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Pharmacological inhibition of CLK2 activates YAP by promoting alternative splicing of AMOTL2

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Abstract

Yes-associated protein (YAP), the downstream effector of the evolutionarily conserved Hippo pathway, promotes cellular proliferation and coordinates certain regenerative responses in mammals. Small molecule activators of YAP may therefore display therapeutic utility in treating disease states involving insufficient proliferative repair. From a high-throughput chemical screen of the comprehensive drug repurposing library ReFRAME, here we report the identification of SM04690, a clinical stage inhibitor of CLK2, as a potent activator of YAP driven transcriptional activity in cells. CLK2 inhibition promotes alternative splicing of the Hippo pathway protein AMOTL2, producing an exon-skipped gene product that can no longer associate with membrane-bound proteins, resulting in decreased phosphorylation and membrane localization of YAP. This study reveals a novel mechanism by which pharmacological perturbation of alternative splicing inactivates the Hippo pathway and promotes YAP dependent cellular growth.

eLife assessment

This paper reports **important** findings on a potent activator of the YAP pathway, demonstrating its mechanism through alternative splicing changes. The authors provide **convincing** evidence to support their claims. This research is of interest to biologists studying alternative splicing or the Hippo pathway, with significant implications for medical research.

Introduction

The evolutionarily and functionally conserved Hippo signaling pathway functions as a master regulator of cellular proliferation and tissue growth in animals. Molecularly, the Hippo pathway is composed of a kinase cascade that catalyzes a series of phosphorylation events at the plasma membrane. The core pathway includes Ste20-like kinase 1 (MST1) and MST2, which phosphorylate and activate large tumor suppressor kinase 1 (LATS1) and LATS2[1,2]. LATS1/2 phosphorylation

results in the phosphorylation of downstream effector Yes-associated protein 1 (YAP), leading to its cytoplasmic retention and inactivation[3]. Several other proteins work together to regulate the core kinase cascade, such as Salvador homolog 1 (SAV1) and MOB kinase activator 1A and 1B (MOB1A/B), binders of MST1/2 and LATS1/2, respectively[4,5]. In addition to phosphorylation-dependent regulation of the Hippo pathway, YAP is also regulated by protein-protein interactions, such as with the angiomotin family of proteins (AMOT, AMOTL1, and AMOTL2), which directly bind to and cause the localization of YAP at tight junctions[6]. Adherens junctions and tight junctions are important sites of Hippo pathway regulation that link to the core kinases of the Hippo pathway through the AMOT proteins. When the Hippo pathway is inactivated, YAP translocates to the nucleus, acting as a transcriptional co-activator of TEA domain transcription factors (TEADs), which increases the transcription of genes involved in cellular proliferation and survival.

Regulation of the Hippo pathway by cell-cell contacts, metabolic changes, and diverse extrinsic inputs results in decreased activity of YAP, which prevents organ overgrowth and abnormal cellular proliferation[7]. On the other hand, YAP activity is also critical in healing injury by augmenting cellular proliferation and survival. While many multicellular organisms can repair wounded or damaged organs, mammalian regeneration is more limited, as many adult tissues lack the capacity to regenerate. Genetic activation of YAP, either by genetic inactivation of upstream Hippo pathway proteins or by forced overexpression of YAP, has been shown to enhance endogenous repair in wounded tissues. For example, hepatic knockdown of MST1/2 with small interfering RNA (siRNA) in the mouse after partial hepatectomy augments regeneration of the liver via YAP dependent hepatocyte proliferation[8]. Likewise, in the skin, YAP is essential for regenerative wound repair, as siRNA-mediated knockdown of YAP markedly delays wound closure[9]. Conversely, conditional hyperactivation of YAP enhances proliferation of epidermal keratinocytes, thickening the epidermal layer of neonatal mice[10]. Lastly, in the heart, an organ previously considered to have no regenerative potential post-development, conditional SAV deletion promotes proliferation of cardiomyocytes and reverses deficits in ejection fraction volume post myocardial infarction[11]. Collectively, these studies suggest that small molecule activators of YAP may therefore display therapeutic utility in treating diseases etiologically defined by an insufficient proliferative response.

We recently reported the identification of PY-60, a small molecule that activates YAP by binding to a membrane-binding scaffolding protein, annexin A2 (ANXA2), which we defined as a central component of the Hippo pathway[12]. This study suggested to us that likely many other druggable mechanisms exist for activating YAP including those associated with previously drugged cellular proteins. Here, to explore the hypothesis that known drugs might activate YAP driven transcriptional activity, we performed a reporter-based screen of the comprehensive drug repurposing library ReFRAME. We found that SM04690, a small molecule kinase inhibitor in clinical trials for the treatment of osteoarthritis, activates YAP with nanomolar potency in cells. Mechanistic studies revealed the relevant target kinase of SM04690 to be CDC-like Kinase 2 (CLK2), inhibition of which induces alternative splicing of Hippo signaling component AMOTL2 that can no longer localize YAP to the plasma membrane, in effect activating YAP.

Results

Screening of the ReFRAME library

To identify small molecule activators of YAP, we screened the Repurposing, Focused Rescue, and Accelerated Medchem (ReFRAME) small molecule library using a HEK293A cell line with a stably integrated cassette of eight copies of the TEAD-binding element upstream of luciferase (293A-TEAD-LUC). This library contains ~13,000 small molecules that have undergone substantial preclinical evaluation, clinical development, or are FDA-approved (**Fig. 1A**)[13]. The screen was

conducted using a previously validated assay in 1,536-well format at three compound concentrations (200 nM, 2 μM, and 10 μM) to maximize the potential number of mechanisms surveyed. Hit compounds were defined if they displayed increases of TEAD-LUC signal of more than 5 robust Z scores above mean plate signal (**Fig. 1B**). Identified hits could be grouped into several classes of inhibitors including known nonspecific modulators of transcription (e.g., bromodomain inhibitors, histone deacetylase inhibitors), ion channel modulators, and kinase inhibitors, among others (**Fig. 1C**). Of these, we identified 15 kinase inhibitors that did not have any associated YAP augmenting activity and are not characterized inhibitors of MST1/2 and LATS1/2. Evaluating these 15 hits in dose response using the original screening assay with library material, we confirmed 9 displayed reproducible YAP activation (**Fig. 1D**). Of these, three were inhibitors of non-receptor tyrosine kinases including Cerdulatinib (inhibitor of SYK and JAK paralogs[14]; TEAD-LUC EC_{50} =0.31 μM), Peficitinib (a pan JAK inhibitor[15]; TEAD-LUC EC_{50} = 2.4 μM), and KX02 (an inhibitor of SRC family kinases[16]; TEAD-LUC EC_{50} = 3.7 μM). Also identified as efficacious YAP activators were XL-999 (a pan RTK inhibitor, TEAD-LUC EC_{50} = 1.5 μM) and CEP-1347 (a JNK family inhibitor; TEAD-LUC EC_{50} = 2.5 μM). Two inhibitors of the AGC family of kinases were also identified, namely GSK690693[17] (TEAD-LUC EC_{50} > 20 μM) and AT13148[18] (TEAD-LUC EC_{50} > 20 μM). Lastly, we identified two inhibitors of CLK and DYRK family kinases including SM04690[19] (TEAD-LUC EC_{50} = 5.7 μM) and SM04646 (TEAD-LUC EC_{50} = 2.1 μM), which had been initially described as agents which inhibit canonical Wnt signaling and induce differentiation of chondrocytes. Among these, we chose to further characterize SM04690, which is in clinical trials for the treatment of osteoarthritis of the knee, as no association of its target kinases had previously been reported as related to Hippo pathway signaling[19,20].

SM04690 is a YAP activator

We first confirmed the YAP-stimulating activity of SM04690 in 384-well format using 293A-TEAD-LUC cells with fresh, commercially obtained material, finding that the compound dose-dependently increased TEAD-LUC reporter activity with an EC_{50} of 1.2 nM, a value considerably more potent than that observed in the screening assay (**Fig. 2A**). We next confirmed that SM04690 functionally activated a YAP driven transcriptional program, as SM04690 was found to significantly increase the levels of YAP-controlled transcripts *ANKRD1* and *CTGF* (**Fig. 2B**). Likewise, SM04690 treatment markedly decreased phosphorylation of YAP, as SM04690 was found to decrease immunopositivity dose-dependently to anti-phospho-YAP in Western blotting experiments following 24-hour compound treatment (**Fig. 2C**). We next knocked down YAP transcript using shRNA and found that 1 μM compound treatment failed to increase *ANKRD1* and *CTGF* transcript levels in the absence of YAP, suggesting the compound activates a YAP dependent transcriptional program (**Fig. 2D**). We additionally observed by immunofluorescent imaging that YAP localized to the nucleus in MDCK cells treated with SM04690 (1 μM, 24 hours), suggestive that compound treatment promotes nuclear translocation and activation of YAP. This is in comparison to diffuse plasma membrane and cytoplasm YAP localization in DMSO treated cells (**Fig. 2E, F**). Lastly, since pharmacological YAP activators have been shown to promote expansion of cells, we tested 0.2 nM SM04690 treatment in immortalized human keratinocytes (HaCaT) over 7 days and found treatment promoted clonal expansion, as seen with rhodamine B staining and increased nuclei counts (**Fig. S1A, B**). Importantly, these proliferative effects occur at a non-cytotoxic concentration (under 0.5 nM) of SM04690 treatment (**Fig. S1C**).

CLK2 is the relevant cellular target of SM04690

SM04690 was first described in the literature as an inhibitor of Wnt signaling capable of promoting differentiation of mesenchymal stem cells to chondrocytes[19]. Following work defined SM04690 as an inhibitor of several subfamilies of related CMGC family protein kinases[21], including Dual Specificity Tyrosine Phosphorylation Regulated Kinase (DYRK) and CDC Like Kinase (CLK) subfamilies (**Fig. S2A**). Specifically, SM04690 has been shown to inhibit CLK2, CLK3, DYRK1A and B, Homeodomain Interacting Protein Kinase 1 (HIPK1), and HIPK2 with over 90%

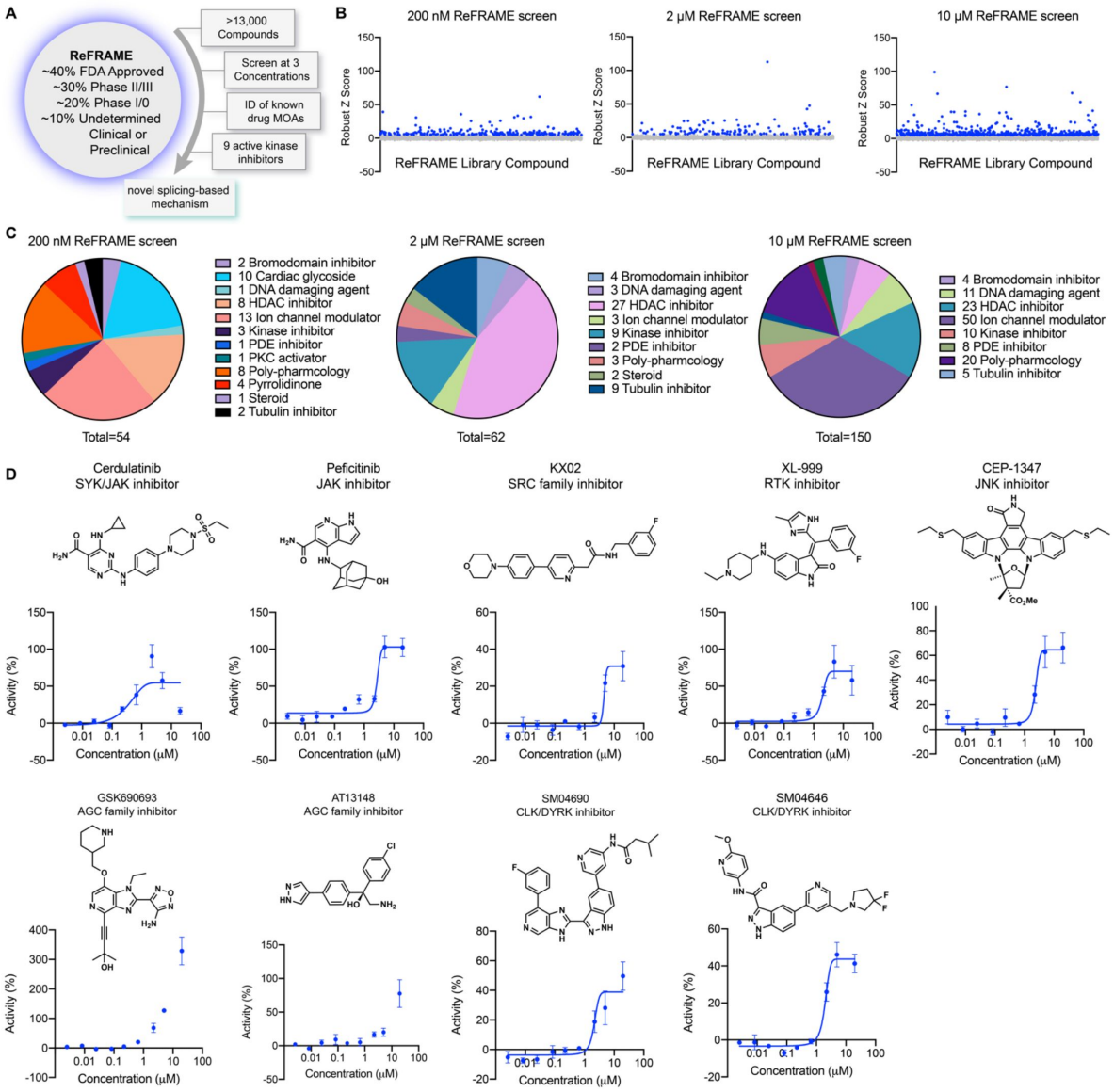

Figure 1.

A screen of ReFRAME identifies pharmacological mechanisms for activating YAP.

(A) Schematic depicting screening workflow, which resulted in the identification of 9 kinase inhibitor-based YAP activating small molecules. (B) Scatter plots of TEAD-LUC activity from each of the screens conducted at the final compound concentrations of 200 nM, 2 µM, and 10 µM. Each data point represents a single compound. Hits were classified as having robust Z scores over five. (C) Pie charts indicating the classes of small molecules characterized as hits in each screen. (D) Structure and dose-response plots of TEAD-LUC activity of the nine identified kinase inhibitors (n=3, mean and s.d.).

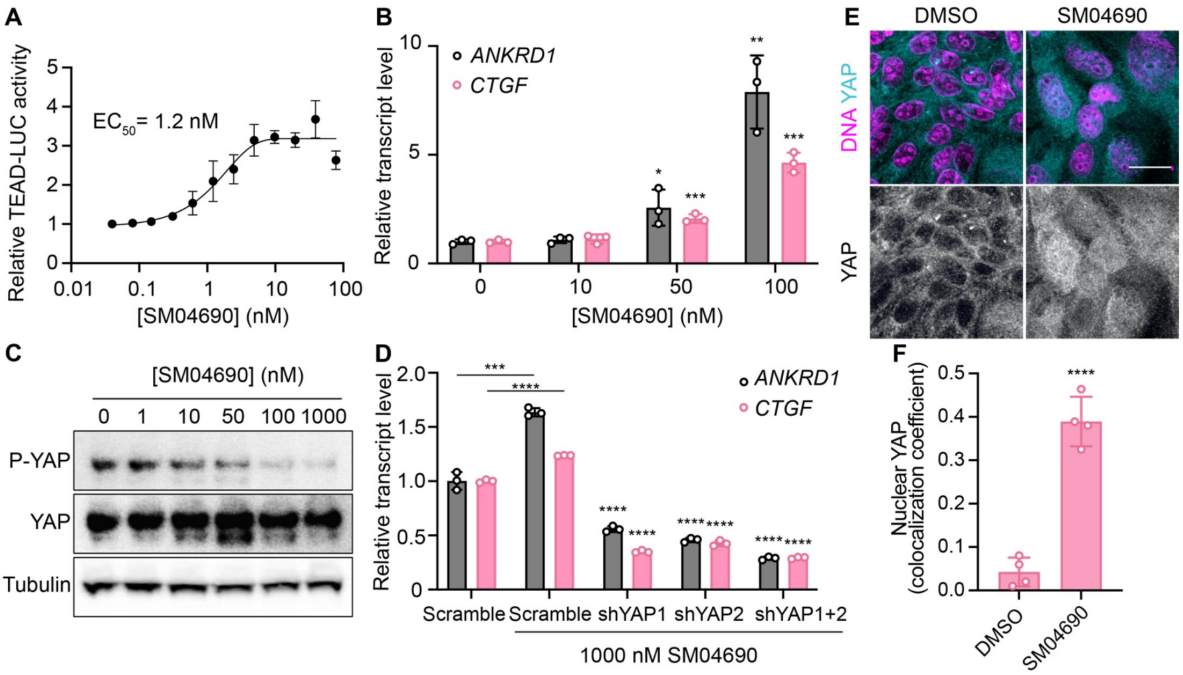

Figure 2.

SM04690 is a pharmacological activator of YAP.

(A) Relative luminance values after 24h treatment of 293A-TEAD-LUC cells with the indicated concentrations of SM04690 (n=3 biological replicates, mean and s.e.m.). (B) Relative transcript levels of YAP-dependent genes (*ANKRD1* and *CTGF*) from HEK293A cells treated for 24h with SM04690, measured by RT-qPCR (n=3, mean and s.d.). (C) Western blot analysis of anti-phospho-YAP and total YAP levels from HEK293A cells treated with the indicated concentrations of SM04690 for 24h. (D) Relative transcript levels of *ANKRD1* and *CTGF* from HEK293A cells expressing the indicated YAP targeting shRNAs and then treated with 1 μM SM04690, measured by RT-qPCR (n=3, mean and s.d.). Asterisks above samples treated with shRNAs to YAP and SM04690 refer to statistical significance as compared to scramble treated with SM04690. (E) Representative images of anti-YAP immunofluorescent staining of MDCK cells following treatment with 1 μM SM04690. Anti-YAP antibody in teal and Hoechst 33342 in pink to visualize nuclei (scale bar = 10 μm). (F) Quantification of anti-YAP and Hoechst 33342 (DNA) correlative immunofluorescent staining (n=4, mean and s.d.). (B, D, F) Statistical analyses are univariate two-sided t-tests (* P <0.0332, ** P <0.0021, *** P < 0.0002, **** P <0.0001).

inhibition at 500 nM[21]. As such, we hypothesized that inhibiting one of these targets was likely responsible for modulating YAP activity. Previously published transcript expression data from our lab indicated that among the targets of SM04690 only CLK2, DYRK1B, HIPK1, and HIPK2 are expressed in HEK293A cells, helping to narrow our search (**Fig. S2B**)[12]. We then treated 293A-TEAD-LUC cells with structurally distinct small molecule inhibitors of these putative targets to see if other treatment of cells with inhibitors to the potential target might recapitulate the YAP activating effect of SM04690. We found that only inhibitors targeting CLK2 (CC671, CLK-in-T3, and T025) elicited a dose-dependent increase in YAP activity (**Fig. S2C**). In contrast, inhibitors targeting DYRKs (Harmine, INDY, and Leucettine L41) and CLK4 (ML167, TG003) showed no activity at concentrations over multiple orders of magnitude (**Fig. S2D**). To confirm CLK2 as the relevant target of SM04690, we knocked down CLK2 by approximately 50% using three different targeting shRNAs, all of which increased YAP-controlled transcripts *ANKRD1*, *CTGF*, and *CYR61* to varying degrees (**Fig. 3A, B**). Collectively, these data strongly suggested that CLK2 was most likely the target of SM04690 responsible for activating YAP.

CLK2 is a protein kinase that phosphorylates serine/arginine-rich (SR) proteins of the spliceosomal complex, which play an important role in exon selection by the spliceosome[22,23]. The phosphorylation states of SR proteins dictate their subcellular localization and interactions with pre-mRNA[24]. When CLK2 is inhibited, SR proteins are not phosphorylated, leading to alternative splicing that is most often exon skipping[25]. Previous work using pharmacological CLK2 inhibitors revealed that inhibiting SR phosphorylation had a marked transcriptome wide effect on altering alternative splicing. Interestingly, the third most skipped exon of the transcriptome in response to CLK2 inhibition corresponded to exon 5 of Angiomotin-like 2 (AMOTL2), as nearly 3,000 new junction reads between non-neighboring exons were observed in this study[26] (**Fig. S3**). Exon 9 of AMOTL2 was also skipped but to a lesser extent with around 700 new junction reads. AMOTL2 is part of the AMOT family of proteins and is crucial to the tight junction localization of YAP through direct interaction with YAP's WW domain[6]. Notably, knockdown of AMOTL2 decreases the content of tight junction-localized YAP, instead promoting YAP translocation to the nucleus[27]. Given the central role that AMOTL2 plays in regulating YAP as well as the observation that no other canonical Hippo pathway member displayed alternative splicing in response to CLK2 inhibition, we hypothesized that SM04690 most likely increased YAP activity through modulating AMOTL2 splicing.

SM04690 induces alternative splicing of AMOTL2

We first confirmed that SM04690 treatment results in alternative splicing of AMOTL2. Consistent with reported literature, quantitative polymerase chain reaction (RT-qPCR) revealed that 24-hour SM04690 treatment at 1 μM induces exon skipping for exon 5 by 629-fold and exon 9 by 80-fold when compared to untreated samples (**Fig. 3C**, **S4B**). The skipping of exons 5 and 9 was observed as low as 10 nM with 24 hour treatment of SM04690 (**Fig. S4A**). Using semi-quantitative reverse transcription PCR, in which primers are designed directly flanking an exon, we could visualize the presence of each splice isoform using gel electrophoresis. Exon 5, which is 93 base pairs long, has a PCR amplicon length of 132 base pairs when primer sequences are added on both ends (39 base pairs for both) (**Fig. S4C**). Indeed, in the untreated samples, the amplicon ran through the gel to just over 100 base pairs. However, in the sample treated with 100 nM of SM04690, there are two PCR amplicons, one which ran the same distance as the untreated sample and a second band that ran well under 100 base pairs, as only the sequences of the primers were amplified, indicating skipping of exon 5 (**Fig. S4D**). Exon 9 is 180 base pairs long with a PCR amplicon length of 218 base pairs with the primer sequences, (**Fig. S4C**) which we see in the untreated sample. Like exon 5, in the 100 nM SM04690 treated sample, a second PCR amplicon unique to the treated sample ran under 100 base pairs, indicating the skipping of exon 9 (**Fig. S4D**). This experiment thus confirmed that SM04690 treatment of HEK293A cells created exon skipping of exons as indicated by shorter PCR amplicons in treated samples differing from the untreated samples by exactly the length of the missing exon. The presence of exon skipped spliceoforms at the protein level was confirmed by anti-AMOTL2 Western blot using an antibody

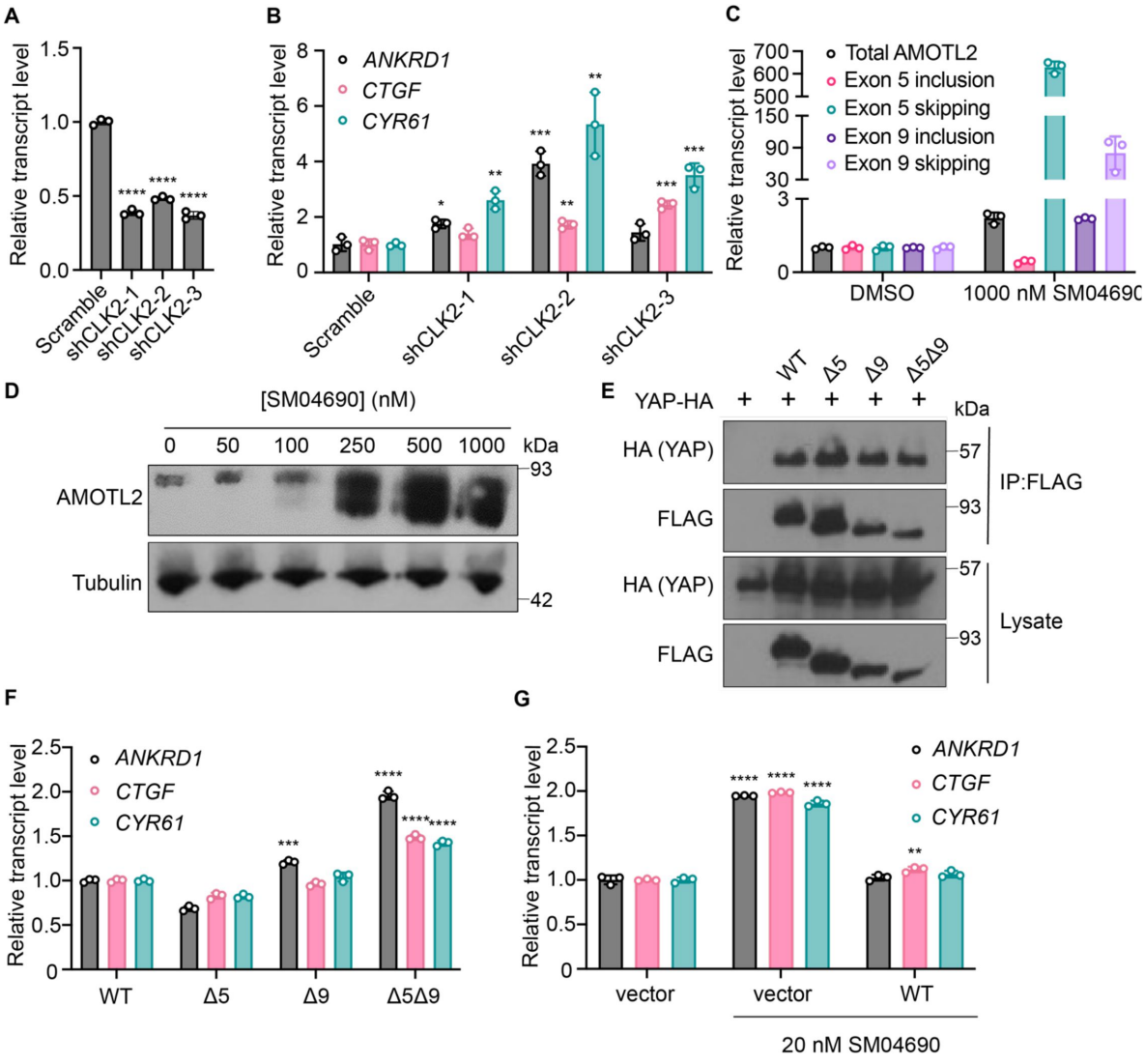

Figure 3.

Inhibition of CLK2 by SM04690 induces alternative splicing of Hippo pathway component AMOTL2.

(A) Relative transcript levels of CLK2 following knockdown by the indicated shRNA, measured by RT-qPCR (n=3, mean and s.d.). (B) Relative transcript levels of YAP-dependent genes (*ANKRD1, CTGF, CYR61*) expressing the indicated shRNA, measured by RT-qPCR (n=3, mean and s.d.). (C) Relative transcript levels of exon-included and exon-skipped spliceoforms following treatment with SM04690 for 24h in HEK293A cells, measured by RT-qPCR (n=3, mean and s.d.). (D) Anti-AMOTL2 immunoblotting from HEK293A cells treated with SM04690 for 24h. (E) Immunoblotting analysis of HA-YAP from anti-FLAG immunoprecipitated content from HEK293T cells. (F) Relative transcript levels of YAP-dependent genes (*ANKRD1, CTGF, CYR61*) following overexpression of 1 µg of AMOTL2 WT, Δ5, Δ9, Δ5Δ9, measured by RT-qPCR (n=3, mean and s.d.). (G) Relative transcript levels of YAP-dependent genes (*ANKRD1, CTGF, CYR61*) following overexpression of 2 µg WT AMOTL2 and treatment with 20 nM SM04690 for 24h (n=3, mean and s.d.). (A, B, F, G) Statistical analyses are univariate two-sided t-tests (* P <0.0332, ** P <0.0021, *** P <0.0002, **** P <0.0001).

that can detect each possible spliceoform of AMOTL2. We found that with increasing SM04690 treatment in HEK293A cells for 24 hours, there was the appearance of broad lower molecular weight bands, consistent with the buildup of multiple forms of exon skipped AMOTL2 (**Fig. 3D**). It is important to note that both the RT-qPCR and Western blot not only indicate the increase in alternative splicing but also an increase in the total AMOTL2 present with SM04690 treatment, as the relative transcript level of total AMOTL2 in SM04690 treated cells increased 2.3-fold in the RT-qPCR analysis and the Western blot showed increase band width.

Exon-skipped AMOTL2 activates YAP through a dominant mechanism

AMOTL2 interacts directly with YAP, helping to localize it to the plasma membrane. Accordingly, we next assessed if the exon-skipped gene products might interfere with the ability of AMOTL2 to bind YAP. Using FLAG-tagged transient overexpression constructs of wild-type AMOTL2 (WT), exon 5-skipped AMOTL2 (Δ5), exon 9-skipped AMOTL2 (Δ9), and double exon-skipped AMOTL2 (Δ5Δ9) (**Fig. S4E**), we performed co-immunoprecipitation studies with HA-tagged YAP at high cell density in HEK293T cells. We found that the three AMOTL2 spliceoforms retained a similar ability to bind YAP as the WT AMOTL2 transgene (**Fig. 3E**). As such, we next assessed if overexpression of the spliceoform transgenes themselves was sufficient to activate YAP. From experiments in HEK293A cells transiently overexpressing WT AMOLT2 or each of the three transgenes (AMOTL2 Δ5, AMOTL2 Δ9, AMOTL2 Δ5Δ9), AMOTL2 Δ5Δ9 uniquely displayed 1.5 to 2 times higher levels of YAP controlled transcripts in comparison to cell overexpressing WT AMOTL2 (**Fig. 3F**). In contrast, no transcriptional induction was observed by expression of AMOTL2 Δ5 and only a minor induction of *ANKRD1* was observed for AMOTL2 Δ9, suggesting the AMOTL2 Δ5Δ9 spliceoform likely has a stronger YAP activating effect than either skipped exon alone. Given expression of the AMOTL2 spliceoforms mimics the effects of SM04690 treatment, we sought to understand if overexpression of WT AMOTL2 might inhibit the ability of SM04690 to activate YAP. Indeed, overexpression of WT AMOTL2 was found to inhibit the upregulation of *ANKRD1*, *CTGF*, and *CYR61* in response to 20 nM compound treatment (**Fig. 3G**), indicating that inducing alternative splicing is essential to the mechanism of SM04690.

Alternative splicing of AMOTL2 promotes membrane delocalization

Consistent with previous research on the subcellular localization of AMOT family proteins[27,28,29], we found that AMOTL2 localizes to tight junctions at the plasma membrane of MDCK cells in conditions of high cell density (**Fig. S5A, B**). Interestingly, we found that when MDCK cells were plated at high density and then treated with 1 μM SM04690 for 24 hours, AMOTL2 much less efficiently localized to the plasma membrane as assessed by immunofluorescent staining followed by confocal microscopy (**Fig. 4A**). Indeed, SM04690-treated samples displayed a greater than 2-fold loss of colocalization of AMOTL2 with adherens junction protein E-cadherin (**Fig. 4B**). Instead, SM04690 treatment resulted in the noticeable formation of AMOLT2 positive puncta in the cytoplasm of cells. This effect on AMOTL2 puncta formation could be additionally recapitulated in HEK293A cells (**Fig. 4C**, **S6A**), as 1 μM SM04690 resulted in a 10-fold increase in puncta. Likewise, we found that overexpression of AMOTL2 Δ5, AMOTL2 Δ9, and AMOTL2 Δ5Δ9 in HEK293A cells displayed dramatic increases in puncta number in comparison to WT AMOTL2 expression, with over 10-fold increases in puncta for AMOTL2 Δ5Δ9 compared to WT AMOTL2 (Fig. **Fig. 4D**, **S6B**).

We next sought to understand if the change of AMOTL2 localization to the cytoplasm might be responsible for the mechanism of YAP activation by SM04690. AMOTL2 was originally discovered as an interactor of the membrane associated protein Membrane Associated Guanylate Kinase, WW and PDZ Domain Containing 1 (MAGI1), and it has also been shown to interact with tight junction associated proteins such as Protein Associated with LIN7, MAGUK p55 Family Member (PALS1)[30,31]. Through these interactions, AMOTL2 localizes YAP to tight junctions, where YAP is inactivated by phosphorylation. We hypothesized that AMOTL2 spliceoforms created after

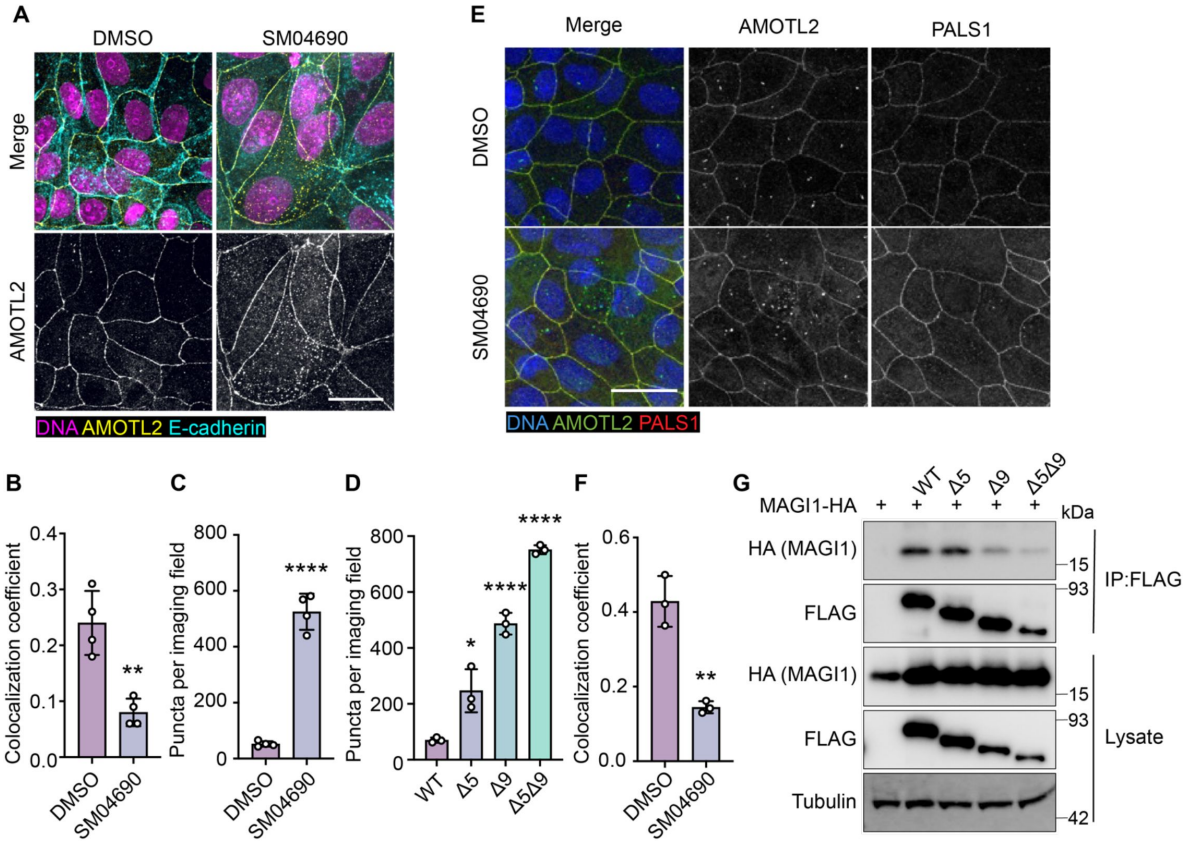

Figure 4.

Alternative splicing of AMOTL2 promotes delocalization from the membrane, essential to the activity of SM04690.

(A) Representative images of anti-AMOTL2 (yellow) and anti-E-cadherin (teal) immunofluorescent staining (with Hoechst 33342 in pink to visualize nuclei) of MDCK cells treated with control or 1 μM SM04690 for 24h (scale bar = 10 μm). (B) Quantification of anti-AMOTL2 and anti-E-cadherin correlative immunofluorescent staining (n=4, mean and s.d.). (C) Number of puncta in HEK293A cells treated with 1μM SM04690 for 24h (n=4, mean and s.d.). (D) Number of puncta per image field in HEK293A cells from 12-well plates overexpressing AMOTL2 spliceoforms (n=3, mean and s.d.). (E) Representative images of anti-AMOTL2 (green) with anti-PALS1 (red) immunofluorescent staining of MDCK cells treated with control or 1 μM SM04690 for 24h. Hoechst 33342 (blue) was used to visualize nuclei (scale bar = 20 μm). (F) Quantification of anti-AMOTL2 and anti-PALS1 correlative immunofluorescent staining (n=3, mean and s.d.). (I) Immunoblotting analysis of HA-MAGI1 WW1,2 from anti-FLAG immunoprecipitated content from HEK293T cells. (B, C, D, F) Statistical analyses are univariate two-sided t-tests (* P <0.0332, ** P <0.0021, **** P <0.0001).

SM04690 treatment can likely no longer interact with these membrane-associated proteins, acting to sequester YAP away from inhibitory Hippo signaling. Indeed, co-immunofluorescent studies in MDCK cells plated at high density indicated that 1 μM SM04690 treatment for 24 hours decreases co-localization of AMOTL2 with PALS1 and MAGI1 by approximately 2-fold (**Fig. 4E, F**, **S7A, B**). While cloning of these large membrane-associated proteins proved difficult, we were able to clone the WW domain regions of MAGI1, which are responsible for its interaction with AMOTL2. From co-immunoprecipitation with the FLAG-tagged AMOTL2 spliceoforms in HEK293T cells, we found that AMOTL2 Δ9 and AMOTL2 Δ5Δ9 lost their ability to interact with the WW domain regions of MAGI1 (**Fig. 4G**). Collectively, these data suggest that alternative splicing of AMOTL2 culminates in gene products less capable of plasma membrane localization, which results in decreased YAP phosphorylation.

Discussion

In this study, we set out to identify repurposed drugs that might activate YAP, an effort we hypothesized would yield new druggable mechanisms for inactivating the Hippo pathway. From a high-throughput screen of the ReFRAME library, we have identified several kinase inhibitors that activate YAP. We specifically examined SM04690 as a novel YAP activator and have demonstrated a connection between CLK2 inhibition and YAP activity. Specifically, CLK2 inhibition induces exon skipping of exons 5 and 9 of AMOTL2, a protein integral to Hippo pathway activity. We propose a working model in which the alternative splicing of AMOTL2 impedes its ability to interact with proteins at the plasma membrane, such as PALS1 and MAGI-1 (**Fig. 5**). The exon skipped AMOTL2 gene product is therefore unable to shepherd YAP to tight junctions at the plasma membrane where it receives inhibitory phosphorylation. Consequently, unphosphorylated YAP is capable of translocating to nucleus, where it interacts with TEADs to promote transcription of genes related to proliferation and survival. While we focused our efforts on understanding the role of SM04690, we identified several other kinase inhibitors as efficacious activators of YAP. Several of the reported targets of these identified inhibitors corresponded to related kinase families, including JAK kinases and AGC kinases. Future investigation into these and other protein kinase-based mechanisms will likely reveal several other druggable kinases that regulate Hippo pathway activity.

AMOTL2 serves a central role in regulating the Hippo pathway. Specifically, AMOTL2 has been shown to inhibit YAP, restraining it to the plasma membrane while concurrently stimulating the enzymatic activity of LATS1/2[32]. These functions are possible due to AMOTL2's activity as a scaffolding protein, bringing LATS1/2 in close proximity to MST1/2 at the plasma membrane[33]. While most research indicates AMOTL2 plays a role in negatively regulating YAP activity, there remains some debate in the field as to whether AMOTL2, and more broadly the AMOT family of proteins, might also exert YAP stimulating activities in the cell[34]. The work presented here adds to the mounting evidence that AMOTL2 acts as a negative regulator of YAP, most prominently displayed through an attenuation of transcription of YAP-controlled genes when wild type AMOTL2 is overexpressed in the context of the YAP-activating compound SM04690 (**Fig. 3G**). A lack of scaffolding functionality by AMOTL2 spliceoforms lacking exons 5 and/or 9 may also help to explain how this alternative splicing is able to activate YAP. It has been shown that the AMOTL2 related protein AMOT regulates Hippo pathway activity through the formation of biomolecular condensates, in contrast to traditional scaffolding proteins[35]. AMOT laden condensates have been shown to concentrate core Hippo pathway kinases, acting to potentially sequester YAP in the cytoplasm. Since there is considerable redundancy between AMOT and AMOTL2[29,36–38], it is conceivable that AMOTL2 may also regulate YAP through biomolecular condensates. The exon skipped AMOTL2 spliceoforms identified here could have impaired ability to phase separate into condensates or fail to incorporate core Hippo pathway kinases into these condensates. Future studies examining the capacity of AMOTL2 to phase separate and interact with Hippo pathway proteins could reveal exactly how the lack of tight junction localization of the AMOTL2:YAP

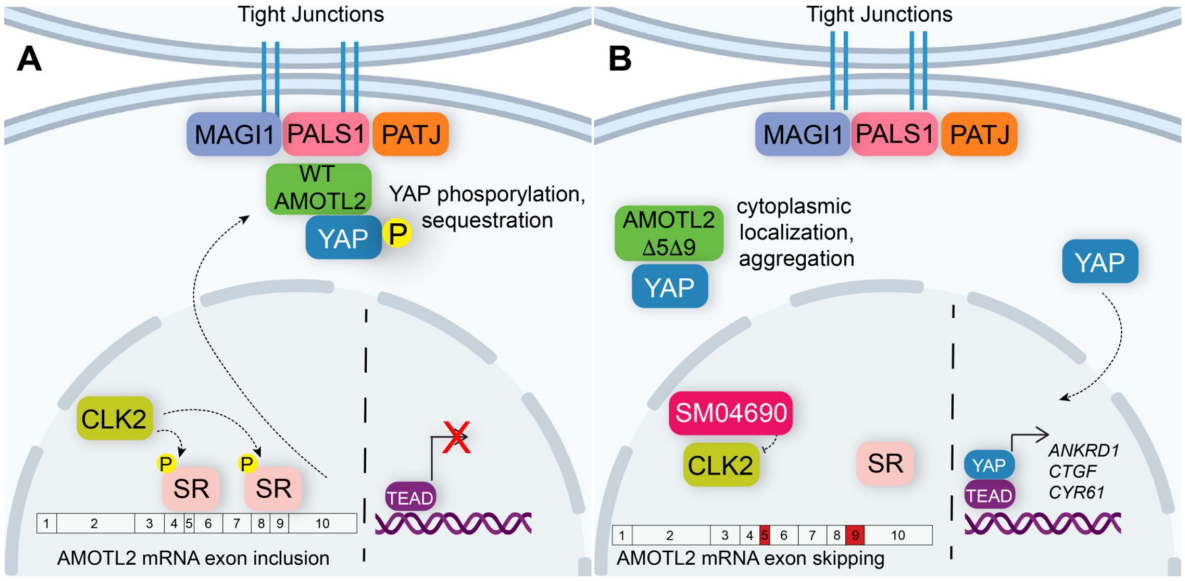

Figure 5.

Alternative splicing of AMOTL2 modulates Hippo pathway activity.

(A) A mechanistic model depicting that in the unperturbed system, AMOTL2 localizes at the membranes with proteins associated at tight junctions, bringing YAP in contact with the core kinase cascade of the Hippo pathway resulting in its phosphorylation and retention in the cytoplasm. (B) Inhibition of CLK2 by SM04690 decreases phosphorylation of serine/arginine-rich splicing factors, leading to exon skipping of exons 5 and 9 of AMOTL2. AMOTL2 lacking exons 5 and 9 cannot interact with membrane and membrane-associated tight junction proteins. As such, AMOTL2 cannot localize YAP to tight junctions, and so YAP localizes to the nucleus, interacts with TEADs, and activates its transcripts.

complex leads to increased YAP activity. More generally, this study highlights the existing need to precisely define the specific roles for each AMOT family member (i.e., AMOT, AMOTL1, and AMOTL2), delineating which functions are redundant and which are unique to each family member.

Others have also shown that alternative splicing influences YAP activity. Indeed, several core Hippo pathway proteins have alternatively spliced isoforms. For example, tissue-specific alternative splicing of YAP itself can lead to eight different isoforms and has been shown to regulate its activity by altering the efficiency with which it interacts with LATS1/2 and TEADs, leading to cell-type specific effects on cellular proliferation[39–41]. In addition, LATS1 and LATS2 exist as 7 and 2 spliceoforms in the cell respectively, affecting their interactions with Hippo pathway scaffolding proteins Neurofibromin 2 (NF2) and MOB1 and modulating their catalytic activity[42,43]. While the exon skipping observed in this study is derived from pharmacological perturbation, it is possible that similar processes exist endogenously, particularly in cell types or physiological scenarios with lower CLK2 activity. For example, several breast cancer cell lines have been found to display highly variable levels of CLK2 expression[44]. As YAP hyperactivity has been characterized as essential in several human tumor types, including breast cancer[45], it follows that cancer cells lines with lower CLK2 activity may display increased aggressiveness by activating YAP through the splicing-based mechanism presented here.

One final consideration raised in this study concerns the use of alternative splicing modulators in the treatment of disease. SM04690 was initially reported in the literature as an inhibitor of canonical Wnt signaling, displaying an EC_{50} of 19.5 nM in reporter-based assays[19]. Here, we have shown that SM04690 activates YAP with an EC_{50} of 1.2 nM in TEAD-LUC assays, suggesting that several transcriptional programs including YAP might be modulated by this compound at therapeutic doses. Indeed, while SM04690 has been used in a localized fashion to treat osteoarthritis of the knee[46,47], our work suggests that the use of broad acting modulators of alternative splicing should be met with caution, as inhibition of the CLK family of kinases induces alternative splicing in many transcripts[26], which may lead to undesired off-target effects. Nevertheless, this work has identified that CLK2 inhibitors are novel pharmacological tools to study the processes governing the regulation of the Hippo pathway and it further underscores the utility of performing unbiased screens for identifying important cellular biological mechanisms.

Materials and Methods

Cell Culture

Reporter cells (293A-TEAD-LUC (HEK-293A-8xGTII-Luc)) were a gift from the laboratory of Xu Wu (Mass General). HEK293A cells were from ThermoFisher. MDCK and HEK293T cells were obtained from the American Type Culture Collection (ATCC). HaCaT immortalized human keratinocytes were from AddexBio. All cell lines were cultured in DMEM (Corning) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) and maintained at 37°C with 5% CO_2.

High-throughput Screening

20 nL of stock compounds solvated in DMSO were dispensed using an Echo Acoustic Liquid Handler instrument (Labcyte) before adding 750 293A-TEAD-LUC cells per well in white 1,536-well plates (Corning). 24 h later, 2 µL of ONE-glo luciferase detection reagent (Promega) was dispensed per well and luminance signal recorded with an Envision plate reader (Perkin Elmer).

Miniaturized Reporter Assays

2,500 293A-TEAD-LUC cells were plated in 50 µL of growth medium without FBS in white 384-well plates (Corning) and allowed to proliferate for 48 h until fully confluent. 100 nL of compound solvated in DMSO was then transferred to each well using a Bravo Automated Liquid Handling Platform (Agilent) affixed with a pintool head (V&P Scientific). For TEAD-LUC activity assays, 30 µL of BrightGlo reagent solution (Promega, diluted 1:3 in water) was added to each well after 24 h of compound treatment. For cytotoxicity assays, 30 µL of CellTiter-Glo reagent solution (Promega, diluted 1:6 in water) was added to each well after 24 h and 72 h of compound treatment. Luminance values were recorded on an Envision plate reader (Perkin Elmer).

shRNA Knockdown Studies

CLK2-targeting shRNA vectors 1, 2, and 3 refer to Sigma Mission shRNA lentiviral clones TRCN0000197205, TRCN0000229970, TRCN0000355628, respectively. YAP-targeting shRNA vectors 1 and 2 refer to pLKO1-shYAP1 (Addgene, #27368) and pLKO1-shYAP2 (Addgene, #27369), respectively. The non-targeting scrambled control vector refers to SHC002 (Sigma). Lentiviruses were generated in HEK293T cells by transient expression of the vectors with pSPAX2 and pMD2.G packaging vectors (Addgene plasmids #12260 and #12259). Viral supernatants were collected after 48 h of expression and passed through a 45 µm syringe filter before exposure to target cells.

Quantitative Reverse Transcription PCR (qRT-PCR)

After 48 h of transgene expression or 24 h of compound treatment, cells were collected by trypsinization and subsequent centrifugation at 1200 x*g*. RNA was isolated using an RNeasy kit (Qiagen) and RNA concentrations were determined using a NanoDrop instrument. 1 µg of RNA was subjected to reverse transcription reaction with High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). Quantitative RT-PCR reactions were measured on a Viia 7 Real-Time PCR system (ThermoFisher) using Power SYBR Green (ThermoFisher) and transcript-specific primers below. Reactions were normalized to GAPDH levels for each biological replicate and relative transcript abundance calculated with the comparative C_t method.

Gene Name	Forward Primer	Reverse Primer
ANKRD1	GTGTAGCACCAGATCCATCG	CGGTGAGACTGAACCGCTAT
CTGF	TGGAGATTTTGGGAGTACGG	CAGGCTAGAGAAGCAGAGCC
CYR61	CCCGTTTTGGTAGATTCTGG	GCTGGAATGCAACTTCGG
GAPDH	AATGAAGGGGTCATTGATGG	AAGGTGAAGGTCGGAGTCAA
AMOTL2 exon 7	GGAATGTCGGATGAGAGTGG	GAAATCCAGCGGCTCTCTGAG
AMOTL2 exon 5 skipped	CTGTTCGTAGCTCTAAGATCCC	GAAATCCAGCGGCTCTCTGAG
AMOTL2 exon 5 included	GTAGGGAGAAAGAGGCTTG	GGTTTCTTCTTCCATGAAACAGGG
AMOTL2 exon 9 skipped	GCTACAGAGCTGTAGTCAG	GTCGGTGCCATCTGTTTTCG
AMOTL2 exon 9 included	CTGGAGACAGACAAGAACACAG	CAGAGCATTGTGCTTGGTTG

Semi-Quantitative PCR

After 24 h of compound treatment, cells were collected by trypsinization and subsequent centrifugation at 1200 x*g*. RNA was isolated using an RNeasy kit (Qiagen) and RNA concentrations were determined using a NanoDrop instrument. 1 µg of RNA was subjected to reverse transcription reaction with High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). 1 µL of cDNA was subjected to PCR with exon-specific primers using Platinum Taq DNA Polymerase (ThermoFisher). PCR reactions underwent 35 cycles of replication. PCR reactions were visualized via horizontal gel electrophoresis using a 2% agarose gel. Gels were imaged using a ChemiDoc (BioRad).

Gene Name	Forward Primer	Reverse Primer
AMOTL2 exon 5	CTCCTGCTGCTGTTCGTAG	CTTCAACCGGGATCTTAGAG
AMOTL2 exon 9	CTCTGGATGTAGCTACAGAG	CTGCTCGGCTGACTACAG

Plasmids

FLAG-tagged wild type AMOTL2 (NM_016201) was obtained from Origene. Codon-optimized sequences encoding truncated, FLAG-tagged transgenes of AMOTL2 and HA-tagged MAGI-1 WW domains were obtained from Integrated DNA Technologies as gBlock HiFi Gene Fragments and cloned into the pCMV6 backbone via Gibson assembly using a HiFi DNA Assembly Cloning Kit (NEB).

Immunoblotting

HEK293A cells were plated in standard growth medium at 500,000 cells per well in six-well plates and allowed to grow for 48 h. Then, cells were treated with the indicated concentrations of SM04690 for 24 h. Cells were washed with PBS and collected by the addition of 250 µL of RIPA buffer (EMD Millipore) with scraping. Lysates were clarified by centrifugation (18,253 x*g*, 5 min, 4°C) and protein concentrations determined by absorbance on a NanoDrop instrument (ThermoFisher). Equal amounts of lysate were mixed with 4x loading dye (2% SDS, 200 mM Tris, 20% glycerol, and 0.01% bromophenol blue) with ß-mercaptoethanol added to a final concentration of 10%. Protein material was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 12-well gels (Invitrogen, 4-12% Bis-tris BOLT gels) in 0.9x MOPS-SDS buffer (Invitrogen). Samples were transferred to polyvinylidene difluoride (PVDF) membrane (ThermoFisher) using a semi-dry transfer apparatus (Bio-Rad Laboratories). Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk (Bio-Rad Laboratories) in Tris-buffered saline (TBS, Corning) containing 0.1% Tween-20. Primary antibodies were incubated with shaking overnight at 4°C in 5% milk in TBST or 5% bovine serum albumin (BSA) in TBST. Membranes were exposed to either fluorophore-conjugated secondary antibodies (Li-Cor; 1:2000) or HRP-conjugated secondary antibodies (Sigma; 1:3000) in TBST with 5% milk for 30 min (Li-Cor) or 1 h (HRP) at room temperature. Signals were recorded with either a Li-Cor fluorescence imager or exposed to HRP substrate (West Dura Substrate, Pierce) and signals recorded using autoradiography film (Genesee Scientific) or by imaging using a ChemiDoc instrument (BioRad).

Antigen	Supplier	Catalog #	Dilution
Phospho-YAP (S127)	Cell Signaling Technologies	13008S	1:1000 (BSA)
YAP (total)	Cell Signaling Technologies	140074S	1:1000 (BSA)
AMOTL2	Sigma	HPA063027	1:1000 (Milk)
HA	Cell Signaling Technologies	3724S	1:1000 (BSA)
FLAG	Sigma	F1804	1:1000 (Milk)
Tubulin	Sigma	T6557	1:2000 (Milk)

Immunoprecipitation Studies

Plasmids were transiently transfected to HEK293T cells for transgene expression using FuGENE (4 µL FuGENE per 1 µg of DNA) in 100 µL of Opti-MEM (Gibco) per well of a six-well plate (2 µg DNA total per well). Media was replaced 24h later. Following an additional 24-hour incubation, cells were washed with PBS and collected by the addition of 250 µL of RIPA buffer (EMD Millipore) or nondenaturing lysis buffer (20 mM Tris, 137 mM NaCl, and 0.5% NP-40) with scraping. For MAGI1 immunoprecipitation, lysates were tip sonicated. Insoluble material was separated by centrifugation (18,253 x*g*, 5 min, 4°C) and the protein concentration in cellular lysates was determined by absorbance on a nanodrop instrument (ThermoFisher). 1 mg of lysate in 1 mL of lysis buffer was incubated overnight at 4°C with 20 µL of anti-FLAG M2 magnetic bead slurry (MilliporeSigma, no. M8823). After three washes with lysis buffer, immunoprecipitated material was eluted with 250 µg per mL of FLAG peptide (DYKDDDDK, Sino Biological). Eluted material from immunoprecipitations and whole-cell lysates were evaluated by immunoblotting analyses as described above.

Imaging Studies

For AMOTL2 localization studies, MDCK cells were plated in 12-well plates with glass coverslips at either 10,000 (sparse) or 400,000 (dense) cells in growth medium and allowed to propagate for 48 h. For treatment studies, 350,000 cells in growth medium propagated for 48 h cells were treated for 24 h with 1 μM SM04690. Growth medium was removed, and cells were fixed with ice-cold methanol for 10 min at -20°C. After washing 5 times with PBS, cells were blocked and permeabilized with 5% FBS, 0.1% Triton-X in PBS for 30 min at room temperature. Primary antibodies in the same blocking buffer were incubated at 1:100 overnight at 4°C. After three washes with PBS, Secondary AlexaFluor-conjugated antibodies (Life Technologies; 1:500 in 5% FBS, 0.1% Triton-X in PBS) with Hoechst 33342 dye were incubated for 1 h at room temperature in the dark. The wells were washed three more times with PBS and then coverslips were removed and mounted onto glass slides using the ProLong Gold Antifade Mount (ThermoFisher). Slides were imaged using a laser-scanning Zeiss confocal LSM 720 microscope and colocalization coefficients calculated using Zeiss Zen software. For AMOTL2 overexpression localization studies, HEK293A cells plated in 6-well plates with glass coverslips were transfected with 1 μg of AMOTL2 constructs and grown for 48 h. Fixing, staining, and imaging proceeded with the same steps as above. Puncta were quantified using Image J (NIH). For YAP localization studies, MDCK cells were plated in 12-well plates with glass coverslips at 250,000 cells per well and allowed to propagate for 48 h. Cells were then treated for 24 h with 1 μM SM04690. Growth medium was removed, and cells were fixed with ice-cold methanol for 10 min at -20°C. After washing 3 times with PBS, cells were permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature. Primary antibodies in 5% BSA in PBS were incubated at 1:100 overnight at 4°C. After three washes with PBS, Secondary AlexaFluor-conjugated antibodies (Life Technologies; 1:500 in 5% BSA in PBS) with Hoechst 33342 dye were incubated for 1 h at room temperature in the dark. The wells were washed three more times with PBS and then coverslips were removed and mounted onto glass slides using the ProLong Gold Antifade Mount (ThermoFisher). Slides were imaged using a laser-scanning Zeiss confocal microscope as above.

Antigen	Supplier	Catalog #	Dilution
YAP (total)	Santa Cruz Biotechnology	SC-101199	1:100
AMOTL2	Sigma	HPA063027	1:100
E-cadherin	Cell Signaling Technologies	14472	1:100
PALS1	Santa Cruz Biotechnology	SC-365411	1:100
MAGI-1	Abnova	H00009223-M02	1:100

Human Keratinocyte Proliferation Assays

Immortalized human keratinocytes (HaCaT cells) were plated at 5 x 10^3 cells per well in 12-well plates in reduced-serum-containing medium (DMEM containing 1% penicillin/streptomycin and 0 or 2% FBS). After 24 h, cells were treated with the indicated concentrations of SM04690. After 7 days of growth under these conditions, cells were fixed in 4% PFA in PBS for 10 min, exposed to 2 μg per mL Hoechst 33342 for 10 min and imaged on a Nikon Eclipse Ti microscope. Nuclei were quantified by an automated ImageJ macro and reported as nuclei per imaging field.

Chemicals

SM04690 was obtained from Selleck chemicals. T025 and CLK-in-T3 were from MedChemExpress. CC671, Harmine, INDY, Leucettine L41, ML167, and TG003 were from Cayman chemical. All chemicals were dissolved in DMSO with no further purification.

Statistics

All statistical analyses were univariate two-sided t-tests.

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Author Contributions

M.L.B. performed semi-quantitative PCR and RT-qPCR, did immunoprecipitation experiments, performed all immunoblotting, did proliferation assays, and worked on imaging studies. M.L.B. and E.M.G. performed reporter assays and did in situ target identification work. E.C., M.H., and K.A.J. performed the high-throughput chemical screens and validated hit compounds. M.L.B. constructed expression vectors and M.L.B. and C.L. prepared expression vectors. M.J.B. performed imaging. M.L.B., E.M.G., E.C., M.H., K.A.J., and M.J.B. analyzed primary data. M.J.B. conceived of the project. M.L.B. and M.J.B. wrote the paper.

Conflicts of Interest

The authors declare no conflicts of interest.

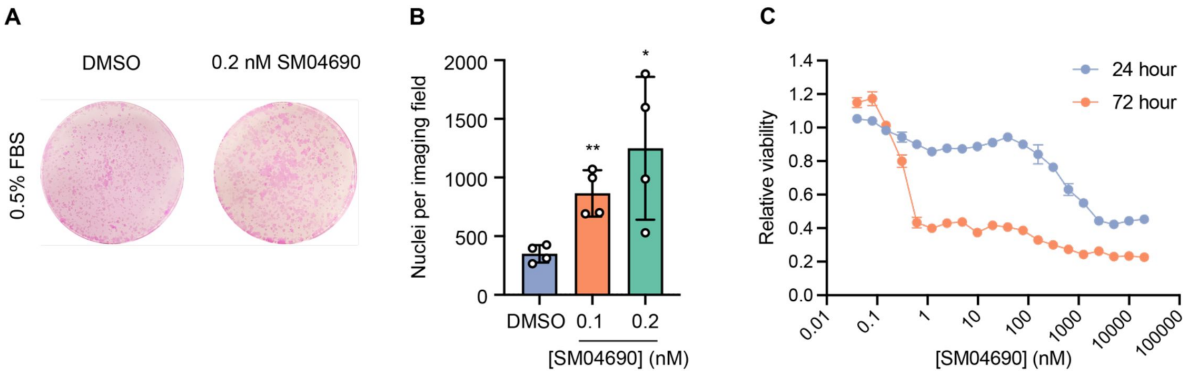

Figure S1.

SM04690 induces YAP dependent proliferation at non-toxic concentrations.

(A) Representative Rhodamine B staining, and (B) number of cells of human keratinocytes (HaCaT) in 0.5% FBS treated with SM04690 for 7 days (n=4, mean and s.d.). Statistical test is a univariate two-sided t-test (* P <0.0332, ** P < 0.0021). (C) Cell viability dose-response of SM04690 in 293A-TEAD-LUC cells treated for 72h with SM04690 evaluated using CellTiter-Glo luminescent cell viability assay (n=3 biological replicates, mean and s.d.).

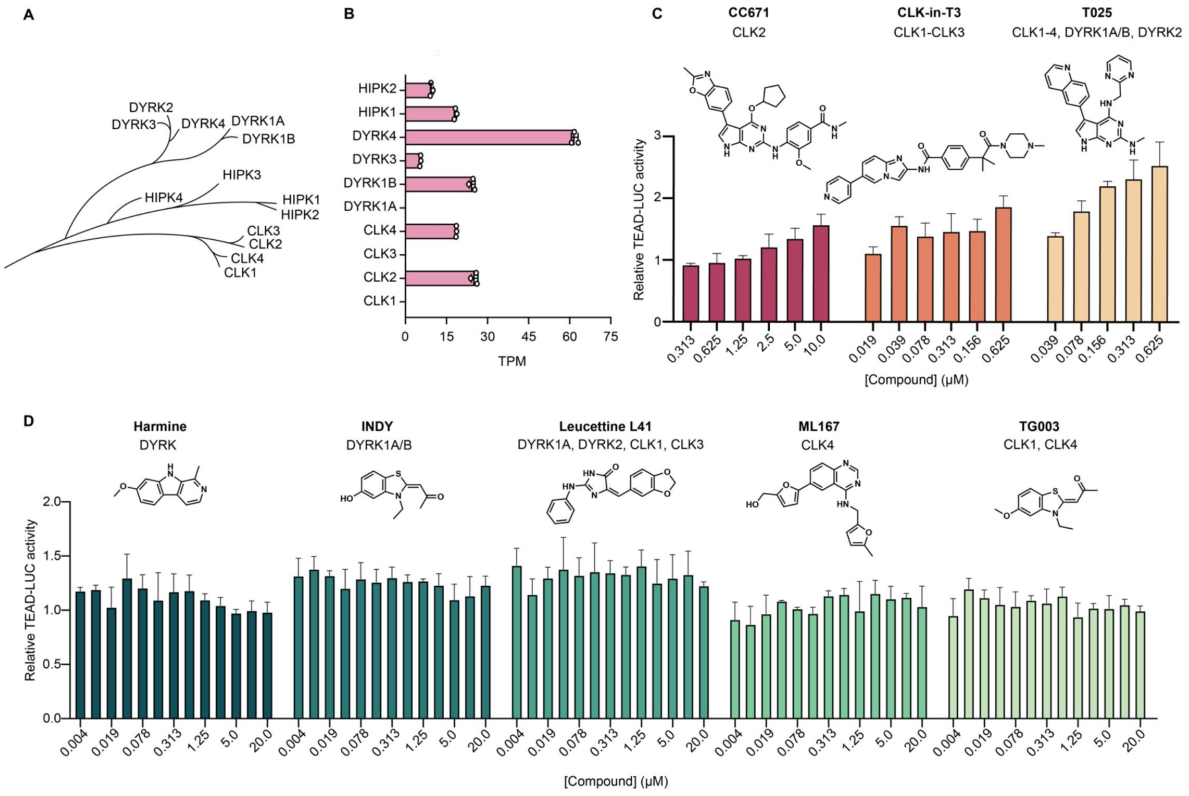

Figure S2.

CLK2 inhibition activates YAP.

(A) Family tree of CLK, DYRK, and HIPK kinases. (B) Transcripts per million (TPM) of the transcripts corresponding to reported targets of SM04690 in HEK293A cells, as determined by RNA-sequencing of unstimulated cells. (C) Relative TEAD-LUC activity of CLK2-targeting inhibitors CC671, CLK-in-T3, and T025 in cells treated for 24h with compound (n=3 biological replicates, mean and s.d.). (D) Relative TEAD-LUC activity of non-CLK2-targeting inhibitors Harmine, INDY, Leucettine L41, ML167, and TG003 in cells treated for 24h with compound (n=3 biological replicates, mean and s.d.).

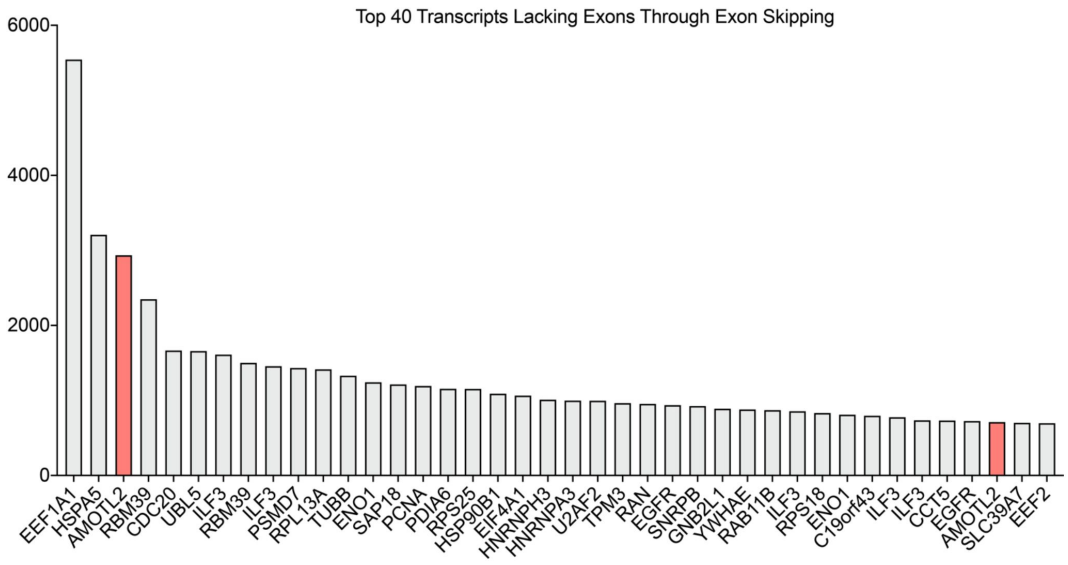

Figure S3.

Top 40 transcripts with skipped exons in response to CLK2 inhibition.

Number of skipped exons of the indicated transcripts in response to CLK2 inhibition. Figure derived from Araki, S. et al., *PLOS One*, 2015.

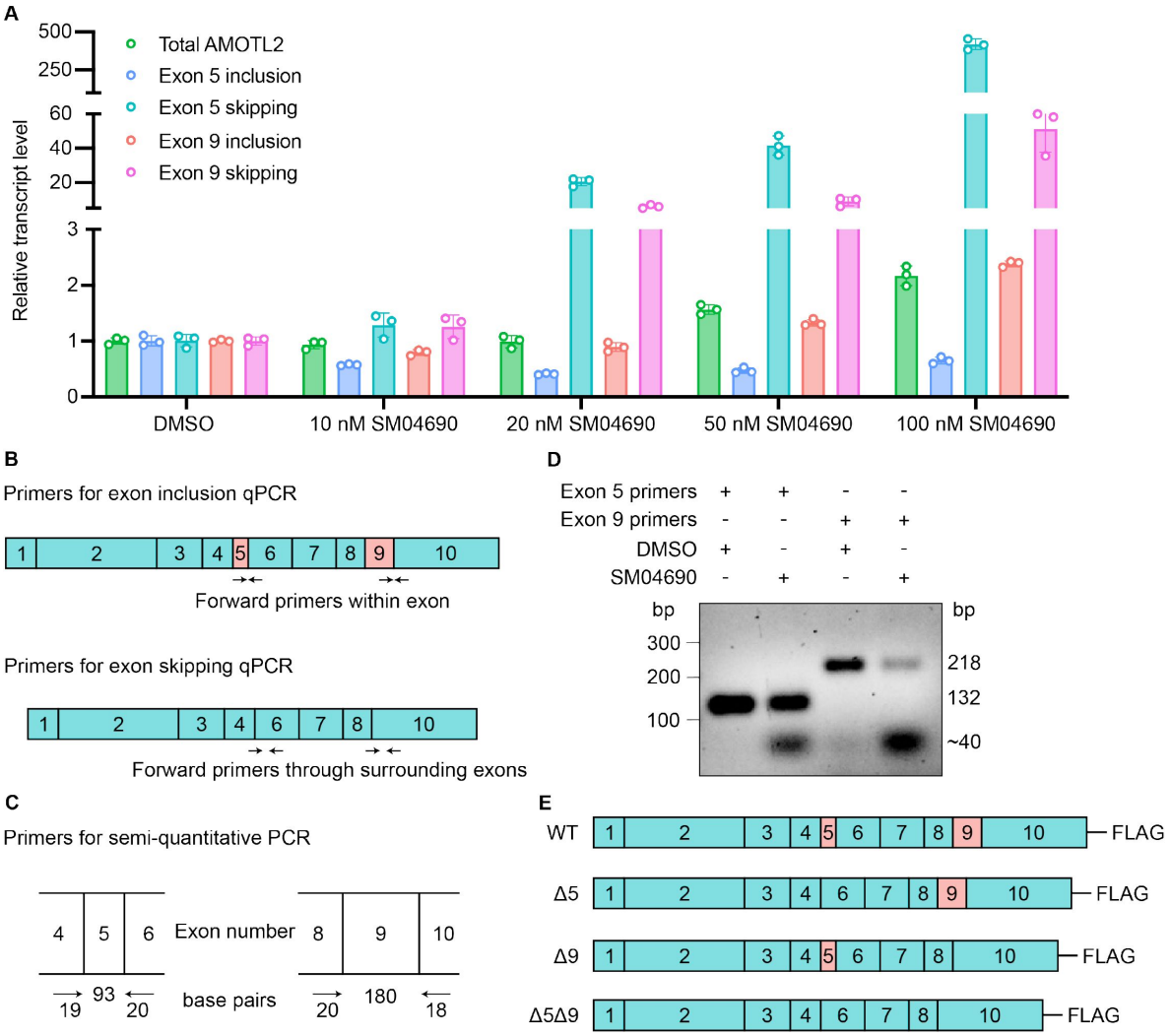

Figure S4.

Inhibition of CLK2 by SM04690 causes alternative splicing of AMOTL2 exons 5 and 9.

(A) Schematic of primer design to determine the presence of exon skipping in AMOTL2 by RT-qPCR. Exon inclusion primers were designed with the forward primer within the exon and the reverse primer at the start of the next flanking exon. Exon skipping primers were designed with the forward primer spanning the junction between the two exons flanking the skipped exon and the reverse primer within the adjacent exon. (B) Schematic of primer design to determine exon skipping in AMOTL2 by semi-quantitative PCR. The forward primer resides in the exon preceding the skipped exon and the reverse primer resides in the exon following the skipped exon. (C) Agarose gel electrophoresis of PCR amplified products using exon-specific primer sets. The primers amplified differently sized PCR amplicons depending on whether the template included the variable exon. Exon-included samples appear in the gel at the size of the exon plus the size of the primers. Exon-skipped samples appear in the gel at the size of only the primers (~40 base pairs). (D) Schematic of C-terminal FLAG-tagged AMOTL2 constructs.

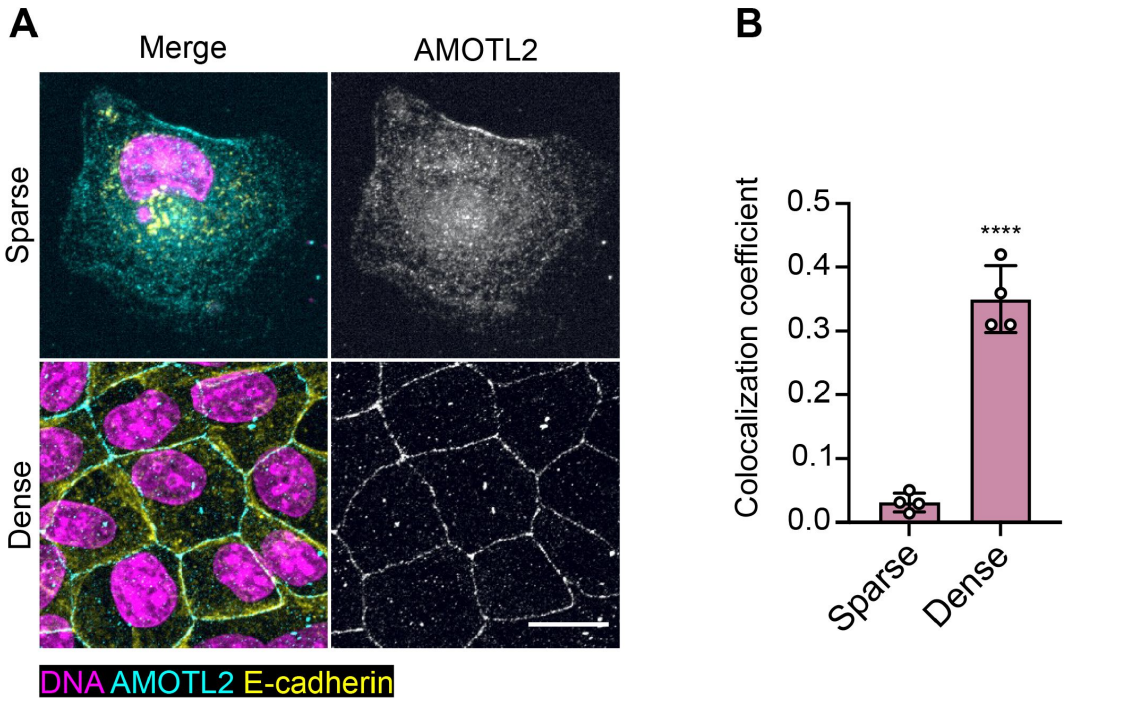

Figure S5.

AMOTL2 displays density dependent localization to the plasma membrane.

(A) Representative images of anti-AMOTL2 (teal) and anti-E-cadherin (yellow) immunofluorescent staining of MDCK cells grown in sparse (10,000 cells) and dense (400,000 cells) cell conditions with Hoechst 33342 (pink) to visualize nuclei (scale bar = 20 μm). (B) Quantification of anti-AMOTL2 and anti-E-cadherin correlative immunofluorescent staining (n=4, mean and s.d.). Statistical analysis is a univariate two-sided t-test (**** $P < 0.0001$).

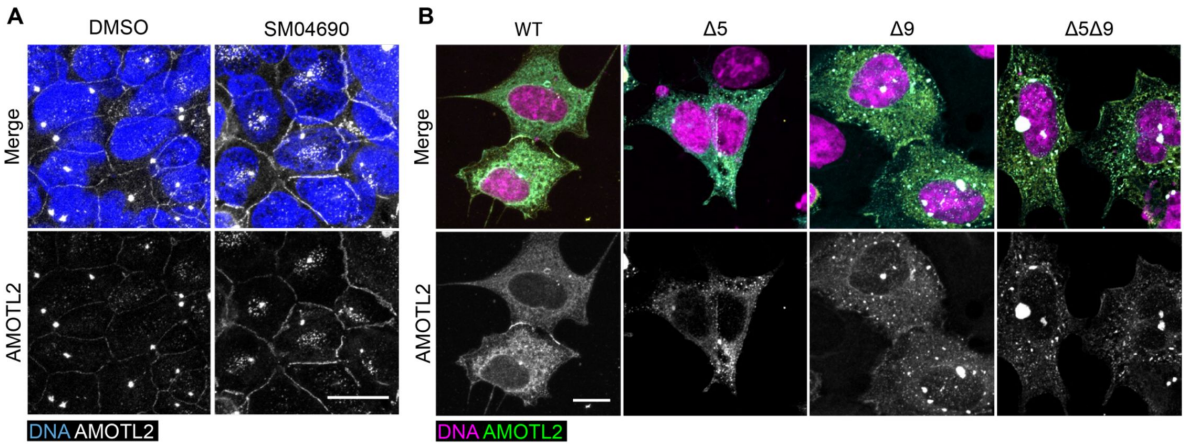

Figure S6.

AMOTL2 spliceoforms are aggregation prone and do not localize to membranes. (A) Representative images of anti-AMOTL2 (white) immunofluorescent staining of HEK293A cells treated with control or 1μM SM04690 for 24h with Hoechst 33342 (blue) to visualize nuclei (scale bar = 20 μm). (B) Representative images of anti-AMOTL2 (green) immunofluorescent staining of HEK293A cells overexpressing AMOTL2 spliceoforms with Hoechst 33342 (pink) to visualize nuclei (scale bar = 10 μm).

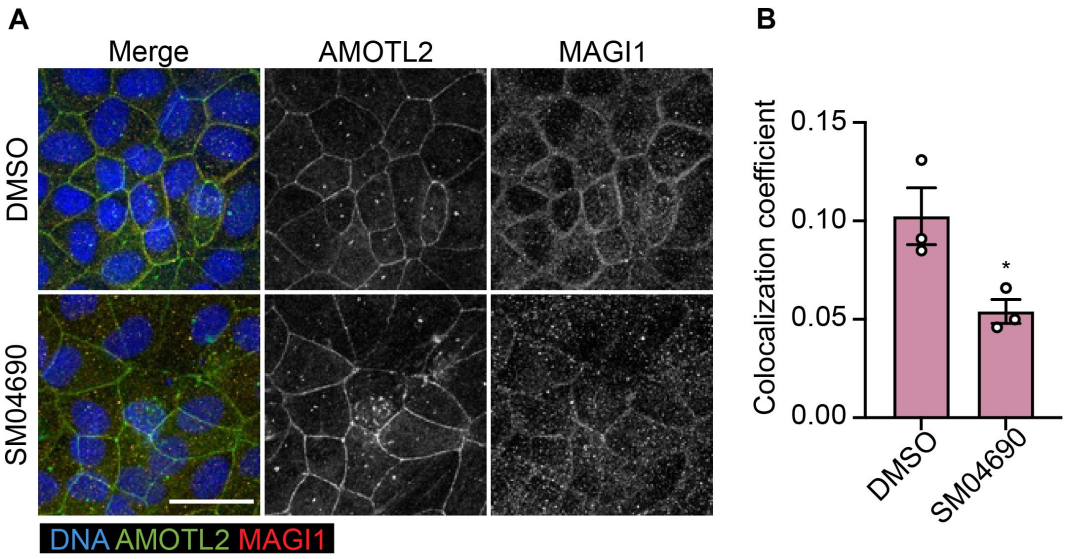

Figure S7.

SM04690 treatment delocalizes AMOTL2 from MAGI1. (A) Representative images of anti-AMOTL2 (green) and anti-MAGI1 (red) immunofluorescent staining of MDCK cells treated with control or 1 μM SM04690 for 24h. Hoechst 33342 (blue) was used to visualize nuclei (scale bar = 30 μm). (B) Quantification of anti-AMOTL2 and anti-MAGI1 correlative immunofluorescent staining (n=3, mean and s.d.). Statistical analysis is a univariate two-sided t-test (*P <0.0332).

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Reviewer #1 (Public Review):

In order to find small molecules capable of enhancing regenerative repair, this study employed a high throughput YAP-activity screen method to query the ReFRAME library, identifying CLK2 inhibitor as one of the hits. Further studies showed that CLK2 inhibition leads to AMOTL2 exon skipping, rendering it unable to suppress YAP.

The novelty of the study is that it showed that inhibition of a kinase not previously associated with the HIPPO pathway can influence YAP activity through modification of mRNA splicing. The major arguments appear solid.

In the revised manuscript, additional discussion was provided regarding drug concentration and molecular mechanisms, which helps clear some of the confusing points in the original manuscript.

Reviewer #2 (Public Review):

In this manuscript, the authors have screened the ReFRAME library and identified candidate small molecules that can activate YAP. They found that SM04690, an inhibitor of the WNT signaling pathway, could efficiently activate YAP through CLK2 kinase which has been shown to phosphorylate SR proteins to alter gene alternative splicing. They further demonstrated that SM04690 mediated alternative splicing of AMOTL2 and rendered it unlocalized on the membrane. Alternatively spliced AMOTL2 prevented YAP from anchoring to the cell membrane which results in decreased YAP phosphorylation and activated YAP. Previous findings showed that WNT signaling more or less activates YAP. The authors revealed that an inhibitor of WNT signaling could activate YAP. Thus, these findings are potentially interesting and important. However, the present manuscript provided a lot of indirect data and lacked key experiments.

Major points:
1. In Figure S3, since inhibition of CLK2 resulted in extensive changes in alternative splicing, why did the authors choose AMOTL2? How to exclude other factors such as EEF1A1 and HSPA5, do they affect YAP activation? Angiomotin-related AMOTL1 and AMOTL2 were identified as negative regulators of YAP and TAZ by preventing their nuclear translocation. It has been reported that high cell density promoted assembly of the Crumbs complex, which recruited AMOTL2 to tight junctions. Ubiquitination of AMOTL2 K347 and K408 served as a docking site for LATS2, which phosphorylated YAP to promote its cytoplasmic retention and degradation. How to determine that alternative splicing rather than ubiquitination of AMOTL2 affects YAP activity? Does AMOTL2 Δ5 affect the ubiquitination of AMOTL2? Does overexpression of AMOTL2 Δ5Δ9 cause YAP and puncta to co-localize?
2. The author proposed that AMOTL2 splicing isoform formed biomolecular condensates,.However, there was no relevant experimental data to support this conclusion. AMOTL2 is located not only on the cell membrane but also on the circulating endosome of the cell, and the puncta formed after AMOTL2 dissociation from the membrane is likely to be the localization of the circulating endosome. The author should co-stain AMOTL2 with markers of circulating endosomes, or conduct experiments to prove the liquidity of puncta to verify the phase separation of AMOTL2 splicing isoform.
3. The localization of YAP in cells is regulated by cell density, and YAP usually translocates to the nucleus at low cell density. In Figure 2E, the cell densities of DMSO and SM04690-treated groups are inconsistent. In Figure 4A, the magnification of t DMSO and SM04690-treated groups is inconsistent, and the SM04690-treated group seems to have a higher magnification.
4. There have been many reports that the WNT signaling pathway and the Hippo signaling pathway can crosstalk with each other. The authors should exclude the influence of the WNT signaling pathway by using SM04690.

Reviewer #3 (Public Review):

This study on drug repurposing presents the identification of potent activators of the Hippo pathway. The authors successfully screen a drug library and identify two CLK kinase inhibitors as YAP activators, with SM04690 targeting specifically CLK2. They further investigate the molecular basis of SM04690-induced YAP activity and identify splicing events in AMOTL2 as strongly affected by CLK2 inhibition. Exon skipping within AMOTL2 decreases the interactions with membrane bound proteins and is sufficient to induce YAP target gene expression. Importantly, inhibitor concentrations that are sufficient to change YAP target gene expression show differential alternative splicing of AMOTL2. Overall the study is well designed, the conclusions are supported by sufficient data and represent an exciting connection between alternative splicing and the HIPPO pathway.

Author Response

The following is the authors' response to the original reviews.

Public Reviews:

> ***Reviewer #1 (Public Review):***
>
> *In order to find small molecules capable of enhancing regenerative repair, this study employed a high throughput YAP-activity screen method to query the ReFRAME library, identifying CLK2 inhibitor as one of the hits. Further studies showed that CLK2 inhibition leads to AMOTL2 exon skipping, rendering it unable to suppress YAP.*
>
> *The novelty of the study is that it showed that inhibition of a kinase not previously associated with the HIPPO pathway can influence YAP activity through modification of mRNA splicing. The major arguments appear solid.*

We thank the Reviewer for their thoughtful assessment of this work. We have fully addressed each comment below in a point-by-point fashion.

> *There are several noteworthy points when assessing the results. In Figure S1C, 100nM drug was toxic to cells at 72 hours and 1nM drug suppressed cell proliferation by 60%. Yet such concentrations were used in Figure 1B and C to argue CLK2 inhibition liberates YAP activity (which one would assume will increase cellular proliferation). In Figure 1C it appears that 1nM drug treatment led to some kind of cellular stress, as cells are visibly enlarged. In Figure 1D, 1nM drug, which would have suppressed cell growth by 60%, did not affect YAP phosphorylation. Taken together, it appears even though CLK2 inhibitor (at high concentrations) liberates YAP activity, its toxicity may override the potential use of this drug as a YAP-activator to salve tissue regenerative repair, which was one of the goals hinted in the background section.*

We do not claim that CLK2 inhibition is useful as a YAP activator, either as a precise pharmacological tool or as a therapeutic mechanism for inducing regenerative repair. Instead, the key finding of this work is to describe a novel, unanticipated cellular mechanism for activating YAP, one that should be considered when optimizing pharmacological candidates that modulate alternative splicing for diseases where potential proliferation is undesirable.

However, to address this point, we have included additional experimentation. Specifically, we show that cytotoxicity with compound treatment at 24 hours, a timepoint at which we perform most evaluation of alternative splicing induced by compound, is considerably less than that observed at 72 hours. Now included as Figure S1C, this panel shows while the compound displays some cytotoxicity at ~1 nM at 72 hours, the half maximal inhibitory potency at 24 hours is ~300 nM. As such, we believe there is not incongruity between YAP activity, cellular proliferation, and SM04690-induced cytotoxicity. It is simply such that higher concentrations of compound, and thus increased engagement of CLK2 and other targets of the inhibitor, result in a cumulative cytotoxic effect over time.

> *In Figure 2D, at 100nM concentration, the drug did not appear to affect AMOTL2 splicing. Even though at higher concentrations it did, this potentially put into question whether YAP activity liberated by this drug at 1nM (Fig 2A), 10-50nM (Fig 2C) concentrations is caused by altered AMOTL2 splicing. Discussions should be provided on the difference in drug concentrations in these experiments. Does the drug decay very fast, and is that why later studies required higher dose?*

We believe this comment is in reference to Fig. 3D, and we argue that, while faint, there is the presence of AMOTL2 splicing at 100 nM SM04690 treatment as seen by a faint lower molecular weight band. However, to further understand the extent to which AMOTL2 is alternatively spliced in response to compound treatment, we performed RT-qPCR analysis of AMOTL2 splicing with an expanded concentration response. These results indicate that high magnitude exon skipping of AMOTL2 occurs starting at 10 nM with 24-hour treatment of compound (now in the manuscript as Fig. S4A). This result matches with our data in Fig. 2C, wherein YAP phosphorylation begins decreasing at 10 nM SM04690 treatment.

> *Likely impact of the work on the field: this study presented a high throughput screen method for YAP activators and showed that such an approach works. The hit compound found from ReFRAME library, a CLK2 inhibitor, may not be actually useful as a YAP activator, given its clear toxicity. Applying this screen method on other large compound libraries may help find a YAP activator that helps regenerative repair. The finding that CLK2 inhibition could alter AMOTL2 splicing to affect HIPPO pathway could bring a new angle to understanding the regulation of HIPPO pathway.*

> ***Reviewer #2 (Public Review):***
>
> *In this manuscript, the authors have screened the ReFRAME library and identified candidate small molecules that can activate YAP. The found that SM04690, an inhibitor of the WNT signaling pathway, could efficiently activate YAP through CLK2 kinase which has been shown to phosphorylate SR proteins to alter gene alternative splicing. They further demonstrated that SM04690 mediated alternative splicing of AMOTL2 and rendered it unlocalized on the membrane. Alternatively spliced AMOTL2 prevented YAP from anchoring to the cell membrane which results in decreased YAP phosphorylation and activated YAP. Previous findings showed that WNT signaling more or less activates YAP. The authors revealed that an inhibitor of WNT signaling could activate YAP. Thus, these findings are potentially interesting and important. However, the present manuscript provided a lot of indirect data and lacked key experiments.*

We thank the Reviewer for their thorough review of this work. We have responded to each comment below.

> *Major points:*
>
> 1. *In Figure S3, since inhibition of CLK2 resulted in extensive changes in alternative splicing, why did the authors choose AMOTL2? How to exclude other factors such as EEF1A1 and HSPA5, do they affect YAP activation? Angiomotin-related AMOTL1 and AMOTL2 were identified as negative regulators of YAP and TAZ by preventing their nuclear translocation. It has been reported that high cell density promoted assembly of the Crumbs complex, which recruited AMOTL2 to tight junctions. Ubiquitination of AMOTL2 K347 and K408 served as a docking site for LATS2, which phosphorylated YAP to promote its cytoplasmic retention and degradation. How to determine that alternative splicing rather than ubiquitination of AMOTL2 affects YAP activity? Does AMOTL2 Δ5 affect the ubiquitination of AMOTL2? Does overexpression of AMOTL2 Δ5Δ9 cause YAP and puncta to co-localize?*

AMOTL2 is the relevant cellular target, because among the entire transcriptome it was the third most alternatively spliced in response to CLK2 inhibition (Fig. S3). No other targets relevant to the Hippo pathway were identified.

We have shown that overexpression of exon skipped AMOTL2 (Fig. 3F) recapitulates the effect of compound, indicating that splicing per se is what drives the YAP activation phenotype. While AMOTL2 is ubiquitinated, these established sites of ubiquitination do not lie within exons 5 or 9. Thus, we anticipate that ubiquitination is less likely a driving factor in the observed phenotype. The manuscript is written as not to exclude this as a possibility, but it is downstream of what we describe, and we believe out of scope to explore this further in this preliminary report.

> 1. *The author proposed that AMOTL2 splicing isoform formed biomolecular condensates. However, there was no relevant experimental data to support this conclusion. AMOTL2 is located not only on the cell membrane but also on the circulating endosome of the cell, and the puncta formed after AMOTL2 dissociation from the membrane is likely to be the localization of the circulating endosome. The author should co-stain AMOTL2 with markers of circulating endosomes or conduct experiments to prove the liquidity of puncta to verify the phase separation of AMOTL2 splicing isoform.*

We do not claim AMOTL2 forms biomolecular condensates. Instead, we hypothesize in the Discussion section that AMOTL2 could possibly phase separate into biomolecular condensates

based on its similarity to AMOT, which has been shown to phase separate and form cytoplasmic puncta (PMID: 36318920). AMOT has also been shown to colocalize with endosomes (PMID: 25995376), which also appear as puncta.

> 1. *The localization of YAP in cells is regulated by cell density, and YAP usually translocates to the nucleus at low cell density. In Figure 2E, the cell densities of DMSO and SM04690-treated groups are inconsistent. In Figure 4A, the magnification of t DMSO and SM04690-treated groups is inconsistent, and the SM04690treated group seems to have a higher magnification.*

In immunofluorescence experiments, cells were plated at the same density and grown for the same amount of time before treatment. Additionally, within an experiment, images were taken at the same magnification. Any apparent differences in cell density are due to effects of the compound.

> 1. *There have been many reports that the WNT signaling pathway and the Hippo signaling pathway can crosstalk with each other. The authors should exclude the influence of the WNT signaling pathway by using SM04690.*

While the WNT pathway has been shown to influence Hippo pathway activity, we have shown a direct effect of CLK2 inhibition by SM04690. Any WNT potential pathway effects are in addition to the splicing-based mechanism we described.

> ***Reviewer #3 (Public Review):***
>
> *This study on drug repurposing presents the identification of potent activators of the Hippo pathway. The authors successfully screen a drug library and identify two CLK kinase inhibitors as YAP activators, with SM04690 targeting specifically CLK2. They further investigate the molecular basis of SM04690-induced YAP activity and identify splicing events in AMOTL2 as strongly affected by CLK2 inhibition. Exon skipping within AMOTL2 decreases the interactions with membrane bound proteins and is sufficient to induce YAP target gene expression. Overall the study is well designed, the conclusions are supported by sufficient data and represent an exciting connection between alternative splicing and the HIPPO pathway. The specificity of the inhibitor towards CLK2 and the mode of action via AMOTL2 could be supported by further data:*

We thank the Reviewer for their close examination of our work. We respond below.

> 1. *The inconsistent inhibitor concentrations and varying results reported in the paper can be distracting. For instance, the response of endogenous targets to 100 nM concentration is described as a >5-fold increase in Figure 2B, whereas it is reported as a 1-1.5-fold response to 1000 nM in Figure 2D. This inconsistency should be addressed and clarified to provide a more accurate and reliable representation of the findings.*

In Figure 2D, we have transduced cells with lentivirus, which most likely suppresses their responsiveness to compound treatment. We have addressed the issue of varying inhibitor concentrations in response to Reviewer 1.

> 1. *In the absence of a strong inhibitor induced YAP target gene expression (Figure 2D), it is difficult to conclude the dependency on YAP expression, as investigated by siRNA mediated knockdown. In a similar experiment, the dependency of the inhibitor on CLK2 expression could be confirmed*

While the sample with Scramble virus does not respond to the same extent that WT HEK293A cells do (e.g., Fig. 2B), there is still responsiveness to compound. Likewise, YAP knockdown cells display statistically significant decreases in YAP-controlled transcripts. This decrease of transcript is therefore sufficient evidence that SM04690 requires YAP for its activity. We have shown that multiple CLK2 inhibitors recapitulate the effect of SM04690, abrogating the need to show dependency of CLK2.

> 1. *To further support the conclusion that CLK2 is the direct target of SM04690, it would be informative to investigate the effects of CLK1/4 inhibition on AMOTL2 exons (for example within RNA-seq data). If CLK1/4 inhibitors do not induce changes in AMOTL2 exons, it would strengthen the evidence for CLK2's role as the direct target. Including the results in the discussion would enhance the comprehensiveness of the study.*

We showed that CLK1/4 inhibition with small molecules ML167 and TG003 does not affect YAP activity in our luciferase reporter assay (Fig. S2D), which we believe is sufficient evidence that CLK1/4 is neither the direct target of SM04690 nor relevant to the splicing mechanism we describe.

> 1. *It would be important to determine the specific dose of SM04690 required to induce changes in AMOTL2 splicing. The authors observe that AMOTL2 protein levels appear unaffected at doses below 50 nM in Figure 3D, while YAP target genes are already affected at 20 nM in Figure 3G. Although Western blotting may not be the most sensitive method to detect minor changes in splicing, performing PCR experiments at lower doses could provide more insight into the splicing changes. Therefore, it is suggested that the authors include PCR experiments at lower doses to determine if changes in splicing are visible and to better establish the relationship between splicing and gene expression changes.*

We agree with the Reviewer that this experiment is essential to better understand splicing changes with SM04690 treatment. Accordingly, we have added RT-qPCR-based analysis of AMOTL2 exon inclusion at lower concentrations between 10 nM and 100 nM (Fig. S4A). We included a similar discussion in response to a point from Reviewer 1.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *As stated in the public review section, it will be helpful to discuss the differences in drug concentration. Although no one should require or expect a perfect drug dose match throughout any study, in this study the drug dose clearly demarcated when CLK2 inhibitor help/hurt proliferation, when CLK2 inhibitor was able to affect YAP phosphorylation, and when CLK2 inhibitor was able to affect AMOTL2 splicing. This is not to challenge the major conclusions of the paper, but it is hard to ignore if no discussion is provided.*
>
> *Several suggestions on data presentation:*
>
> 1. *Scale bar information is missing in Fig. 2E, 4A and 4B.*

We have corrected this mistake in the revised manuscript.

> 1. *For Fig.3 D and 3E, it's better if kD information was labeled alongside the AMOTL2 Western blot.*

Thank you for the suggestion; we have added the appropriate labeling.

> 1. *It's better to label Figure2D as sh YAP-1, sh YAP-2; Figure 3A as sh CLK2-1, sh CLK2-2 etc. Currently they are all labeled shRNA-1, shRNA-2, which can be confusing.*

We have altered the labeling for clarity as requested.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> 1. *The use of asterisks in Figure 2D is unclear, especially their placement on the "Scramble" sample.*

We have amended the asterisks and have also added more detail to the figure legend.

> 1. *When designing primers for splicing-sensitive PCR, it is recommended that the skipping isoform is larger than 100 bp. This will help to avoid quantitative issues with ethidium bromide staining. In the results part, the text reads as if only the skipping isoform is present after SM04690 treatment.*

This experiment was performed to confirm the presence of exon skipping in the treated samples. Accordingly, we did not optimize the ethidium bromide staining of the lower bp bands. We will take the size of the isoform into consideration in any future experiments. We thank the reviewer for catching the textual error and have amended the text in the manuscript.