA-YAP1-2γ. Scale bars, 20 μm. Lower panels, the nuclear/cytoplasmic ratio of the SHP2 expression level, which was determined by measuring fluorescence intensity using ImageJ, is plotted for each sample. The red bar represents the median value (n = 20). *, p < 0.05, analysis of variance post–Bonferroni correction.
density–dependent subcellular localization of SHP2 is primarily regulated by the interaction of SHP2 with YAP1, in which YAP1 acts as a carrier and SHP2 serves as a cargo (18). It was also shown that Shp2 (mouse ortholog of human SHP2) interacts with the shorter form of Yap1 (hereinafter referred to as “Yap1-2α”) but not with the longer form of Yap1 (hereinafter referred to as “Yap1-2γ”). Because these YAP1/Yap1 isoforms are generated by differential splicing, YAP1–SHP2 interaction is considered to be regulated by alternative splicing of YAP1 precursor mRNA (pre-mRNA) (18), suggesting that differential splicing not only influences the co-activator activity but also affects the SHP2-binding capability of YAP1. In the present study, we focused on YAP1-2α and YAP1-2γ as representative isoforms that harbor an intact leucine zipper and a disrupted leucine zipper, respectively. Contrary to our expectations, cell–intrinsic and cell-extrinsic pro-oncogenic activities are reversed between the two YAP1 isoforms, caused by the difference in their SHP2-binding capacity. Furthermore, inclusion of exon 6 into YAP1 pre-mRNA is promoted by the SRSF3 (serine and arginine-rich splicing factor 3) splicing factor, the expression of which is suppressed by oncogenic KRAS. The results reveal a complicated interplay between differentially spliced YAP1 isoforms and SHP2 in the regulation of cell-autonomous and non–cell-autonomous YAP1 activities, perturbation of which contributes to the formation of a differential tumor microenvironment.

Results

Functional leucine zipper of YAP1 is required for SHP2 interaction

To examine whether splicing variations of YAP1 affect SHP2-binding activity (18), SHP2 was ectopically expressed together with each of the YAP1 splicing isoforms in HEK293T human embryonic kidney cells. A co-immunoprecipitation experiment revealed that SHP2 associated most strongly with YAP1-2α and next-most strongly with YAP1-1α. YAP1-1β and 1-2β also bound SHP2 but much less efficiently. On the other hand, all of the γ and δ isoforms of YAP1, which contain the γ-segment, did not interact with SHP2 (Fig. 1B), indicating a critical role of the leucine zipper–based coiled-coil region in the YAP1–SHP2 interaction. Given that the 16-amino acid γ-segment is inserted into and thereby disrupts the leucine zipper motif in YAP1 (Fig. 1A) (8), the results suggested the importance of the leucine zipper in SHP2 binding. To test this idea, a leucine zipper mutant (LZM) of YAP1-2α (YAP1-2αLZM), in which five heptad repeats of leucines (Leu-311, Leu-318, Leu-325, Leu-332, and Leu-339) were all replaced by alanines, was expressed together with SHP2 in HEK293T cells. In contrast to YAP1-2α, YAP1-2αLZM failed to interact with SHP2 (Fig. 1C). A leucine zipper, which comprises a coiled-coil structure, interacts with another leucine zipper–mediated coiled-coil structure (19). Because SHP2 does not possess a leucine zipper, YAP1 was unlikely to bind SHP2 directly through the leucine zipper. In this regard, the YAP homolog TAZ possesses a functional leucine zipper, which is involved in YAP1 binding (20). To test whether YAP1–SHP2 interaction requires YAP1–TAZ heterodimerization, FLAG-tagged YAP1-2α was co-expressed with HA-tagged YAP1-2α, YAP1-2γ, or TAZ in HEK293T cells. Whereas YAP1-2α formed a complex with TAZ, no YAP1-2α homodimer or YAP1-2α–YAP1-2γ heterodimer was detected in the cells (Fig. 1D). Also, co-expression of YAP1-2α or YAP1-2αLZM and TAZ or a mutant TAZ (TAZLZM), in which four heptad repeats of leucines/isoleucines (Ile-240, Ile-247, Leu-254, and Leu-261) were all replaced by alanines, in HEK293T cells showed YAP1-2α–TAZ interaction but not YAP1-2αLZM–TAZ or YAP1-2α–TAZLZM interaction (Fig. 1E).

Hence, YAP1-2α and TAZ underwent heterodimerization through their leucine zippers. Notably, YAP1-2β, made by insertion of the β-segment (VRPQ sequence) into the leucine zipper of YAP1-2α, markedly reduced the ability of YAP1 to interact with TAZ (Fig. 1F). The observation was also supported by the predicted structural model of leucine zipper–mediated YAP1–TAZ interactions (Fig. S1, A and B). Inhibition of endogenous TAZ expression in HEK293T cells by three distinct TAZ-specific shRNAs (#1, #2, and #3) abolished YAP1-2α–SHP2 complex formation (Fig. 1G and Fig. S1C). These results indicated that binding of YAP1 with SHP2 requires heterodimerization between YAP1 and TAZ.

As previously reported, SHP2/Shp2 was localized to both the nucleus and cytoplasm at a low cell density (6.25 × 10^3 cells/cm^2), whereas it was excluded from the nucleus at a high cell density (6.25 × 10^4 cells/cm^2) (18). Because YAP1–SHP2 interaction was regulated by differential splicing of YAP1 pre-mRNA, nuclear translocalization of SHP2 was considered to be specifically mediated by YAP1 isoforms capable of binding with SHP2. To test this idea, constitutively active YAP1 mutants, YAP1-2αS127A and YAP1-2γS127A, both of which are localized to the nucleus independently of cell density (21), were co-expressed in AGS human gastric cancer cells and in nontransformed NIH3T3 mouse fibroblasts in a high–cell density condition. As a result, SHP2/Shp2 and YAP1-2αS127A were both localized in the nucleus (Fig. 1H, white arrows). On the other hand, SHP2/Shp2 was localized to the cytoplasm in cells expressing YAP1-2γS127A in the nucleus (Fig. 1H, yellow arrows). Accordingly, subcellular localization of SHP2 was determined by the SHP2-binding activity of the YAP1 isoforms.

The γ-segment confers stronger cell-intrinsic pro-oncogenic activity upon YAP1

Both SHP2 and YAP1 are known to be pro-oncogenic, positively regulating cell proliferation, as well as cell motility (1, 15). We therefore compared the pro-oncogenic potentials of YAP1-2α and YAP1-2γ. To this end, we established two independent YAP1-knockout cell clones from AGS cells (YAPIKO#1 and YAPIKO#2 AGS cells) using two distinct YAP1-sgRNAs (Fig. S2A). Whereas the level of SHP2 in AGS cells was not significantly changed by YAPI knockout, TAZ expression was slightly elevated in YAPIKO cells as previously reported (Fig. S2B) (22). An expression vector for YAPI-2α or YAPI-2γ was then reintroduced into these YAPIKO#1 or YAPIKO#2 AGS cells to establish AGS-derived stable transfectant clones that exclusively express one of the two YAP1 isoforms, referred to as YAPIKO#1/1-2α AGS and YAPIKO#2/1-2α AGS cells (Fig. 2A) or YAPIKO#2/1-2γ AGS and YAPIKO#2/1-2γ AGS cells (Fig. S2C), at comparable...
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Figure 2. Cell-intrinsic pro-oncogenic activities of YAP1-2a and YAP1-2y. A, YAP1-knockout AGS cells (YAP1KO#1 AGS) were established by using CRISPR–Cas9 system. Control indicates AGS cells transfected with the CRISPR-Cas9 empty vector (pX330). YAP1KO#1/1-2a AGS cells and YAP1KO#1/1-2y AGS cells were made by stably transfecting expression vectors for sgRNA-resistant HA-YAP1-2a and HA-YAP1-2y, respectively, into YAP1KO#1 AGS cells. The total cell lysates prepared were subjected to immunoblot analysis with the respective antibodies. B, left panel, YAP1KO#1, YAP1KO#1/1-2a, and YAP1KO#1/1-2y AGS cells were stained with anti-SHP2 antibody (green), anti-HA antibody (YAP1, red), and 4',6-diamino-2-phenylindole (DAPI, nuclei, blue). Scale bars, 20 μm. Right panel, the nuclear/cytoplasmic ratio of SHP2 is plotted for each sample. The red bar represents the median value. The nuclear/cytoplasmic ratio of SHP2 distribution at low cell density (6.25 × 10^3 cells/cm^2) was determined by using ImageJ (n = 20). *, p < 0.05, analysis of variance post–Bonferroni correction. C, growth curves of control, YAP1KO#1, YAP1KO/1-2a, and YAP1KO/1-2y AGS cells were made by counting viable cells at days 1, 2, 3, and 4. Control indicates AGS cells transfected with the CRISPR–Cas9 empty vector (pX330). Error bars indicate means ± S.D. (n = 3). *, p < 0.05, Student's t test. D, total cell lysates from YAP1KO#1, YAP1KO/1-2a, and YAP1KO/1-2y AGS cells were subjected to immunoblot analysis with the respective antibodies. 

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levels. In \(YAP1^{ko}\#1\) AGS or \(YAP1^{ko}\#1/1-2\gamma\) cells, SHP2 was localized to the cytoplasm at a low cell density because of the lack of interaction with YAP1 (Fig. 2B, bottom row). On the other hand, SHP2 was accumulated in the nucleus of \(YAP1^{ko}\#1/1-2\alpha\) cells even at a low cell density because of the YAP1–SHP2 complex formation (Fig. 2B, middle row). Consistent with the notion that YAP1 is pro-mitogenic, growth of \(YAP1^{ko}\#1\) or \(YAP1^{ko}\#2\) AGS cells was substantially retarded compared with that of parental AGS cells, and the retarded growth was restored by re-expressing YAP1-2\(\alpha\) or YAP1-2\(\gamma\) in \(YAP1^{ko}\#1\) or \(YAP1^{ko}\#2\) AGS cells (Fig. 2C and Fig. S2D). Notably, \(YAP1^{ko}\#1\) or \(YAP1^{ko}\#2\) AGS cells reconstituted with YAP1-2\(\alpha\) grew faster than did \(YAP1^{ko}\#1\) or \(YAP1^{ko}\#2\) AGS cells reconstituted with YAP1-2\(\alpha\) (Fig. 2C and Fig. S2D). Because a luciferase reporter assay using AGS or NIH3T3 mouse fibroblasts revealed that YAP1-2\(\alpha\) exhibits stronger transcriptional co-activator activity toward TEAD than YAP1-2\(\gamma\) does as previously described (Fig. S2E) (10, 11), the difference in growth stimulation could not be explained by their differential co-activator activities. Because cytoplasmic SHP2 is critically involved in activation of the RAS–ERK signaling pathway (16, 23), a reduction in the level of cytoplasmic SHP2 by YAP1-2\(\alpha\) was suspected to be involved in the diminished mitogenic activity of YAP1-2\(\alpha\) compared with that of YAP1-2\(\gamma\). In fact, the level of RAS–ERK signal activation, determined by the phosphorylated/active form of ERK (pERK) (AGS cell), was reduced in \(YAP1^{ko}\#1\) or \(YAP1^{ko}\#1/1-2\alpha\) AGS cells but was elevated in \(YAP1^{ko}\#1/1-2\gamma\) AGS cells compared with that in \(YAP1^{ko}\#1\) AGS cells (Fig. 2D). As in the case of YAP1, knockdown of Taz expression in NIH3T3 cells by specific siRNA gave rise to an increase in the level of cytoplasmic Shp2 at a low cell density (Fig. S2F), which was concomitantly associated with the increase in the active form of Erk (pErk) (Fig. S2G). To substantiate this idea, WT SHP2 or an SHP2 mutant that cannot bind YAP1 (SHP2-PA) and thus is retained in the cytoplasm (18) was reintroduced into \(YAP1^{ko}\#1/1-2\alpha\) AGS or \(YAP1^{ko}\#2/1-2\alpha\) AGS cells. The cell growth curve revealed that ectopic expression of SHP2, especially SHP2-PA, gave rise to a substantial increase in cell growth (Fig. 2E and Fig. S2H). A wound-healing assay was also conducted to compare the effects of YAP1-2\(\alpha\) and YAP1-2\(\gamma\) on cell motility (24, 25). Both YAP1-2\(\alpha\) expression and YAP1-2\(\gamma\) expression enhanced the motility of \(YAP1^{ko}\#1\) AGS cells, and again, the motogenic activity of YAP1-2\(\gamma\) was stronger than that of YAP1-2\(\alpha\) (Fig. 2F). Next, to investigate the oncogenic potential of the YAP1 splicing isoforms in vitro, a colony formation assay was performed to examine whether these YAP1 isoforms are capable of conferring anchorage-independent growth, the well-recognized neoplastic trait of cells in vitro (5, 26), using nontransformed NIH3T3 fibroblasts. To do so, NIH3T3 cells were transduced with a lentivirus expressing YAP1-2\(\alpha\) or YAP1-2\(\gamma\) and subjected to a soft agar assay. No colonies were developed by NIH3T3 cells, and a few colonies with small sizes were formed by NIH3T3 cells expressing YAP1-2\(\alpha\) (Fig. 2G). The weak colony-forming activity of YAP1-2\(\alpha\) was substantially potenti-ated by co-expressing SHP2-PA (Fig. S2I), indicating that cytoplasmic SHP2 is critically associated with the colony-forming activity of YAP1. From these observations, we concluded that YAP1-2\(\gamma\) exhibited greater in vitro pro-oncogenic activities than YAP1-2\(\alpha\) did, most likely being caused by the differential SHP2-binding activity between YAP1-2\(\alpha\) and YAP1-2\(\gamma\).

**In vivo tumorigenicity of NIH3T3 cells expressing YAP1-2\(\alpha\) or YAP1-2\(\gamma\)**

To consolidate the in vitro observations that YAP1-2\(\gamma\) is more oncogenic than YAP1-2\(\alpha\) in an in vivo situation, a tumor formation assay was performed in nude mice again using nontransformed NIH3T3 mouse fibroblasts. Parental NIH3T3 cells are nontumorigenic in nude mice and have long been used to evaluate in vivo oncogenic potential of genes of interest, especially those constituting the RAS signaling pathway. To this end, five pairs of stable NIH3T3 transfectants (pairs 1–5) that express comparable levels of YAP1-2\(\alpha\) or YAP1-2\(\gamma\), as determined by the HA-tag levels, were established (Fig. 3A, upper panels). All of the mice injected with the pairs of NIH3T3 transfectants developed palpable tumors, whereas injection of parental NIH3T3 cells did not result in tumor development, indicating that both YAP1-2\(\alpha\) and YAP1-2\(\gamma\) conferred in vivo tumorigenicity upon NIH3T3 cells. In striking contrast to the results obtained in in vitro studies, however, the sizes of the tumors induced by YAP1-2\(\alpha\) were much larger than those induced by YAP1-2\(\gamma\) in all five pairs (Fig. 3A, bottom panels).

Histopathological examination revealed massive infiltration of F4/80- and CD11b-positive macrophages into YAP1-2\(\gamma\)-induced NIH3T3 tumors but not into YAP1-2\(\alpha\)-induced NIH3T3 tumors (Fig. 3B). To examine whether the macrophage infiltration was responsible for the reduced in vivo tumorigenicity, nude mice were pretreated with clodronate liposome, which specifically depletes macrophages in vivo (27). In mice treated with clodronate, YAP1-2\(\gamma\)-expressing NIH3T3 cells gave rise to tumors with sizes that were comparable with or even larger than those of tumors formed from YAP1-2\(\alpha\)-expressing NIH3T3 cells (Fig. 3C, upper panel and lower left panel). Reduced macrophage infiltration into YAP1-2\(\gamma\)-induced NIH3T3 tumors in clodronate-treated mice was confirmed by immunohistochemical analysis (Fig. 3C, lower right panel). On the other hand, YAP1-2\(\alpha\)-induced NIH3T3 tumors consistently grew in vivo without inducing macrophage infiltration regardless of clodronate treatment.

**Mechanism underlying macrophage recruitment by NIH3T3 tumor cells expressing YAP1-2\(\gamma\) in vivo**

To elucidate the mechanism by which YAP1-2\(\gamma\)-expressing NIH3T3 cells recruited macrophages in vivo, total RNAs were extracted from two pairs of NIH3T3 transfectants (pairs 4 and 5) and subjected to RNA-Seq analysis using a mouse cancer-targeted RNA panel (28) to identify genes differentially expressed between the cell pairs. Fig. 4A shows the top four genes for which the relative mRNA levels were higher in YAP1-2\(\gamma\)-expressing NIH3T3 cells than in YAP1-2\(\alpha\)-expressing NIH3T3 cells (Ccl2, Pdk4, Bbc3, and Nrp1) and the top four genes for
which the relative mRNA levels were higher in YAP1-2α–expressing NIH3T3 cells than in YAP1-2γ–expressing NIH3T3 cells (Edn1, Ctgf, Col5a2, and Wnt5a) by RNA-Seq analysis. Among those genes, the greatest difference in expression was observed in mRNA for C-C motif chemokine ligand 2 (CCL2 in humans, Ccl2 in mice), a major chemoattractant of monocytes/macrophages (29). In both pairs 4 and 5, YAP1-2γ–expressing NIH3T3 cells gave rise to an approximately 2-fold increase in Ccl2 mRNA compared with that in cells expressing YAP1-2α (Fig. 4A and Fig. S3A). CCL2/Ccl2 has been shown to be a direct transcriptional target of TEAD/Tead (30, 31). As previously reported (11), YAP1-2α was more potent in activating TEAD/

Figure 3. In vivo tumorigenicity of NIH3T3 cells transformed by YAP1-2α or YAP1-2γ. A, upper panel, immunoblot analysis of HA-YAP1-2α or HA-YAP1-2γ expression in five pairs of NIH3T3 transformants (pairs 1–5; each NIH3T3 transformant pairs express comparable levels of ectopic YAP1-2α and YAP1-2γ). Lower panel, each of the NIH3T3 transformant pairs was subcutaneously inoculated into the left and right flanks of the same nude mice. Tumors were dissected from the mice for visual inspection. Scale bars, 1 cm. B, left panel, formalin-fixed and paraffin-embedded serial sections of the NIH3T3-derived tumor tissues were immunostained with an anti-F4/80 antibody and an anti-CD11b antibody. F4/80- or CD11b-positive cells were visualized by 3,3′-diaminobenzidine staining. Scale bars, 20 μm. Right panel, 3,3′-diaminobenzidine (DAB)–stained areas were quantitated by ImageJ analysis. S.D. and averages were calculated from technical triplicate. Error bars indicate means ± S.D. (n = 3). **, p < 0.01, Student’s t test. C, upper panel, nude mice pretreated with control liposome or clodronate liposome were subcutaneously inoculated with the NIH3T3 transformants stably expressing YAP1-2α or YAP1-2γ. Tumors were dissected from the mice for visual inspection. Scale bars, 1 cm. Lower left panel, tumor volumes derived from NIH3T3 cells transformed by YAP1-2α and YAP1-2γ were calculated by use of the modified ellipsoid formula 1/2 (length × width²). The sizes of tumors were plotted for each sample. Tumor volumes for each pair of YAP1-2α–expressing and YAP1-2γ–expressing NIH3T3 cells, which were injected into the same mice, are shown as circles with the same color and connected with dotted lines. Lower right panel, immunohistochemical staining of tumor with an anti-F4/80 antibody. Scale bar, 10 μm.
Figure 4. YAP1-2α and YAP1-2γ opposingly regulate CCL2/Ccl2 expression at transcriptional level via SHP2. A, the results of RNA-Seq using next-generation sequencing are shown as bar graphs, in which relative mRNA expression levels of each gene in YAP1-2α–expressing NIH3T3 cells are shown as 1 (dotted lines). Top four genes in which the relative mRNA levels were higher in YAP1-2γ–expressing NIH3T3 cells and top four genes in which the relative mRNA level were lower in YAP1-2γ–expressing NIH3T3 cells are presented. The data are obtained using pair 4 and pair 5 cells. B, qRT-PCR analysis of Ccl2 mRNA expression in NIH3T3 cells transiently transfected with YAP1-2α, YAP1-2γ, YAP1-2αLZM, or control empty vector. S.D. and averages were calculated from technical triplicate. Error bars indicate means ± S.D. (n = 3). *, p < 0.05; **, p < 0.01, Student’s t test. C, qRT-PCR analysis of Ccl2 mRNA expression in control NIH3T3 cells, Shp2KO#1 NIH3T3 cells, Shp2KO#1 NIH3T3 cells reconstituted with sgRNA-resistant SHP2, NIH3T3 cells reconstituted with sgRNA-resistant SHP2-PA, or NIH3T3 cells reconstituted with sgRNA-resistant SHP2C459S. S.D. and averages were calculated from technical repeats (three repeats per sample at one experiment). Control indicates NIH3T3 cells transfected with the CRISPR–Cas9 empty vector (pX330). S.D. and averages were calculated from technical triplicate. Error bars indicate means ± S.D. (n = 3), **, p < 0.01, Student’s t test. Lower panels, cell lysates were subjected to immunoblot analysis with an anti-SHP2 antibody and an anti-actin antibody. D, Left panel, HARP plot analysis of the human CCL2 and mouse Cc12 enhancer/promoter regions. Right panel, the conserved M-CAT sequence motif between human CCL2 and mouse Cc12 enhancer region. E, upper panel, chromatin was prepared from NIH3T3 cells fixed with formaldehyde. The ChIP was performed with an anti-SHP2 antibody or anti-IgG antibody (as a control). The immunoprecipitated (IP) DNAs were quantified by qRT-PCR using the primer set for the Ccl2 -1040 bp/-950 bp promoter region. S.D. and averages were calculated from technical triplicate. Error bars indicate means ± S.D. (n = 3), **, p < 0.01, Student’s t test. Lower panel, the immunoprecipitated DNAs were detected by RT-PCR using the primer set for the Ccl2 -1040 bp/-950 bp promoter region. F, A luciferase reporter plasmid for the Ccl2 promoter was transfected with or without a Myc-SHP2 vector in NIH3T3 cells. The bar graph shows the relative luciferase activity. S.D. and averages were calculated from technical triplicate. Error bars indicate means ± S.D. (n = 3), **, p < 0.01, Student’s t test. The protein expression levels in these cells were determined by immunoblot analysis with the respective antibodies. G, luciferase reporter assay was conducted to determine the Ccl2 promoter activity in parental NIH3T3 or Shp2KO#1 NIH3T3 cells transfected with a YAP1-2α, YAP1-2γ, or control empty vector (pSP65SRA). The bar graph shows the relative luciferase activity. S.D. and averages were calculated from technical triplicate. Error bars indicate means ± S.D. (n = 3), *, p < 0.05; **, p < 0.01. Student’s t test. Lower panel, the protein expression levels in these cells were determined by immunoblot analysis with the respective antibodies. n.s., not significant.
Tead reporter than YAP1-2γ was (Fig. S2E), and indeed YAP1-2α transactivated Cytb1 and Cigf, the unique Tead target genes, more strongly than YAP1-2γ by qRT-PCR analysis (Fig. S3B). In striking contrast, YAP1-2γ was more potent than YAP1-2α in Ccl2 activation (Fig. 4B). Given this, we hypothesized that TEAD-mediated CCL2 induction was repressed by binding of YAP1 with SHP2. Indeed, disruption of the leucine zipper in YAP1-2α (YAP1-2αLZΔ) converted YAP1-2α from a repressor to an activator of Ccl2 expression, indicating that SHP2 binding repressed the co-activator function of YAP1 (Fig. 4B). To corroborate the conclusion, Shp2-knockout NIH3T3 (Shp2KO#1 NIH3T3) cells were generated by using the CRISPR–Cas9 system. Whereas Shp2 knockout did not alter the levels of Yap1 and Taz protein expression (Fig. S3C), it caused a marked increase in the level of Ccl2 mRNA (Fig. 4C). Re-expression of SHP2 not only promoted cell growth (data not shown) but also restored the Ccl2 mRNA to the original level (Fig. 4C). Although the phosphatase-dead SHP2C459S mutant retained the ability to repress Ccl2 transactivation, SHP2-PA, which cannot bind YAP1, failed to do so (Fig. 4C). These results indicated that YAP1-2α–SHP2 complex formation but not SHP2 phosphatase activity is required for the repressor function of SHP2. The results provided compelling evidence for the transrepressional role of SHP2/Shp2 in CCL2/Ccl2 expression.

TEAD transcription factors recognize and bind to the M-CAT element (5′-CATTTCC/T/A-3′) (32). A canonical M-CAT element was found between 989 and 983 bp residues upstream of the transcription start site of the mouse Ccl2 promoter, which was also conserved in the human CCL2 promoter (Fig. 4D). To determine whether Shp2 is physically associated with the M-CAT element in NIH3T3 cells, a ChIP experiment was performed using an anti-Shp2 antibody. The M-CAT sequence was amplified using a primer set for the M-CAT–containing Ccl2 promoter region (−1040 bp/−950 bp) (Fig. 4E). Furthermore, ectopically expressed FLAG–SHP2 was co-precipitated with Tead in Yap1KO#1/1-2α NIH3T3 cells but not in Yap1KO#1/1-2γ NIH3T3 cells, indicating the presence of the SHP2/YAP1-2α/Tead heterotrimeric complex (Fig. S3D). Likewise, ectopically expressed FLAG-YAP1 was co-precipitated with Shp2 in Yap1KO#1/1-2α NIH3T3 cells but not in Yap1KO#1/1-2γ NIH3T3 cells, indicating the presence of the Shp2/YAP1-2α/Tead heterotrimeric complex (Fig. S3E). Next, we generated three distinct luciferase reporter constructs by introducing mouse Ccl2 promoter sequences with various lengths (−1200 bp/+85 bp, −600 bp/+85 bp, −300 bp/+85 bp) upstream of the luciferase gene. The reporters were transfected into NIH3T3 cells and luciferase activity was measured. As a result, the activity of the Ccl2−1200 bp/+85 bp reporter, but not that of the Ccl2−600 bp/+85 bp or Ccl2−300 bp/+85 bp reporter, was diminished by co-expression of SHP2 (Fig. 4F), indicating the presence of an SHP2-dependent repressor element between −1200 bp and −600 bp of the Ccl2 promoter, in which the M-CAT element was also present. Transfection of the Ccl2−1200 bp/+85 bp reporter together with YAP1-2α, YAP1-2γ or a control empty vector into NIH3T3 cells revealed that the reporter activity was inhibited by YAP1-2α but was potentiated by YAP1-2γ (Fig. 4G and Fig. S3F). WT Shp2 inhibited the Ccl2−1200 bp/+85 bp luciferase reporter activity more strongly than did SHP2-PA, which cannot bind YAP1 (Fig. S3G). Co-activator function of YAP1-2α on the Ccl2−1200 bp/+85 bp luciferase reporter was not observed in Shp2KO#1 NIH3T3 cells (Fig. 4G). Importantly, in this experiment, YAP1-2α (shown in a blue bar) was converted from a repressor (2nd bar from the left) to a co-activator (2nd bar from the right) toward the Ccl2−1200 bp/+85 bp promoter as was the case with YAP1-2γ in Shp2KO NIH3T3 cells. Accordingly, YAP1-2γ/TEAD, which cannot bind SHP2, stimulated CCL2 transcription, whereas YAP1-2α/TEAD repressed CCL2 transcription when YAP1-2α formed a complex with SHP2.

**Mechanisms regulating alternative exon 6 splicing in YAP1 pre-mRNA**

To gain insights into the mechanism regulating the alternative splicing of exon 6 in YAP1 pre-mRNA, we searched for splicing factor-binding motifs in YAP1 exon 6 using the online database SpliceAid (http://www.introni.it/splicing.html) (33), and we identified SRSF3 consensus binding motifs in the database (Fig. S4A). To quantitatively evaluate the frequency of exon 6 inclusion/skipping in YAP1 pre-mRNA, a primer set selectively amplifying the exon 6–containing YAP1 isoforms by RT-PCR was designed (Fig. S4B, red arrows). An additional primer set, which amplifies a sequence encoded by exon 1 and exon 2, was also made to quantify the total YAP mRNA levels (Fig. S4B, blue arrows). To test whether SRSF3/Srsf3 was involved in the exon 6 splicing of YAP1 pre-mRNA, SRSF3-knockout AGS (SRSF3KO#1 AGS, SRSF3KO#2 AGS) cells (Fig. 5A) or Srsf3 knockout NIH3T3 (Srsf3KO#1 NIH3T3, Srsf3KO#2 NIH3T3) cells (Fig. S4C) were generated by using the CRISPR–Cas9 system. The levels of YAPI/Yapi mRNAs containing exon 6 were markedly reduced in this SRSF3/Srsf3-knockout cells (Fig. 5B and Fig. S4D). Notably, Srsf3 knockout did not influence the levels of Yap1 and Shp2 protein expression in NIH3T3 cells (Fig. S4E).

We next examined whether SRSF3/Srsf3 was actually involved in the regulation of YAP1/Yap1-mediated CCL2/Ccl2 expression by measuring Ccl2 mRNA levels in NIH3T3 or Srsf3KO#1 NIH3T3 cells by qRT-PCR analysis. As a result, Srsf3-knockout NIH3T3 cells displayed a substantially reduced level of Ccl2 mRNA, which was restored and even further boosted by ectopic re-expression of SRSF3. (Note that human SRSF3 and mouse Srsf3 are 100% identical at the amino acid level) (Fig. 5C). Furthermore, SRSF3 knockout did not substantially influence the growth potentials of Yap1KO#1 AGS, Yap1KO#1/1-2α AGS, and Yap1KO#1/1-2γ AGS cells (Fig. S4F), indicating that SRSF3-mediated splicing regulation of YAP1 was responsible for the differential growth of these cells. Reciprocal regulation of CCL2 expression by YAP1-2α and YAP1-2γ was also shown at the protein level in AGS cells, although the effect was rather weak, possibly because intracellular CCL2 levels were measured by immunoblotting (Fig. S4G). Ectopic expression of SRSF3 also stimulated Ccl2−1200 bp/+85 bp reporter activity in parental NIH3T3 cells but not in Yap1KO#1 NIH3T3 cells (Fig. 5D). Collectively, these observations indicated that SRSF3/Srsf3 elevated CCL2/Ccl2 mRNA by promoting incorporation of exon 6 into YAP1/Yap1 mRNA, the
Oncogenic KRAS signal influences YAP1 exon 6 splicing via SRSF3

Oncogenic KRAS has been shown to promote nuclear trans-localization of SHP2 (34), raising the possibility that it enhances exon 6 skipping in YAP1/Yap1 pre-mRNA. To test this idea, NIH3T3 cells stably expressing moderate and comparable levels of oncogenic KRAS were established (Fig. 6A and Fig. S5A). qRT-PCR analysis revealed that inclusion of exon 6 in Yap1 mRNA was markedly decreased in NIH3T3 cells upon expression of oncogenic KRAS (Fig. 6B). Consistently, expression of Srsf3 was also reduced in NIH3T3 cells stably expressing oncogenic KRAS (Fig. 6C). As a result, oncogenic KRAS promoted
exon 6 skipping in YAP1 pre-mRNA by reducing the SRSF3/Srsf3 protein level. Because treatment with a MEK inhibitor or a proteasome inhibitor partially restored the oncogenic KRAS-mediated reduction in Srsf3 expression in NIH3T3 cells (Fig. S5A), Ras-Erk signal activation might be involved at least partly in the reduction of Srsf3 (35).

KRAS is most frequently mutated in pancreatic and colorectal cancers (36). Kaplan–Meier analysis of the TCGA database (37, 38) revealed that survival of patients with a low level of SRSF3 expression was inferior to that of patients with high SRSF3 expression for both cancers (Fig. S5B). The survival of patients with a low level of CCL2 expression was also inferior to

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**Figure 6. Activation of KRAS signaling alters YAP1 exon 6 splicing by suppressing SRSF3 expression.**

A, establishment of NIH3T3 cells stably expressing KRAS<sup>G12D</sup> and KRAS<sup>G12V</sup>. Empty indicates NIH3T3 cells transfected with the empty vector (pSP65SR<sup>a</sup>). The protein levels expressed in these cells were determined by immunoblot analysis with the respective antibodies. The values shown below the second row (Srsf3) indicate relative densities of the Srsf3 bands. B, relative ratio of exon 6-containing Yap1 mRNAs versus total Yap1 mRNAs in NIH3T3 cells, KRAS<sup>G12D</sup>-expressing NIH3T3 cells, or KRAS<sup>G12V</sup>-expressing NIH3T3 cells was determined by qRT-PCR. S.D. and averages were calculated from technical triplicates. Error bars indicate means ± S.D. (n = 3), **, p < 0.01, Student’s t test. C, relative expression of Srsf3 mRNA in NIH3T3 cells, KRAS<sup>G12D</sup>-expressing NIH3T3 cells, or KRAS<sup>G12V</sup>-expressing NIH3T3 cells was determined by qRT-PCR. S.D. and averages were calculated from technical triplicates. Error bars indicate means ± S.D. (n = 3), *, p < 0.05, Student’s t test. D, the expression level of CCL2 mRNA is lower in patients with KRAS mutations in both pancreatic cancer (left panel) and colon (right panel) cancer patients.
that of patients with a high level of CCL2 expression for pancreatic cancer (Fig. S5C). A co-expression study showed a significant positive correlation between SRSF3 and CCL2 in colon cancer (Fig. S5D). Reciprocally, a negative correlation was observed between SHP2 expression and CCL2 expression (Fig. S5E). CCL2 expression levels were lower in pancreatic and colon cancer patients carrying KRAS mutations (Fig. 6D). These clinical data also support the notion of interplay between KRAS and SRSF3 in regulating YAP1 mRNA splicing that influences the prognosis of patients with cancers harboring KRAS mutations.

Discussion

YAP1-2α and YAP1-2γ are two representative isoforms of human YAP1 that are generated by alternative splicing of exon 6, which encodes the γ-segment (8). Inclusion of the γ-segment disrupts the leucine zipper motif in YAP1. Thus, YAP1-2α, lacking exon 6, possesses a functional leucine zipper, whereas YAP1-2γ, containing exon 6, does not. In the present study, we found that YAP1-2α interacts with SHP2 in a manner that depends on the functional leucine zipper. We also found that YAP1-2α forms a heterodimer with TAZ via functional leucine zippers. Taken together with the results of previous work (18, 21), our results suggest that the proline-rich tail of SHP2 may interact with one of two WW domains in YAP1-2α only when YAP1-2α and TAZ are heterodimerized. A reduced YAP1-1α association with SHP2 compared with YAP1-2α is most likely because YAP1-1α has only a single WW domain. Upon complex formation, YAP1-2α/TAZ and SHP2 act as a carrier and a cargo, respectively, so that SHP2 is efficiently translocated from the cytoplasm to the nucleus.

The present study revealed that YAP1-2γ stimulates cell proliferation and motility, as well as anchorage-independent growth of nontransformed cells, more strongly than YAP1-2α does. The difference is due to differential binding of the YAP1 isoforms with SHP2. Because YAP1-2γ does not bind SHP2, YAP1-2γ expression does not diminish cytoplasmic SHP2 below a certain threshold level and thereby potentiates RAS–ERK signaling that stimulates cell proliferation and cell motility. Consequently, YAP1-2γ confers greater cell-intrinsic pro-oncogenic potential upon nontransformed cells than YAP1-2α does. In contrast to these in vitro observations, cells expressing YAP1-2α are more potent in vivo tumorigenesis than are cells expressing YAP1-2γ. The inverted oncogenic potential between YAP1-2α and YAP1-2γ in vivo is due to the difference in the magnitude of macrophage recruitment to the tumor tissue, which is explained by the finding that YAP1-2γ–predominant tumor cells secrete CCL2, the chemokine that potently recruits macrophages to the tumor site (39), whereas YAP1-2α–predominant tumor cells do not. Mechanistically, CCL2 is transactivated by the YAP1/TEAD complex (30, 31), and we identified the TEAD-binding cis-element in the CCL2 promoter in the present study. YAP1-2γ/TEAD transactivates CCL2 by binding to the identified cis-element and constitutively transactivates CCL2 in YAP1-2γ–predominant tumor cells, allowing infiltration of tumor-inhibitory macrophages into tumors in vivo. On the other hand, YAP1-2α/TEAD is converted from a transactivator to a transrepressor upon binding of YAP1-2α with SHP2. The lack of CCL2 production/secretion in YAP1-2α–predominant tumor cells dampens macrophage accumulation in vivo. Our study therefore revealed that YAP1-2α and YAP1-2γ have opposing cell-extrinsic roles in establishing a tumor microenvironment. It is not unprecedented that cell-autonomous and non–cell-autonomous biological activities of a single molecule can lead to opposing outcomes in tumorigenesis. The Hippo signal kinase LATS acts as a tumor suppressor by preventing nuclear translocalization of YAP1. However, a recent study showed that LATS inhibits acquired tumor immunity in vivo (40). Also notably, whereas CCL2 recruits macrophages, polarization of infiltrated macrophages into a tumor-inhibitory (M1) or a tumor-stimulatory (M2) subtype is context-dependent, determined by combined effects of multiple humoral and cellular factors (41). In fact, several studies have shown that CCL2 recruits M2 macrophages rather than M1 macrophages (31).

Alternative splicing is mediated by the spliceosome, which is composed of small nuclear ribonucleoproteins (snRNPs) and non-snRNPs. Non-snRNPs include serine/arginine-rich splicing factor (SRSF) family proteins, which bind to pre-mRNA and regulate the interaction between snRNPs and pre-mRNA, thereby stimulating or suppressing the recognition of splicing sites (42). SRSF3, also known as Srp20, is the smallest member of the SRSF family. It is variably expressed in many different cell types and has been shown to play a key role in the regulation of alternative exon skipping/inclusion in a variety of genes, including cancer-associated genes such as CD44, PKM, TP53, and RAC1 (43, 44), and the present work adds YAP1 to this list. In the present study, SRSF3-knockout cells showed ~50% reduction in YAP1 mRNA isoforms containing exon 6, which was concomitantly associated with markedly reduced CCL2 mRNA expression, corroborating the role of the SRSF3/YAP1/SHP2 repressor complex. Consequently, SRSF3 is overexpressed in various cancers and has been considered to be pro-oncogenic (44). This notion is not inconsistent with the results of the present study showing that YAP1-2γ production, which is promoted by SRSF3, exhibits greater cell-intrinsic pro-oncogenic potential upon YAP1 by retaining SHP2 in the cytoplasm, which strengthens RAS–ERK signaling. Intriguingly, however, oncogenic KRAS was found to inhibit SRSF3 expression, a finding that is associated with the dominant expression of YAP1 isoforms lacking exon 6 over those containing exon 6 in cells expressing oncogenic KRAS (Fig. 6B). Given that innate immune stimulation is crucial for subsequent activation of acquired immunity, oncogenic KRAS may facilitate the creation of an immunologically “cold” microenvironment in which tumor cells do not stimulate innate immunity by inhibiting CCL2 production via YAP1-2α/SHP2 repressor complex. Consistently, SHP2 negatively regulates proinflammatory genes (45). Mice with intestinal epithelial-specific deletion of Shp2 develop severe colitis with elevated expression of proinflammatory cytokines (46). Hepatocyte-specific deletion of Shp2 induces hepatic inflammation, which predisposes to liver cancer (47). The present study therefore indicates that SHP2 influences inflammation/tumorigenesis through transcriptional
regulation of cytokine/chemokine expression via splicing-dependent interaction with YAP1 (Fig. S6).

The relevance of using mouse cells for analyzing YAP1–SHP2 interaction and vice versa should be noted here. In the present study, we showed that YAP1-2α but not YAP1-2γ bound to Taz (Fig. S3D). We also showed that YAP1-2α but not YAP1-2γ bound to Shp2 (Fig. S3E). Furthermore, TAZ bound to YAP1-2α but not YAP1-2γ (Fig. 1D). In our previous study, we demonstrated that NIH3T3 cells predominantly express two endogenous Yap1 isoforms, the short-form (Yap1-2α) and long-form (Yap1-2γ), and that Shp2 selectively interacts with Yap1-2α (18). That study also showed that Yap1-2α interacted with SHP2, whereas Yap1-2γ (the long form of Yap1) did not (18). Taken together, these observations indicated that the species difference (i.e. human versus mouse) does not hamper the analysis of physical/functional interaction of YAP1/Yap1 isoforms with TAZ/Taz and SHP2/Shp2. The notion was also supported by the strong amino acid sequence conservations in these proteins: YAP1 and Yap1 exhibit 88.7% identity and 92.7% similarity, TAZ and Taz exhibit 91.2% identity and 94.8% similarity, and SHP2 and Shp2 exhibit 99.5% identity and 99.8% similarity. Furthermore, the leucine zipper regions display 92.5% identity and 95.0% similarity between YAP1-2α and Yap1-2α, and the leucine zipper regions of TAZ and Taz are 100% identical. In addition, the γ-segments, which disrupt the leucine zipper, are 100% identical in YAP1 and Yap1.

Although the present study has a limitation of experiments being primarily performed by overexpression of particular splicing isoforms of YAP1 (~5-fold greater than that of the endogenous one), the results provide the first experimental evidence for the presence of context-dependent alterations in the function of differentially spliced isoforms, which are distinct from those made by simple loss- or gain-of-function splicing variants. The results therefore extend the importance of alternative splicing in further diversifying the biological functions of a single gene product. Notably, mutations in genes encoding splicing regulators in hematological malignancies indicate that abnormal splicing is also associated with oncogenesis (48). In the case of YAP1, the balance between splicing isoforms with and those without exon 6 is important for determining the direction and magnitude of cell-autonomous and non–cell-autonomous YAP1 activities that could act oppositely in terms of oncogenesis. The present work points to the notion that artificial manipulation of YAP1 pre-mRNA splicing will lead to the development of novel cancer therapeutics targeting a tumor microenvironment.

**Experimental procedures**

**Cells**

AGS human gastric epithelial cells (ATCC CRL1739) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. HEK 293T human embryonic kidney cells (ATCC CRL3216) and mouse embryonic fibroblast NIH3T3 cells (ATCC CRL1658) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. The cells were transiently transfected with expression vectors using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instruction. For transient expression experiment, the cells were harvested 24 h after transfection for assay unless otherwise stated. In several experiments, the cells were cultured at a low cell density (6.25 × 10^3 cells/cm^2) or a high cell density (6.25 × 10^4 cells/cm^2).

**Antibody**

The following antibodies were used as primary antibodies for immunostaining, immunoprecipitation, ChIP, immunohistochemistry, and immunoblotting: mouse monoclonal anti-HA antibody (Cell Signaling Technologies catalog no. 2367 (6E2)); rabbit monoclonal anti-HA antibody (Cell Signaling Technologies catalog no. 3724 (C29F4)); rabbit monoclonal anti-YAP/TAZ antibody (Cell Signaling Technologies catalog no. 8418 (D24E4)); rabbit monoclonal anti-Pan-TEAD antibody (Cell Signaling Technologies catalog no. 13295 (D3F7L)); mouse monoclonal anti-YAP antibody (Cell Signaling Technologies catalog no. 12395 (1A12)); mouse monoclonal anti–β-actin (Cell Signaling Technologies catalog no. 3700 (8H10D10)); mouse monoclonal anti-FLAG antibody (Sigma catalog no. F3165 (M2)); mouse monoclonal anti-SRSF3 antibody (Invitrogen catalog no. 33-4200 (7B4)); rat anti-mouse F4/80 antibody (Bio-Rad catalog no. MCA497RT (CI:A3-1)); rabbit polyclonal anti-CD86 antibody (BIOSS catalog no. ab182422 (EPR19518)); biotin monoclonal anti-CD163 antibody (Abcam catalog no. ab1035R); rabbit monoclonal anti-CD163 antibody (Abcam catalog no. ab182422 (EPR19518)); biotin monoclonal anti-CD11 antibody (Biolegend catalog no. 101203 (M1/70)); rabbit polyclonal anti-p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technologies catalog no. 9102s); and rabbit polyclonal anti-phospho-p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technologies catalog no. 9101s). **Allograft transplantation experiments**

Female athymic nude mice (BALB/c; nu/nu) at 6 weeks of age were used for tumor-formation assay. All of the mice were maintained in micro isolator cages. NIH3T3 cells prepared for injection were culture in log phase at the time of harvest, and 1.5 × 10^6 cells were injected into both flanks of nude mice. The mice were maintained under aseptic barrier conditions until they were sacrificed at 6 weeks. For macrophage depletion, clodronate liposome or control liposome (Small, Hygience Bioscience) was injected into nude mice (16.5 mg/kg) before transplantation. Clodronate liposome or control liposome was injected once a week until the mice were sacrificed. All the animal experiments were carried out according to the protocol approved by the Ethics Committee for Animal Experiments at the Graduate School of Medicine of the University of Tokyo.

**Soft agar assay**

NIH3T3 cells were used for soft-agar assay. 4 × 10^4 NIH3T3 cells in 1.5 ml of DMEM containing 10% bovine serum and 0.3% agarose–ME (Iwai Chemicals Company) were seeded onto the solidified bottom layer (2 ml of DMEM containing 10% bovine serum and 0.5% agarose) in a 6-well plate. 500 µl of medium was added once a week. The cells were cultured for 4 weeks. The colonies were stained with crystal violet (Nacalai
Trated following the PEG-it™ (SBI) protocol. Lentivirus titer pernatant containing the lentivirus was collected and concentration was determined using the infection

Establishment of the stable transfectants using lentivirus

The cDNAs encoding human YAP1 isoforms (1α, 1β, 1γ, 1δ, 2α, 2β, 2γ, and 2δ), two sets, one N-terminally tagged by HA and the other N-terminally tagged by FLAG, were subcloned into the pSP65SRx or pCDH-CMV-MCS-EF1-Puro (CD510B-1, SBI) vector. Expression vector for C-terminally FLAG-tagged human SHP2 (FLAG–SHP2) was described previously (18). The cDNA encoding TAZ was described previously (18). The cDNAs for human KRASG12D and KRASG12V mutants were subcloned into the pCDH-CMV-MCS-EF1-Puro vector. The human SRSF3 cDNA was cloned into the pCDH-CMV-MCS-EF1-Puro vector. Gene knockout was conducted by using the CRISPR/Cas9 system. For human SHP2 (FLAG, SRSF3, and the other N-terminally tagged by FLAG, were subcloned into the pSP65SRx vector. The sgRNA list is as follows: SRSF3-sgRNA#1, 5′-GCGAAGCCGCAT-3′; SRSF3-sgRNA#2, 5′-CTCCGCG-3′; SRSF3-sgRNA#1, 5′-GTGGTTGTGGA-3′; YAP1-sgRNA#1, 5′-CATCAGATCGTGCACGTCCG-3′; YAP1-sgRNA#2, 5′-CGGGGACTCGGAGACCCGAC-3′; Yap1-sgRNA#1, 5′-GGCAGCTT-3′; and Shp2-sgRNA#1, 5′-CTCCGGCGGGTACCCTACA-3′.

Immunoprecipitation and immunoblotting

The cells were harvested and lysed in the lysis buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% Brij35, 1 mM β-glycerophosphate, 10 mM NaF, 2 mM Na3VO4, and 2 mM phenylmethylsulfonyl fluoride. For anti-FLAG immunoprecipitation, the cell lysates were incubated with anti-DYKDDDDK antibody-conjugated beads (WAKO). For immunoprecipitation with HA-tagged proteins or endogenous proteins, the lysates were treated with respective antibodies and protein G-Sepharose 4TM Fast Flow (GE Healthcare). The beads were then washed with lysis buffer four times, and the immune complex was eluted with SDS-PAGE sample buffer. The proteins were visualized using Western Lighting Plus ECL (PerkinElmer). The obtained chemiluminescence was exposed to X-ray film (Fuji Film) or quantified using LAS4000 system (Life Technologies).

Wound-healing assay

The cells (6 × 105 cells/3.5 cm-dish) were seeded and incubated for 24 h at 37°C. The cellular layer in each plate was scratched using a plastic pipette tip (P20). The migration of the cells at the edge of the scratch was analyzed at 0 and 8 h, when the microscopic images of the cells were captured. The images were analyzed by ImageJ software.

RNA isolation and quantitative PCR

Total RNAs were purified from cells or tumors using TRIzol (Invitrogen). 1 µg of RNA was used for reverse transcription with SuperScript II (Invitrogen). Quantitative PCR was performed using SYBR Premix Ex Taq II (TaKaRa) and the One-Step real-time PCR system (Life Technologies). The qRT-PCR primers were designed using human YAP1 exon 6, 5′-ATGCGGAATATATCCAATCCCAGCAC-3′ (forward) and 5′-CGGGGACTCGGAGACCCGAC-3′ (reverse); human total Ccl2 mRNA, 5′-CTCCTTCTTCAGGCCGGAGA-3′ (forward) and 5′-CAGTGTCGAGAGAAACAG-3′ (reverse); mouse Yap1 exon 6, 5′-ATACCGGAATATATCCAATCAGCAC-3′ (forward) and 5′-CTCGGAGAGATGATGTCGACT-3′ (reverse); human total Hprt mRNA, 5′-GCGGGACTCGGAGACCCGAC-3′ (forward) and 5′-CTCGGAGAGATGATGTCGACT-3′ (reverse); mouse Yap1 exon 6, 5′-ATACCGGAATATATCCAATCCCAGCAC-3′ (forward) and 5′-CTCCTTCTTCAGGCCGGAGA-3′ (reverse); mouse Srsf3, 5′-TCGTC-CTCCTGAGAGATGATGTCGACT-3′ (forward) and 5′-CTCCTTCTTCAGGCCGGAGA-3′ (reverse); mouse Srsf3, 5′-CTCCTGAGAGATGATGTCGACT-3′ (forward) and 5′-CTCCTTCTTCAGGCCGGAGA-3′ (reverse); mouse Hprt, 5′-ACTGTAATGATCGATCAAGCGG-3′ (forward) and 5′-GCCCTGTATCTCAACACTTCC-3′ (reverse).
Dual-Luciferase assay

The promoter region of mouse Ccl2 (sequence NM_011333) covering 1.2 kb (position $-1200\text{bp}+/+85\text{bp}$ relative to the transcription initiation site, referred to as Ccl2$^{−1200bp/+85bp}$) was cloned into pGL3 luciferase reporter vector (Promega AG). With the same approach, Ccl2$^{−600bp/+85bp}$ and Ccl2$^{−300bp/+85bp}$ reporters were made. All constructs were verified by sequencing. The cells ($1 \times 10^5$ cells/well) were plated in 12-well plates and transfected 24 h later using empty vector (pGL3) as a negative control. For normalization, the cells were co-transfected with 10 ng of a Renilla luciferase plasmid (pRL-TK). 24 h post-transfection they were lysed and assayed for luciferase activity using the Dual-Glo Luciferase reporter system (Promega AG).

ChIP assay

NIH3T3 cells ($1 \times 10^5$) were plated and cross-linked after 24 h with 1% formaldehyde (Thermo Fisher Scientific AG) for 10 min at room temperature. Formaldehyde was quenched by adding 1 mM glycine (Sigma) for 5 min. The cells were collected, washed twice with cold PBS, and lysed in SDS-lysis buffer I (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5% NP-40, 1.0% Triton X-100). The crude extract was sonicated 5 cycles (1 min on, 1 min off). The samples were then incubated overnight at 4°C with an anti-SHP2 antibody (B-1) or an anti-IgG antibody (Cell Signaling Technology) and subjected to immunoprecipitation analysis.

Transcriptome analysis on cancer-related genes using a next-generation sequencer

Mouse cancer-targeted RNA panel profiles the expression of 395 cancer-associated genes (including predefined housekeeping genes as internal control) (Qiagen). Total RNAs were extracted using RNeasy Plus Micro. RNA concentration and quality were analyzed by RNA highly sensitive tape using the TapeStation 4200 (Agilent). Total RNA (400 ng) was used to construct cDNA library with Qiaseq mouse cancer transcriptome panel (RMM-003Z) and Qiaseq Targeted RNA 12 index I (Qiagen). 10 pm library was used for denaturing and subjected to next-generation sequencing using Miseq (Illumina).

Harr plot analysis

YASS (49) was used to generate dot plots for Ccl2 promoter regions between human and mouse using the following score parameter settings: scoring matrix (match: $+5$, transition: $−4$, composition bias correction: $−4$); gap costs (opening: $−16$, extension: $−4$); E-value threshold of 0.01; and X-drop threshold of 30.

Statistical analysis

All samples represent biological replicates. Sample sizes are indicated in the figure legends. No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. The variance similar between the groups has been tested before statistically compared. Unpaired two-tailed Student's $t$ tests were used for the statistical analysis of the data obtained by qRT-PCR and luciferase assay. Paired two-tailed Student's $t$ tests were used for the analyses of growth curve, wound-healing assay, soft-agar colony formation, in vivo tumor formation, and immunohistochemical staining data. Kaplan–Meier curves were generated using data downloaded from TCGA database (http://www.oncolnc.org). Log-rank test was used to compare the prognosis of the high-expression and low-expression groups.

Data availability

All the data and methods necessary to reproduce this study are included in the article and supporting information.

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Author contributions

C. B. and M. H. conceptualization; C. B., X. W., and C. T. K. data curation; C. B., X. W., C. T. K., T. H., and M. H. formal analysis; C. B., X. W., C. T. K., and M. H. validation; C. B., X. W., A. T.-K., C. T. K., and M. H. investigation; C. B. and T. H. visualization; C. B., X. W., T. H., and M. H. methodology; C. B., X. W., and M. H. writing–original draft; C. B. and M. H. writing–review and editing; A. T.-K. and C. T. K. resources; A. T.-K., C. B., X. W., and M. H. writing–review and editing; A. T.-K. and M. H. supervision; T. H. software; M. H. funding acquisition; M. H. project administration.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations

The abbreviations used are: ERK, extracellular regulated protein kinases; L Zam, leucine zipper mutant; SHP2, Src homology region 2 domain-containing protein tyrosine phospha- tase-2; TEAD, TEA domain; TAZ, transcriptional co-activator with PDZ-binding motif; pre-mRNA, precursor mRNA; RNA-Seq, RNA sequencing; qRT-PCR, quantitative RT-PCR; snRNP, small nuclear ribonucleic protein; DMEM, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin; sgRNA, single-guide RNAs.

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