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Title: Cooperation between MYC and β-catenin in liver tumorigenesis requires Yap/Taz

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List of Abbreviations: HCC, hepatocellular carcinoma; AAV, Adeno-Associated viral; TAM, Tamoxifen; mHCCs, mouse hepatocellular carcinomas; 4-OHT, 4-hydroxytamoxifen; DEG, differentially expressed genes; GSEA, Gene Set Enrichment Analysis; wt, wild-type; Doxy, Doxycyline; TCGA, The Cancer Genome Atlas; NES, Normalized Enrichment score; FDR, False Discovery Rate.

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Abstract

Background & Aims. Activation of *MYC* and *CTNNB1* (encoding β-catenin) can co-occur in liver cancer, but how these oncogenes cooperate in tumorigenesis remains unclear.

Approach & Results. We generated a mouse model allowing conditional activation of MYC and WNT/β-catenin signaling (through either β-catenin activation or Apc loss) upon expression of CRE recombinase in the liver, and monitored their effects on hepatocyte proliferation, apoptosis, gene expression profiles and tumorigenesis. Activation of WNT/β-catenin signaling strongly accelerated MYC-driven carcinogenesis in the liver. Both pathways also cooperated in promoting cellular transformation *in vitro*, demonstrating their cell-autonomous action. Short-term induction of MYC and β-catenin in hepatocytes followed by RNA-seq profiling allowed the identification of a "Myc/β-catenin signature", composed of a discrete set of Myc-activated genes whose expression increased in the presence of active β-catenin. Notably this signature enriched for targets of Yap and Taz, two transcriptional co-activators known to be activated by WNT/β-catenin signaling, and to cooperate with MYC in mitogenic activation and liver transformation. Consistent with these regulatory connections, Yap/Taz accumulated upon Myc/β-catenin activation and were required not only for the ensuing proliferative response, but also for tumor cell growth and survival. Finally, the Myc/β-catenin signature was enriched in a subset of human hepatocellular carcinomas characterized by comparatively poor prognosis.

Conclusions. Myc and β-catenin show a strong cooperative action in liver carcinogenesis, with Yap and Taz serving as mediators of this effect. These findings warrant efforts toward therapeutic targeting of Yap/Taz in aggressive liver tumors marked by elevated Myc/β-catenin activity.

Introduction

Liver cancers, among which hepatocellular carcinoma (HCC) is the predominant form, are the fourth leading cause of cancer-related mortality worldwide. Heterogeneity in genomic alterations in HCCs, as revealed by recent large-scale genome sequencing efforts, renders the understanding of driving molecular events and the design of targeted therapeutic strategies very challenging, with HCC patients still having limited therapeutic options (1, 2).

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One of the most frequently altered pathways in HCC is WNT/β-catenin signaling (1-3). In this pathway, also known as "canonical" WNT signaling, the exposure of cells to WNT ligands leads to stabilization of the transcriptional coactivator β-catenin, which is then free to translocate to the nucleus and activate target genes in association with DNA-binding factors of the TCF family (4). β-catenin turnover is regulated by the so-called "destruction complex", a cytoplasmic assembly that includes - among others - Axin1, APC, the ubiquitin ligase β-TrCP and the kinases CK1α/δ and GSK3α/β. In the absence of Wnt ligands, these kinases phosphorylate β-catenin at several sites, creating a discrete phospho-degron motif that acts as a docking site for β-TrCP, thus triggering ubiquitination and proteasome-mediated degradation of β-catenin (4). Activation of the WNT/β-catenin pathway in cancer can occur either via enhanced exposure to WNT ligands, or through genetic lesions in its core components: the latter include loss of APC, most frequent in colorectal cancer (4), or activating mutations in the β-catenin gene *CTNNB1*, as commonly observed in HCC (1-3).

The *MYC* proto-oncogene is one of the transcriptional targets of β-catenin/TCF, and functions as a key downstream effector of WNT/β-catenin signaling in several tissues, such as the small intestine, T cells and lung (5-8). In the liver, however, this epistatic relationship may not hold true: in particular, APC loss and the consequent activation of β-catenin do not induce *Myc* expression (9) and *Myc* deletion does not suppress the effects of APC loss on either hepatocyte proliferation (10) or liver zonation (11). Moreover, there are indications that the two pathways can be independently activated and cooperate in tumorigenesis (12). In particular, *MYC* amplification and *CTNNB1* mutations showed a tendency toward co-occurrence in either adult HCC or aggressive childhood hepatoblastoma (3, 5, 13), and MYC-driven HCC in experimental mouse models frequently acquired activating mutations in *Ctnnb1* (5, 14, 15).

In order to unravel the functional cross-talk between MYC and WNT/β-catenin signaling in liver tumorigenesis, we generated a new mouse model allowing conditional activation of MYC and β-catenin in hepatocytes. This model demonstrated a strong cooperativity between the two oncogenes in promoting liver tumorigenesis. Our data indicate that this cooperation occurs mainly through unrestrained proliferation of liver cells, and requires activation of the transcriptional co-factors Yap and Taz.

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Materials and Methods

Mice

Alb-CreER^{T2} mice (termed SA-Cre-ERT2 in the original publication (16)) were a kind gift from Pierre Chambon, Ctnnb1$^{\Delta Ex3}$ mice were a kind gift from Makoto Taketo (17), Taz$^{f/f}$ mice (18) were a kind gift from Stefano Piccolo, Apc$^{f/f}$ (19) mice were a kind gift of Eduard Battle, R26-lsl-CAG-MYC-ires-hCD2* (20) mice were purchased from Jackson Laboratory (Stock No: 020458), R26-lsl-EYFP mice were purchased from Jackson Laboratory (Stock No: 006148) and backcrossed into the C57BL/6 background, and Yap$^{f/f}$ mice were purchased from the KOMP Knockout mouse project (https://www.komp.org). For tumor-free survival analysis Alb-CreER^{T2};β-cat^{Ex3};R26-lslMYC mice were monitored 3 times per week and sacrificed when showing abdominal enlargement, indicative of hepatomegaly due to tumor formation. The same procedure was applied to β-cat^{Ex3};R26-lslMYC mice injected at 6-8 weeks of age by tail vein injection with low-titer (10^9) AAV8-TBG-CRE particles (University of Pennsylvania Vector Core, #AV-8-PV1091). For short-term liver-specific activation of the various alleles, high-titer (10^{11}) AAV8-TBG-CRE particles were given to 6- to 8-week-old mice, and the mice sacrificed after 2, 4, or 8 days, as specified in the text or figures. Tumor nodules or liver parenchyma were dissected and either processed freshly, or frozen and stored at -80°C until further analysis. CD1-nude mice (purchased from Charles River Laboratories) were injected subcutaneously with $3*10^6$ 3T9$^{MycER;S33Y}$ cells, and provided with food containing 400 ppm/kg Tamoxifen (Envigo, #TD.55125.I) and/or drinking water with 2 mg/ml doxycyline hydrate (Sigma-Aldrich, #D9891-100G), as indicated in the text. Mice were monitored 3 times per week and sacrificed when showing tumor masses of 1 cm^3. Experiments involving animals were done in accordance with the Italian Laws (D. lgs. 26/2014), which enforces Dir. 2010/63/EU (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes) and authorized by the Italian Minister of Health with projects 81/2016-PR and 726/2017-PR.

Additional Methods can be found in Supplementary Material and Methods.

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Results

MYC and β-catenin cooperate in liver tumorigenesis

In line with previous observations (21) analysis of the TCGA database showed that *MYC* amplification and *CTNNB1* mutations co occur in a fraction of human HCCs (Supplementary Figure 1A) (2, 3). To model this scenario, we took advantage of the CRE-activated alleles R26-lsl-CAG-MYC-ires-hCD2* (hereafter R26-lslMYC) (20) and Ctnnb1$^{\Delta Ex3}$ (hereafter *β-cat*^{Ex3}) (17) (Supplementary Figure 1B). Recombination of the *β-cat*^{Ex3} allele leads to loss of residues 14-89, spanning the phospho-degron of mouse β-catenin, thus mimicking the cancer-associated mutations observed in the human gene. To circumvent the known lethal effects of β-catenin activation in the whole liver (22), we sought to obtain a sporadic activation of our alleles in a minority of the hepatocytes, which was achieved by two complementary means: (i.) exploiting the basal leakiness of the CreER^{T2} fusion protein in the absence of its pharmacological activator, and (ii.) infection with low titer CRE-expressing Adeno-Associated viral (AAV) particles.

The Alb-CreER^{T2} transgene drives constitutive expression of the CreER^{T2} fusion protein in the liver, allowing controlled post-translational activation of the recombinase by exposure of the animals to Tamoxifen (TAM) (16). CreER^{T2} can occasionally show leakiness (23), causing sporadic recombination events: indeed, even if never exposed to TAM, compound Alb-CreER^{T2};R26-lslMYC mice developed highly penetrant, gender-independent multi-nodal liver tumors, with a median survival of 6 months (Figure 1A, Supplementary Figure 2A-B). Activation of the *β-cat*^{Ex3} allele had no effect on its own, as previously reported (22), but significantly accelerated tumorigenesis when combined with R26-lslMYC (Figure 1A; supplementary Figure 2A). As expected, the resulting tumors expressed MYC at high levels, the associated hCD2 reporter, as well as the shorter form of β-catenin encoded by the recombined *β-cat*^{Ex3} allele (Figure 1B; supplementary Figure 1B, 2C). We further assessed the expression of known MYC- and β-catenin-activated genes, confirming the activation of the two oncoproteins (Supplementary Figure 2D, E). The tumors also showed reduced expression of markers typically associated with differentiated hepatocytes (e. g. Ck-18, Albumin, and Hnf-4a) and increased expression of fetal liver markers (e. g. Afp; Supplementary Figure 2F). Finally, pathological analysis revealed no major differences between MYC-only and MYC/β-catenin tumors, both resembling human HCC with a trabecular pattern (Figure 1C; see Supplementary Table 1 for a detailed pathological

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description). We will thus generally refer to these tumors as mouse hepatocellular carcinomas (mHCCs).

As a second approach to jointly activate MYC and β-catenin in hepatocytes, *β-cat*^{Ex3};R26-lslMYC and control mice were injected with recombinant AAV8-TBG-CRE particles, which show a high tropism for liver cells and express CRE recombinase under control of the hepatocyte-specific TBG promoter (24). This system can be used to achieve either sporadic (~5-10% of the cells) or almost complete (>90%) infection of hepatocytes, as measured by CRE-induced activation of the R26-lsl-EYFP reporter allele 4 days after injection of 10^9 (low titer) or 10^{11} (high titer) particles (Supplementary Figure 3A). When infected with AAV8-TBG-CRE at low titer, *β-cat*^{Ex3};R26-lslMYC mice developed tumors that resembled those arising in Alb-CreER^{T2};*β-cat*^{Ex3};R26-lslMYC animals, albeit with longer latency (Supplementary Figure 3B, C; Supplementary Table1) – owing most likely to activation of the oncogenes at a later stage of liver development.

Finally, as an alternative means to activate the WNT/β-catenin pathway, we used a conditional knockout allele of *APC (19)*, bred this to homozygosity (*Apc*$^{f/f}$) in combination with Alb-CreER^{T2} and R26-lslMYC, and monitored the mice over time in the absence of TAM: as observed above with activation of *β-cat*^{Ex3}, deletion of APC had no effect on its own, but accelerated tumorigenesis in the presence R26-lslMYC (Supplementary Figure 2G). Altogether, our data show that oncogenic activation of MYC and WNT/β-catenin signaling cooperate in liver tumorigenesis.

We next wondered whether the cooperation between MYC and β-catenin is peculiar to hepatocytes or may be recapitulated in other cells. To address this question, we infected 3T9^{MycER} fibroblasts, which constitutively express a MycER^{T2} chimaera (25), with a doxycycline-inducible lentiviral vector encoding a 6xMyc-tagged S33Y-mutant version of β-catenin resistant to GSK3β-induced phosphorylation and proteasomal degradation (hereafter β-cat^{S33Y}) (26). Immunoblot and RT-qPCR analysis of 3T9$^{MycER;S33Y}$ cells confirmed the induction of β-cat^{S33Y} by doxycycline, as well as an increase in MycER levels – most likely due to stabilization of the protein – upon 4-hydroxytamoxifen (4-OHT) treatment (Supplementary Figure 4A). Importantly, β-catenin- and MYC-activated genes were induced upon doxycycline and 4-OHT treatment, respectively, indicating that the two transcription factors were active (Supplementary Figure 4B, C). The induction of β-cat^{S33Y} reduced cell proliferation in adherent cultures, independently from MycER

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activation (Supplementary Figure 4D); most importantly, however, co-activation of MycER^{T2} and β-cat^{S33Y} allowed colony formation in methylcellulose, indicating that the cells had acquired the capacity to proliferate in suspension (Figure 1D). 3T9$^{MycER;S33Y}$ cells were then implanted subcutaneously in CD1-nude mice: in this setting, combined treatment of the animals with Doxycycline and Tamoxifen, but neither alone, led to tumor formation (Figure 1E). Thus, co-activation of MYC and β-catenin supports the malignant transformation of immortalized mouse fibroblasts.

Altogether, the above results demonstrate that MYC and WNT/β-catenin signaling cooperate in cellular transformation. In particular, we showed that the aberrant activation of MYC in the mouse liver promoted the development of undifferentiated hepatocellular carcinomas, whose latency was strongly reduced upon either activation of β-catenin, or deletion of APC. Most importantly, MycER and β-cat^{S33Y} also cooperated in the transformation of fibroblasts *in vitro*. Thus, besides its recently reported impact on immune surveillance (21), β-catenin also cooperates with MYC through cell-autonomous mechanisms.

Concomitant activation of Myc and β-catenin induces of a pro-proliferative, Yap/Taz-related, transcriptional signature

Considering the prevalence of secondary transcriptional responses in established Myc-driven mHCCs (27), we sought to profile the short-term effects of MYC and β-cat^{Ex3} activation in hepatocytes. In this regard, short-term activation of CreER^{T2} in young Alb-CreER^{T2};*β-cat*^{Ex3};R26-lslMYC mice did not provide a clean model, owing to the possible confounding effects of pre-existing tumoral lesions. In order to circumvent this caveat, we focused on the short-term activation of R26-lslMYC and/or β-cat^{Ex3} with injection of high-titer AAV8-TBG-CRE particles. RNA-seq profiles were established 4 days after infection, a time at which both MYC and β-cat^{Ex3} were expressed in hepatocytes, showing clear nuclear localization and transcriptional activity (Supplementary Figure 5A-E). Of note, activation of *β-cat*^{Ex3} did not increase *Myc* mRNA levels (Supplementary Figure 5C), indicating that *Myc* is not regulated by WNT/β-catenin in hepatocytes, as previously reported (9, 10).

Activation of β-cat^{Ex3} alone induced limited transcriptional changes relative to AAV-infected wild-type livers, with only 98 differentially expressed genes (DEGs), while MYC regulated almost 3000 genes, either up or down (Figure 2A). This MYC-driven transcriptional

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program was largely unchanged upon co-activation of β-cat^{Ex3} (Figure 2B, Supplementary Figure 6A). Gene ontology analysis showed that MYC, either alone or in combination with β-catenin, elicited not only MYC-, but also E2F-, mTOR- and WNT/β-catenin-associated gene signatures: the latter was mobilized by either β-cat^{Ex3} or MYC alone, but responded with higher statistical significance in presence of both oncogenes (Figure 2C, Supplementary Table 2). In line with these findings, direct comparison between MYC/β-cat^{Ex3} and MYC-overexpressing livers yielded only 37 DEGs (33 up and 4 down, Supplementary Figure 6A), comprising canonical WNT/β-catenin targets (Axin2, Lgr5, Notum, Sp5, Tbx3 and Tcf7; Supplementary Table 2).

To address whether the cooperation between MYC and β-cat^{Ex3} in liver tumorigenesis may be associated with more subtle transcriptional changes, we focused on genes that were induced both by MYC and MYC/β-cat^{Ex3} overexpression, but whose fold change in expression relative to wild type livers was at least 1.5 times higher in MYC/β-cat^{Ex3} compared to MYC (Figure 2D): this led to the identification of a group of 125 genes that we will refer to as the "MYC/β-catenin signature". Importantly, these genes were also induced in advanced mHCC tumors relative to wild type livers, and with a higher magnitude in MYC/β-cat^{Ex3} than in MYC-only tumors. Gene ontology analysis revealed that the MYC/β-catenin signature enriched - among others - for Yap/Taz transcriptional targets (Supplementary Table 3). Moreover, 59 out of 125 genes (~45%) in our MYC/β-catenin signature (as opposed to ~14% of all active genes) scored as direct Yap targets by chromatin immunoprecipitation (28, 29) (Biagioni et al., manuscript in preparation) (Supplementary Table 3). Finally, Gene Set Enrichment Analysis (GSEA) revealed that several Yap/Taz signatures were also enriched in the transcriptional profiles of either MYC/β-cat^{Ex3} or MYC-overexpressing livers (Supplementary Figure 6B) and appeared, together with E2F targets, among the top enriched datasets when ranking all the genes by their fold-change between MYC/β-cat^{Ex3} and MYC-overexpressing livers (Figure 2E). Altogether, these data indicated that Yap and/or Taz are transcriptionally active in the liver upon MYC/β-cat^{Ex3} activation.

The enrichment of Yap/Taz-regulated genes in our dataset was particularly intriguing and, based on previous observations, might represent a possible mechanistic link between MYC and β-catenin: first, Yap/Taz are activated by WNT/β-catenin signaling owing to their association with the destruction complex (30); second, these factors cooperate with MYC in integrating mitogenic stimuli and promoting liver tumorigenesis (31); third, Yap/Taz promote proliferation by activating

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genes involved in either G1/S (28) or G2/M progression (29), consistent with the composition of our MYC/β-catenin signature. We thus focused on the possible involvement of Yap/Taz in the cooperative activity MYC and β-cat^{Ex3}.

While the expression of Yap and Taz was barely detectable by immunohistochemistry analysis in normal liver, with the exception of cholangiocytes as previously reported (24), both proteins became detectable in a sizeable fraction of hepatocytes after AAV8-TBG-CRE-mediated activation of either MYC or β-cat^{Ex3} for 4-8 days, and were most strongly induced by concomitant activation of MYC and β-cat^{Ex3} (Figure 3A, B and Supplementary Figure 7A), paralleled by consistent changes in bulk protein levels (Figure 3C and Supplementary Figure 7B). In contrast, RT-qPCR analysis did not show any significant variation in *Yap/Taz* mRNA levels (Supplementary Figure 7C). In line with these observations, immunohistochemical analysis of established tumors confirmed increased expression of Yap and/or Taz relative to wild-type livers, albeit this occurred in either Myc-only or MYC/β-cat^{Ex3} mHCCs (Supplementary Figure 8). Finally, Taz (but not Yap) was induced and localized to the chromatin fraction also in in $3T9^{MycER;S33Y}$ fibroblasts upon induction of β-cat^{S33Y} (but in this system independently from MycER activation), correlating with increased expression of the canonical Yap/Taz transcriptional targets *Ctgf* and *Cyr61* (Supplementary Figure 9A and 9B).

Altogether our data support a model in which β-catenin promotes the stabilization of the Yap and Taz proteins, as previously reported (30), thus enhancing transcription of a subset of common Yap/Taz and MYC targets involved in promoting cell-cycle progression (31). In the liver, as opposed to fibroblasts, MYC also contributed to the up-regulation of Yap/Taz: while this effect remains to be explained at the mechanistic level, it further emphasizes the importance of Yap/Taz in Myc-dependent transformation (31).

High expression of the MYC/β-catenin signature correlates with worst prognosis in HCC patients.

Having defined a distinct MYC/β-catenin signature in mouse liver, we went on to address its significance in human cancer. In either of two independent HCC datasets (TCGA and LCI/FUDAN, see Supplementary Material & Methods), querying for the enrichment of our MYC/β-catenin signature allowed us to identify a distinct subgroup of patients whose tumors expressed high levels of the corresponding mRNAs (Figure 4A, B). In agreement with data from

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our mouse model, most of these patients also showed high expression of the MYC (32, 33), WNT (32, 33) and Yap/Taz (28) transcriptional signatures (Figure 4A, B, top; Fisher test on the TCGA cohort: odds ratio 192.90, $p < 2.2e^{-16}$; on the LCI/FUDAN cohort: odds ratio 17.16346, $p = 3.378e^{-10}$). Most relevant here, HCC patients with high level of expression of the MYC/β-catenin signature (from 5% in the TCGA to 29% in the LCI/FUDAN cohort) showed a significantly shorter survival compared with the ones with low expression (Figure 4C, D): in the TCGA dataset, where information about tumor grade was available (Figure 4A, top), the MYC/β-catenin signature also enriched for the most aggressive cases (grades 3 and 4, compared to grades 1 and 2; Fisher test: odds ratio 3.97, $p = 5.5e^{-08}$).

Yap/Taz are required for hepatocyte proliferation and tumor growth upon MYC/β-catenin activation.

In order to address the role of Yap/Taz in MYC/β-cat^{Ex3}-driven mHCCs, we generated a cohort of mice, in which Alb-CreER^{T2}, β-cat^{Ex3} and R26-lsl-MYC were combined with conditional knockout alleles of Yap (34) and Taz (18) (hereafter Yap$^{f/f}$;Taz$^{f/f}$) and monitored those animals in the absence of TAM. Surprisingly, the presence of the Yap$^{f/f}$;Taz$^{f/f}$ alleles did not affect tumor development (Supplementary Figure 10A). However, PCR analysis showed that all tumors had retained at least one non-recombined Yap^{f} or Taz^{f} allele (Supplementary Figure 10B-C). Moreover, immunohistochemical analysis revealed expression of Yap and/or Taz in tumor sections (Supplementary Figure 10D). We surmise that Yap/Taz-null cells are counter-selected during tumorigenesis, pointing to an essential function of these proteins in MYC/β-cat^{Ex3}-driven tumors. To corroborate this interpretation, we grew tumor cells *in vitro* and treated them with 4-OHT to activate CreER^{T2} and induce acute deletion of the remaining Yap^{f} or Taz^{f} alleles (Supplementary Figure 11A-E): as expected, this led to complete loss of the Yap and Taz proteins, while leaving MYC and β-cat^{Ex3} unaffected (Supplementary Figure 11D). In this setting, 4-OHT treatment caused a strong impairment in cell growth, which was not observed in cells bearing at least one wild-type allele of Yap and/or Taz (Figure 5A and Supplementary Figure 11E-G).

We then sought to address whether MYC and β-cat^{Ex3} might cooperatively induce proliferation when acutely induced in the liver, and whether this activity might be dependent upon Yap/Taz. To this aim, we combined the β-cat^{Ex3}, R26-lslMYC, Yap$^{f/f}$ and Taz$^{f/f}$ alleles, infected mice with high-titer AAV8-TBG-CRE, and sacrificed animals at different time-point (2, 4, 8 days)

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to monitor proliferation (by Ki67 staining) and liver weight (Figure 5B-D): mice co-expressing MYC and β-cat^{Ex3} (with wild-type Yap and Taz) showed increased hepatocyte proliferation over the time-course, preceding a marked increase in liver/body weight ratio at day 8. Strikingly, these responses were abrogated by deletion of Yap/Taz (Figures 5B-D). It is noteworthy here that WNT-1 overexpression and β-catenin activation were reported to dampen MYC-induced apoptosis in rat fibroblasts and intestinal epithelial cells (35). However, this mechanism was not verified in our model: as judged by TUNEL staining, MYC-induced apoptosis was not decreased, but rather increased in the presence of β-cat^{Ex3} (Supplementary Figures 12A, B), most likely associated with the hyper-proliferative response.

Altogether our results support an essential, but mutually redundant role for Yap/Taz as downstream effectors of β-cat^{Ex3}, driving the cooperation with MYC through activation of a set of genes, which in turn promote proliferation in hepatocytes.

Discussion

In this study, we describe a mouse model that recapitulates the co-activation of MYC and WNT/β-catenin observed in a fraction of human HCCs (2, 3). While confirming that Myc is not a downstream transcriptional target of WNT/β-catenin in the liver (9), as opposed to other tissues (5-8), our data revealed that concomitant activation of both pathways in mouse hepatocytes promoted unscheduled cell proliferation and cooperated in the onset of HCC-like tumors. This cooperativity between MYC and WNT/β-catenin can be extended to other tissues, as reported in T-cells (12) and as verified here in immortalized fibroblasts. While our work was under completion, others reported a cooperative action of Myc and β-catenin in liver tumorigenesis, based on hydrodynamic-tail vein injection of plasmids expressing both oncogenes (21). These authors showed that the Myc/β-catenin cooperation relies on the ability of β-catenin to promote immune escape: in particular, β-catenin suppressed expression of the chemokine Ccl5, resulting in defective recruitment of dendritic cells and impaired T-cell activity (21). Consistent with this scenario, our MYC/β-cat^{Ex3} mHCCs showed a slight reduction in *Ccl5* mRNA levels compared to MYC-driven mHCCs, while short term activation of MYC and/or β-catenin had no significant impact on *Ccl5* (data not shown). While the possible contribution of immune-surveillance - or

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other systemic effects - remains to be investigated in our model, our results demonstrate that MYC and β-catenin have a direct, cell-autonomous effect on cellular transformation.

In order to address the mechanisms underlying the cooperativity of MYC and β-catenin, we profiled gene expression shortly after their co-activation in hepatocytes. Our data did not reveal any major interference between MYC- and β-catenin-regulated transcriptional programs, but unveiled the existence of a discrete MYC/β-catenin signature of 125 genes, which were more induced upon co-activation of both oncogenes and mainly encoded products involved in cell cycle control and proliferation. Strikingly, this signature enriched for genes previously reported to be under the direct control of the transcriptional co-activators Yap/Taz. In subsequent experiments, we showed that Yap and Taz were induced upon short-term activation of MYC and β-cat^{Ex3} in the liver, and were essential for the proliferative response of hepatocytes. In line with these observations, Yap/Taz were also abundant in MYC/β-cat^{Ex3} tumors and were required for the growth and survival of tumor cells.

Our findings on the role of Yap/Taz as effectors in the cooperative action of MYC and β-catenin connects two apparently unlinked regulatory cross-talks, including *(i.)* the activation of Yap/Taz by WNT/β-catenin signaling (18, 30, 36) and *(ii.)* the cooperation between Myc and Yap in supporting either cell proliferation in serum-stimulated fibroblasts, or tumorigenesis in the liver (31). First, besides signaling cues such as the Hippo pathway, mechanotransduction (37) or a non-canonical (β-catenin-independent) WNT pathway (38), Yap/Taz have been linked to WNT/β-catenin signaling (18, 30, 36). Taz in particular was shown to be recruited to - and degraded by the destruction complex in a β-catenin-dependent manner (30). Moreover, Yap/Taz were stabilized and activated following either knock-out of Apc in mouse tissues and human cell lines (18, 30, 36), or expression of the stable β-catenin mutant β-cat^{Ex3} in the small intestine (36). It is noteworthy here that additional context-dependent mechanisms may be involved in the aforementioned effects: in fibroblasts, activation of β-cat^{S33Y} (but not Myc) induced accumulation of Taz; in hepatocytes, instead, either β-cat^{Ex3} or Myc alone caused mild increases in Yap/Taz levels, which were markedly enhanced by the co-activation of both oncogenes. Altogether, while the molecular basis for these additional effects remains to be addressed, the above findings establish Yap/Taz as downstream effectors of WNT/β-catenin signaling. As a second connection, Yap was shown to cooperate with Myc in supporting either cell proliferation or tumorigenesis, and

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did so through the joint activation of a subset of proliferation-associated genes (31). Indeed, almost half of the genes included in our MYC/β-catenin signature were independently identified as direct targets of Yap, and were induced upon co-activation of MYC and Yap in the liver. Altogether, we conclude that Yap/Taz link WNT/β-catenin activation to a MYC-regulated proliferative program, underlying the cooperativity between these two oncogenic pathways.

The mechanisms by which Yap/Taz and MYC co-regulate transcription remain to be addressed: most noteworthy in this regard, these factors interact with an overlapping set of general co-regulators (e.g. BRD4, p300, p400, RUVBL1, KMT2D, SAP18 or the SWI/SNF complex) (39, 40). As exemplified by BRD4, these co-regulators may be involved in wider transcriptional programs than the transcription factors themselves (41, 42), but may nonetheless be particularly critical for transcription of genes activated by either Yap/Taz (39), MYC (43) or – even most likely – both together (31).

While the involvement of MYC, WNT/β-catenin and Yap/Taz in liver tumorigenesis was amply documented (3, 13, 31, 44-48), their mutual interplay – if any – remained to be unraveled. Toward this aim, we re-analyzed gene expression profiles from two independent patient cohorts, and observed that the MYC/β-catenin signature identified in our work was enriched in a subset of HCC patients, in which it correlated with worse prognosis.

In recent years, Yap/Taz have emerged as key player in tumorigenesis in different tissues (37), and several approaches for the inhibition of their expression and/or activity have been proposed as potential therapeutic options (49). Our results add a new element in this regard, indicating that a therapeutic strategy aimed at tackling Yap/Taz may be particularly effective in tumors with aberrant activation of MYC and WNT/β-catenin. Moreover, targeting Yap and Taz may be therapeutically safe, given that their deletion showed limited short-term effects in normal tissues, including the liver (34, 50). In this regard, an attractive class of molecules – already in use in the clinic – are statins, which have been demonstrated to (i) suppress Yap/Taz function by impairing their nuclear import and promoting their degradation (51, 52), (ii) reduce Taz-dependent HCC proliferation (53) and (iii) decrease the risk of cancer mortality, in particular for HCC (54, 55).

Altogether, our work in mice has revealed a novel functional interplay between MYC, WNT/β-catenin and Yap/Taz in liver tumorigenesis. Most importantly, these interactions hold true

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in human HCC, with important prognostic implications. Future work will address whether targeting Yap/Taz may hold potential for personalized therapy in patients showing oncogenic activation of Myc and β-catenin.

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Figure Legends

Figure 1. MYC and β-cat^{Ex3} activation cooperate in transformation in liver and fibroblasts. **(A)** Kaplan Meyer disease-free survival curves for mice of the indicated genotypes (all in the presence of the Alb-CreER^{T2} transgene). The number of mice (n) and the median survival are indicated. p-values were calculated with the log-rank test. **(B)** Western blot analysis of β-catenin and MYC protein expression in wild-type (wt) liver and representative R26-lslMYC and *β-cat*^{Ex3};R26-lslMYC liver tumor samples. Vinculin was used as loading control. Each tumor is identified by its unique reference number. **(C)** Hematoxylin and Eosin staining of representative liver sections from the indicated genotypes. Bars: 400 µm (H&E 5x) or 100 µm (H&E 5x). **(D)** Left: representative pictures of the colony forming assay of 3T9$^{MycER;S33Y}$ cells plated in 50% (v/v) methylcellulose and cultured for 7-10 days in the presence of 1 µM Doxycycline and/or 400 nM 4-OHT, as indicated. This experiment was performed four times, each with technical triplicates. Bar: 1000 µm. Right: quantification of the total number of colonies with a diameter >10 µM in each condition. Bar plot represent the average and standard deviation for the 4 biological replicates. **(E)** Representative pictures of CD1-nude mice 6 weeks after injection of 3x10^{6} 3T9$^{MycER;S33Y}$ cells and fed with Tamoxifen (Tam) and/or Doxycyline (Doxy), as indicated. The fraction of tumor-bearing mice in each experimental group is indicated below each photograph.

Figure 2. MYC and β-cat^{Ex3} promote the induction of a pro-proliferative Yap/Taz signature in the liver. RNA-seq profiling was performed in the livers of mice with activated R26-lslMYC and/or *β-cat*^{Ex3}, and wild-type (wt) mice as controls. All samples were collected 4 days after high-titer AAV8-TBG-CRE injection. **(A)** Venn diagrams representing the overlap between differentially expressed genes (DEGs, qval <0.05) identified as induced (top) or repressed (bottom) relative to the wt control in each of the indicated groups. **(B)** Heatmap representation of the same DEGs. The left part (columns 1-3) shows up- and down-regulated genes, respectively marked in red and blue; the right part illustrates the z-score of the change in expression relative to wt for each DEG in each condition. **(C)** Bar-plots showing the False Discovery Rate (–log10) of the most significantly enriched gene ontology categories in at least one of the indicated genotypes. **(D)** Boxplot showing the fold-change in mRNA expression for the 125 genes of the MYC/β-catenin signature in each of the indicated conditions relative to the wt genotype (expressed as log2FC). p-values were calculated using Wilcoxon's test. **(E)** Gene set enrichment plots for 3 examples (top) and complete

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list of Normalized Enrichment score (NES) and False Discovery Rate (FDR) values for all the significantly enriched hallmark gene sets (FDR<0.05) (bottom). Custom curated Yap/Taz signatures were added manually to the group of queried gene sets (see Methods for details). Genes were sorted from left to right according to the log2FC in their expression when comparing MYC/β-cat^{Ex3} versus MYC-overexpressing livers.

Figure 3. Increased Yap/Taz expression upon MYC and β-cat^{Ex3} activation in hepatocytes. **(A)** Immunohistochemical detection of Yap and Taz in representative liver samples from mice of the indicated genotypes, injected with high-titer AAV8-TBG-CRE 8 days prior to collection. Bar: 300 µm. **(B)** Quantification of the fraction of liver cells showing either cytoplasmic or nuclear positivity for Yap (left) and Taz (right) in mice of the different genotypes, 8 days after AAV8-TBG-CRE injection. Bar plot represent average and standard deviation for at least 3 biological replicates. **(C)** Representative Western blot analysis of Yap, Taz, β-catenin and MYC protein expression in lysates from livers sections from mice treated as in (**A**). Vinculin was used as loading control. Each sample is identified by its unique reference number.

Figure 4. Clinical significance of the MYC/β-catenin signature in HCC. Patients in The Cancer Genome Atlas (TCGA, **A**) or LCI/FUDAN (**B**) cohorts were stratified according to the MYC/β-catenin gene expression signature. The level of expression of the MYC (HALLMARK_MYC_TARGETS_V1 from the Broad Institute repository), WNT/β-catenin (HALLMARK_WNT_BETA_CATENIN_SIGNALING from the Broad Institute repository) and YAP/TAZ (28) signatures for each sample is shown on top, with the addition of tumor grade for the TCGA cohort. The heatmap shows the z-score for the expression of each gene of the MYC/β-catenin signature in each patient. Kaplan-Meier plots showing the overall survival of HCC patients in the TCGA (**C**) and LCI/FUDAN (**D**) cohorts according to their level of expression of genes of the MYC/β-catenin signature. The indicated p-values were computed using the log-rank test. Number at risk: live patients in each cluster at the indicated time points.

Figure 5. Yap/Taz are required for hepatocytes proliferation and liver tumorigenesis. **(A)** Growth curves for two independent Alb-CreER^{T2};*β-cat*^{Ex3};R26-lslMYC;Yap$^{f/f}$;Taz$^{f/f}$ mHCCs cultivated in the absence (red line) or presence (blue line) of 400nM 4-OHT to activate the recombinase activity of the CreER^{T2}. Relative cell numbers were determined through Luminescence measurement with the Cell Titer Glo assay. A.U.: Arbitrary Units. **(B)** Immunohistochemical detection of Ki67

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expression in representative liver sections from mice of the indicate genotypes, injected with AAV8-TBG-CRE 8 days prior to collection. Bar: 100 µm. **(C)** Fractions of Ki67-positive hepatocytes, and **(D)** Liver/body weight ratios (as %) for mice of the indicated genotypes, measured as in (B) at different time-points (2, 4 or 8 days) after AAV8-TBG-CRE injection. The bar plots represent average and standard deviation for at least 3 mice per group.

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Disclosure of Potential Conflicts of Interest: The authors declare that they have no conflict of interest.

Authors' Contributions: AB, GPGF, GB, FB, GC, NT, FC and AS performed experiments. MD provided technical support. DO conducted pathological analyses. MF, MJM, VP and AS performed bioinformatic data analysis. DP supervised the work of FC. AB, SC, AS and BA designed experiments, supervised the work, and wrote the manuscript.

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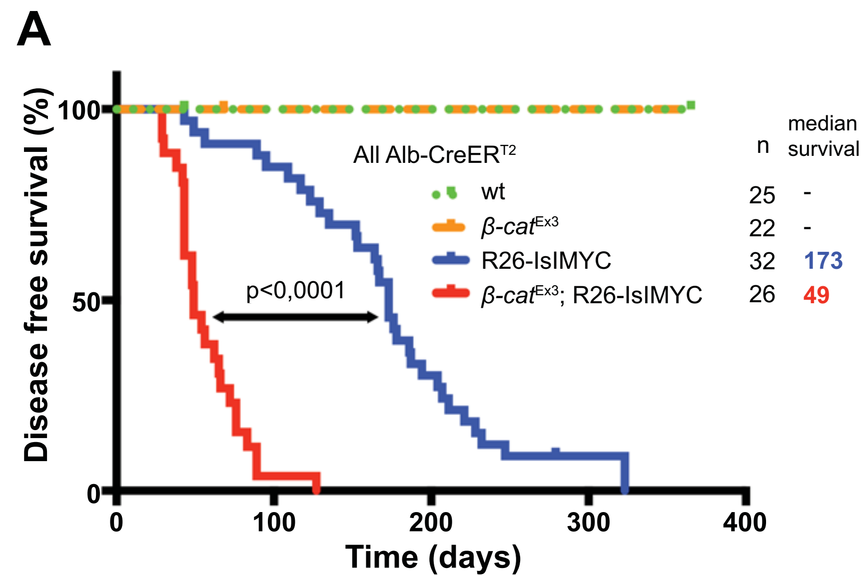
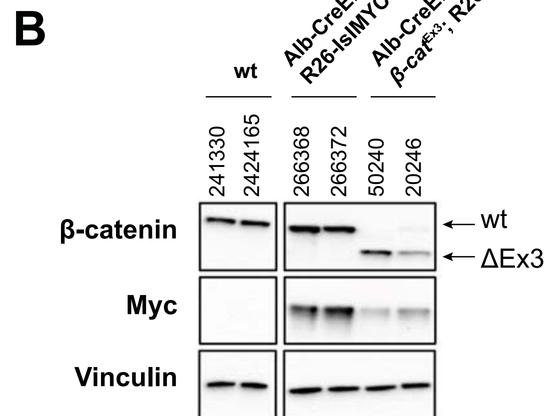
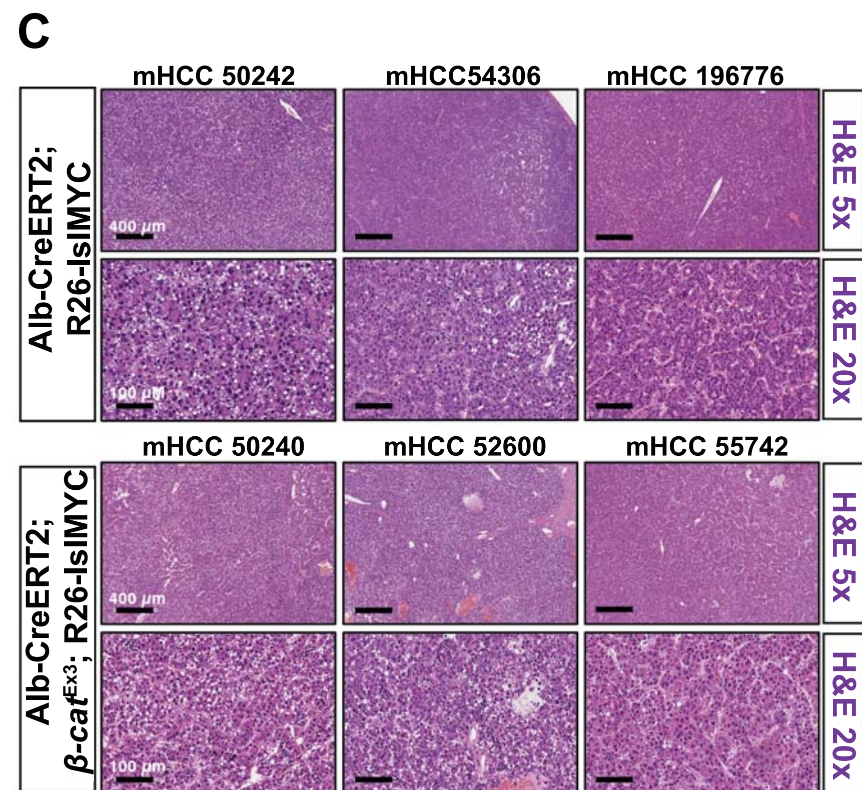
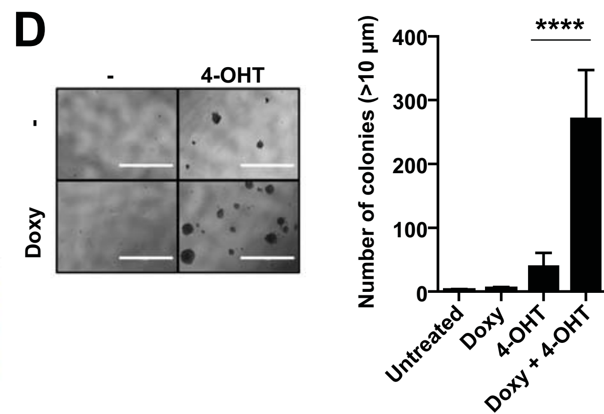
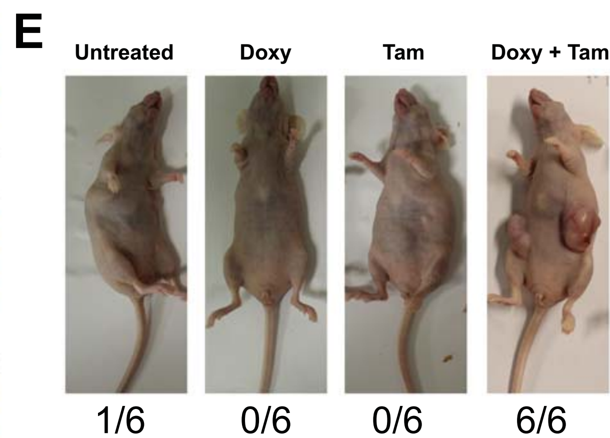


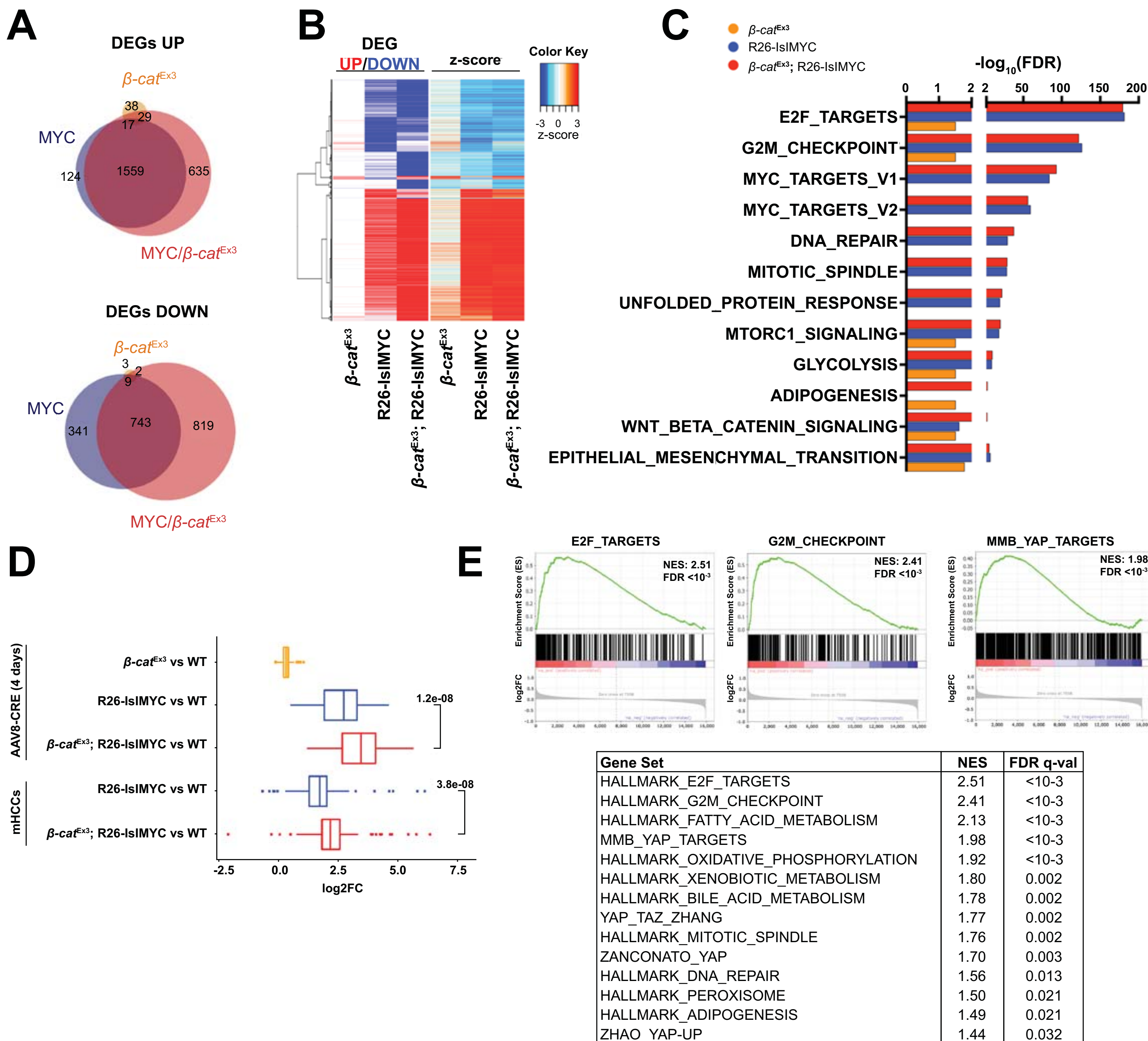






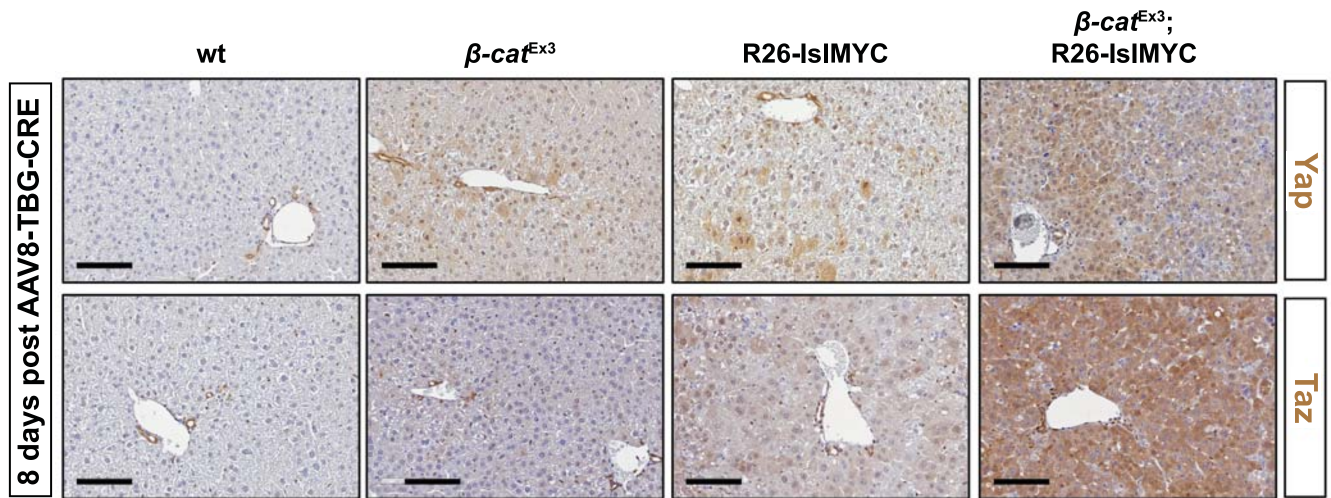


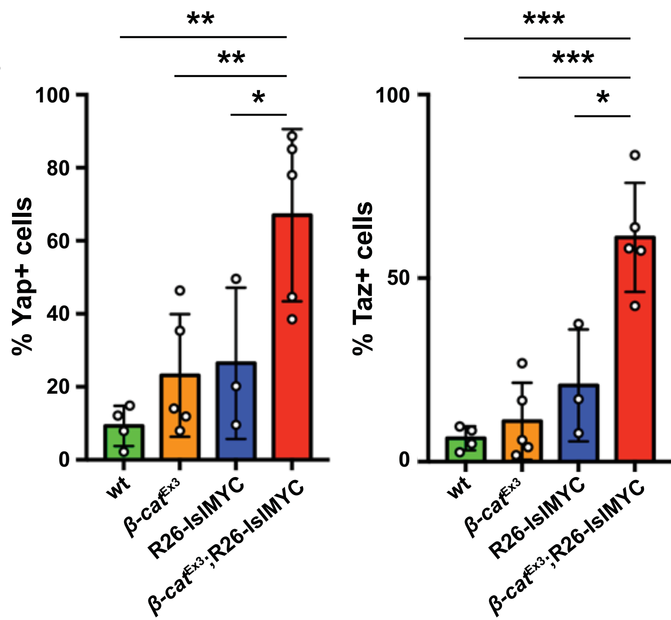
Bisso et al - Figure 1

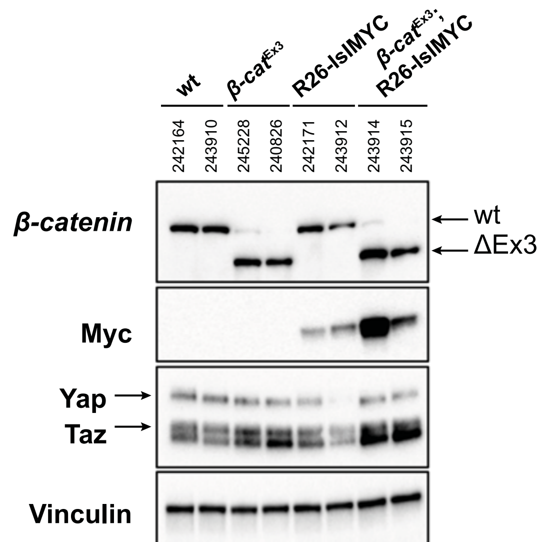


Gene Set	NES	FDR q-val
HALLMARK_E2F_TARGETS	2.51	<10-3
HALLMARK_G2M_CHECKPOINT	2.41	<10-3
HALLMARK_FATTY_ACID_METABOLISM	2.13	<10-3
MMB_YAP_TARGETS	1.98	<10-3
HALLMARK_OXIDATIVE_PHOSPHORYLATION	1.92	<10-3
HALLMARK_XENOBIOTIC_METABOLISM	1.80	0.002
HALLMARK_BILE_ACID_METABOLISM	1.78	0.002
YAP_TAZ_ZHANG	1.77	0.002
HALLMARK_MITOTIC_SPINDLE	1.76	0.002
ZANCONATO_YAP	1.70	0.003
HALLMARK_DNA_REPAIR	1.56	0.013
HALLMARK_PEROXISOME	1.50	0.021
HALLMARK_ADIPOGENESIS	1.49	0.021
ZHAO_YAP-UP	1.44	0.032

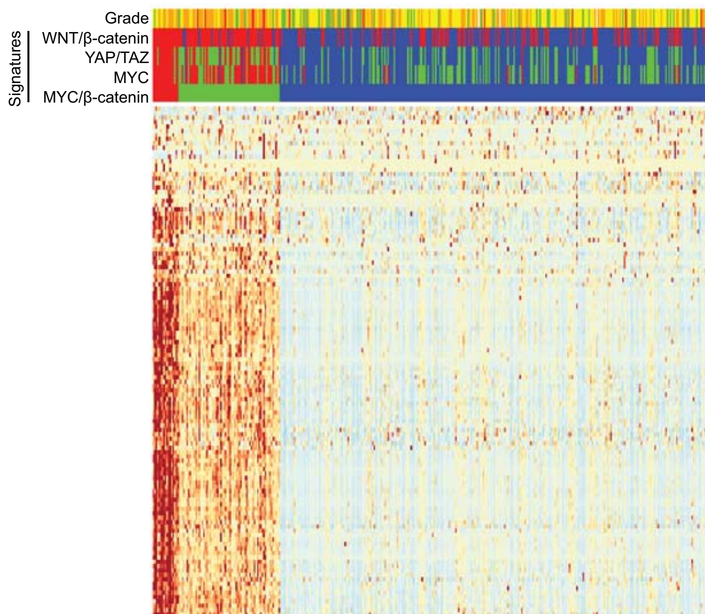
Bisso et al - Figure 2

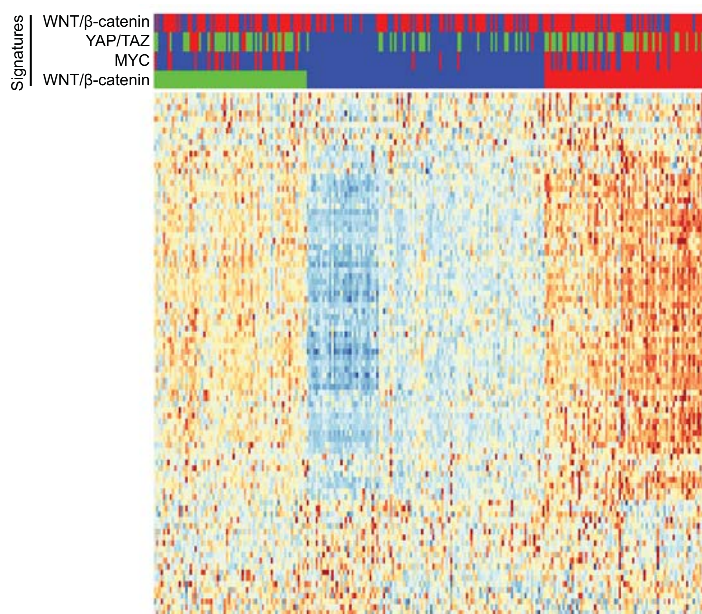






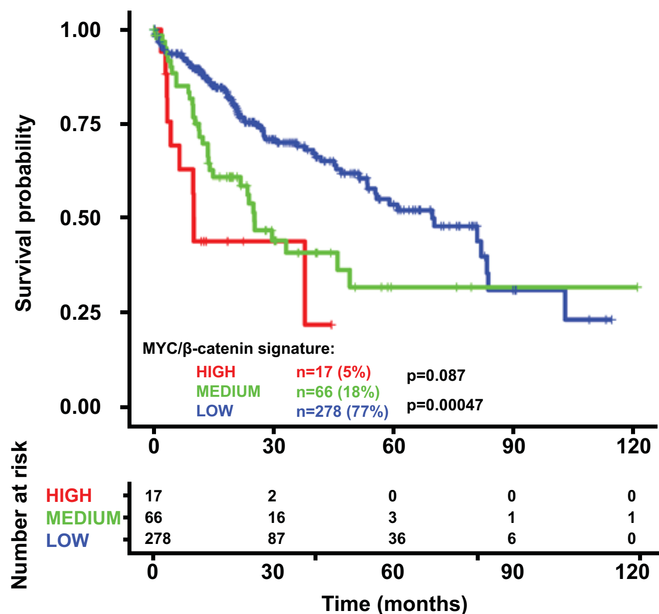
Bisso et al - Figure 3

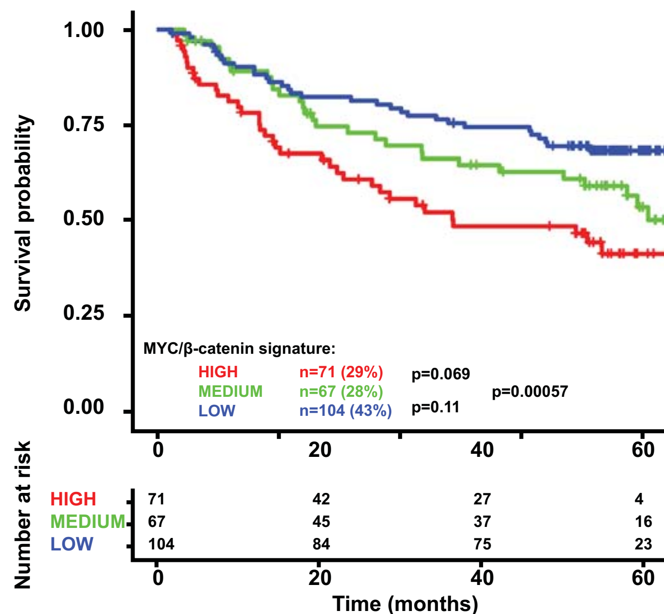




z-score
-3 -2 -1 0 1 2 3

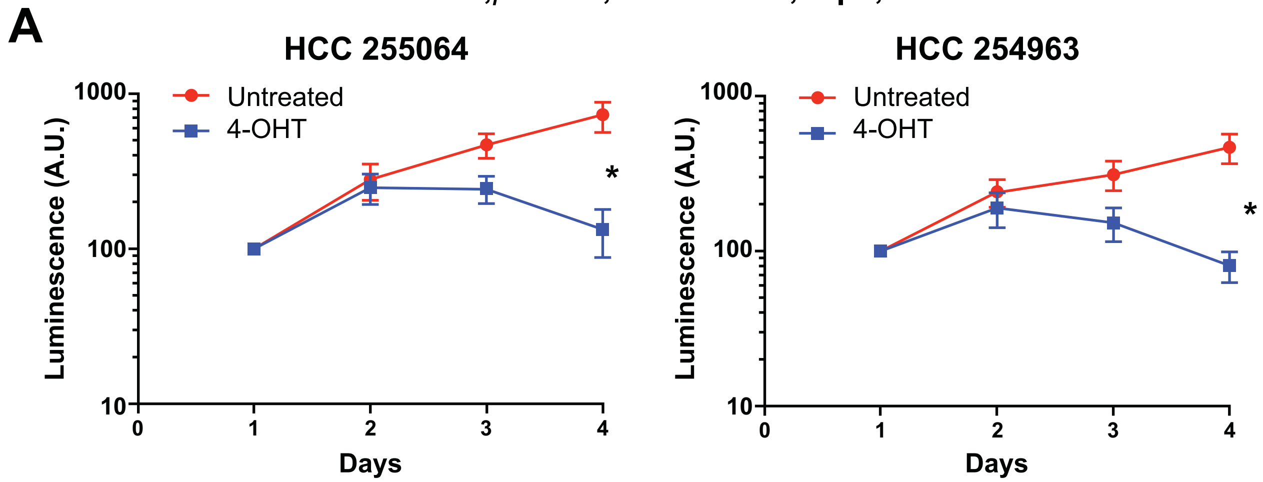
Grade: G1 G2 G3 G4
Signatures: High Medium Low

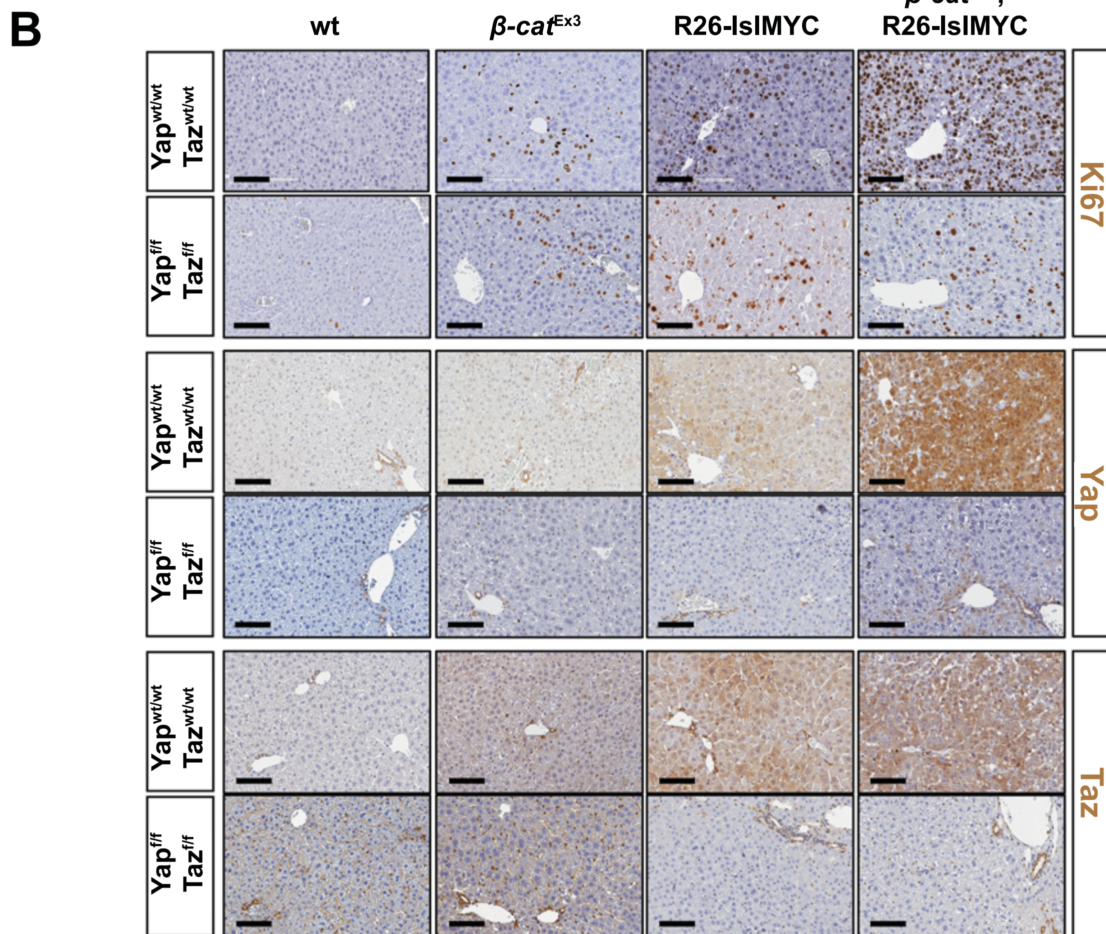


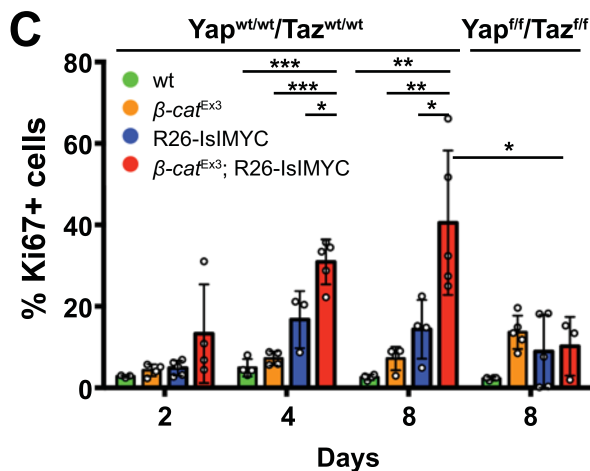


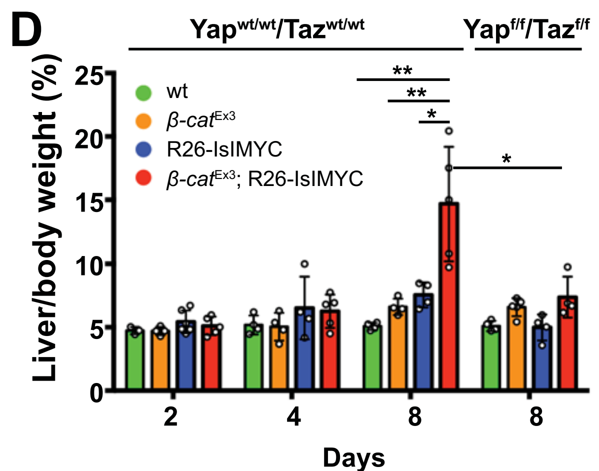
Bisso et al - Figure 4

Alb-CreER^{T2};*β-cat*^{Ex3};R26-lsIMYC;Yap$^{f/f}$;Taz$^{f/f}$









Bisso et al - Figure 5