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# A novel genetic model to study mechanosignaling and its impact on YAP/TAZ activity in mammalian tissues.

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# Publications

Elena Enzo, Giulia Santinon, **Arianna Pocaterra**, Mariaceleste Aragona, Silvia Bresolin, Mattia Forcato, Daniela Grifoni, Annalisa Pession, Francesca Zanconato, Giulia Guzzo, Silvio Bicciato & Sirio Dupont. "Aerobic glycolysis tunes YAP/TAZ transcriptional activity." The EMBO Journal, 2015 DOI: 10.15252/embj.201490379

Giulia Santinon, **Arianna Pocaterra**, and Sirio Dupont. "Control of YAP/TAZ Activity by Metabolic and Nutrient-Sensing Pathways." Trends in Cell Biology, 2016. DOI: 10.1016/j.tcb.2015.11.004

# Abstract

Regulation of F-actin dynamics is key for multiple aspects of cell biology. Recent work indicates that this is particularly significant for cells to correctly sense the mechanical properties of the microenvironment, and to respond by regulating the YAP/TAZ transcriptional coactivators, which ultimately control cell behavior in response to mechanical cues. Yet, whether the connection between F-actin dynamics and YAP/TAZ signaling is physiologically relevant in mammalian tissues, and dominant over the pleiotropic effects of F-actin, remains unaddressed. We will present data on a novel mouse knockout system enabling cell-autonomous regulation of F-actin dynamics, where we observe a potent upregulation of YAP/TAZ activity, leading to tissue hyperproliferation and organ overgrowth. This unveils an unprecedented signaling function for the F-actin cytoskeleton in the regulation of mammalian tissue homeostasis.

# Introduction

## The Hippo signalling pathway: central regulator of organ size

The animal kingdom comprises an astonishing variety of organisms of different size and shape. The form of any adult body is reached through a series of developmental stages, which require the coordination of complicate yet very precise differentiation events and morphogenetic processes. How embryonic and adult cells know when the appropriate stage of organ or tissue growth is reached, i.e. the mechanisms underlying regulation of organism and organ size, remains a key question in biology. Multiple regulators of organs size have been identified: these include both organ-extrinsic regulators providing systemic information (such as nutrient viability, hormones and chalones) and organ-intrinsic regulators providing local information (cellular environment, cell-cell contacts, autocrine/paracrine signals) (Irvine and Harvey 2015; Hafen and Stocker 2003). All these complex inputs are integrated at the cellular level to define output programs, and are sufficiently robust and plastic to allow the correct and stereotypic definition of organ size (Irvine and Harvey, 2015).

One of the most important and established models for the study of organ size is the Drosophila imaginal disc (Diaz-Benjumea and Cohen, 1993). Classical studies in this system suggested that the definition of organ size is intimately linked to the balance between cell proliferation and cell apoptosis (Simpson and Morata 1981; de la Cova et al. 2004 and reviewed in Yang and Xu, 2011). Only years later, with the introduction of mosaic genetic screenings, it was possible to identify genes whose mutation induced over-growth phenotypes (St Johnston, 2002). One of the first genes identified by such genetic screenings was named *warts* (*wts*) (Justice et al., 1995), but it received relatively poor attention until 2003, when other mutants displaying similar phenotypes were isolated (*hpo, sav, mats, exp, mer*), and shown to genetically interact with *wts*. This led to the definition of the Hippo pathway, whose inactivation leads to tissue over-growth (Fig. 1A). Later, biochemical studies led to the identification of the downstream transducer of the pathway,

*Yorkie* (*Yki*), whose activity is inhibited by the Hippo pathway. Flies bearing gain-of-function mutations of *Yki* display tissue over-growth phenotypes, and *yki* activity is enhanced in *hpo* mutants, confirming *Yki* as downstream target of the Hippo pathway (Huang et al., 2005).

## The Core Kinase cascade

All the main members of the Hippo pathway are conserved in animals. In mammals, the pathway is formed of a kinase cascade involving two serine/threonine kinases (*Mst1/2* and *Lats1/2,* homologous genes to *hpo* and *wts* in *Drosophila*) and their adaptor proteins *Sav1/WW45* and *Mob1* (*sav* and *mats* in fly), that inactivates the *Yki* homologues YAP and TAZ (Dong et al. 2007; Piccolo, Dupont, and Cordenonsi 2014). Mechanistically, the Hippo pathway inhibits the activity of YAP/TAZ by promoting their export from the nucleus. In particular, YAP/TAZ are phosphorylated directly by LATS1/2 kinases at multiple sites (5 in YAP, 4 in TAZ). The phosphorylation sites important for inactivation of *Yki*/YAP/TAZ have been mapped in flies and in mammalian systems (Dong et al., 2007; Zhao et al., 2010). YAP S127 and TAZ S89 when phosphorylated serve as a 14-3-3-binding sites, promoting nuclear-cytoplasmic translocation (Zhao et al. 2010). Another important site (YAP S381 and TAZ S311) regulates ubiquitin-mediated degradation, mostly of TAZ (Liu et al. 2010). Thus, when the Hippo pathway is inactive, YAP/TAZ are not phosphorylated and freely accumulate into the nucleus; here, together with the TEAD family of DNA-binding transcription factors (TEAD1-4 in mammals, *scalloped* in flies), they regulate gene transcription to promote proliferation and oppose cell death (Yu and Guan, 2013).

In the mouse, the genetic inactivation of the Hippo pathway, or transgenic overexpression of YAP, powerfully promotes cell proliferation in multiple embryonic and adult tissues; in organs such as the liver and heart, this leads to striking organ overgrowth phenotypes (Fig.1A). Moreover, activation of YAP leads to tissue hyperplasia often progressing into some form of tumor, which reinforced to the idea that the Hippo pathway bears tumor-suppressive functions, and that YAP/TAZ are pro-oncogenic factors (Harvey et al. 2013). Surprisingly, inactivation of YAP/TAZ in adult tissues has very little consequence on normal

tissue homeostasis, even in highly proliferating tissues such as the skin and intestine, while it very strongly inhibits proliferation of cancer cells, or during tissue regeneration. This led to the idea that YAP/TAZ are specifically required for cell growth in "stressed" conditions (Yu and Guan, 2013), making them ideal targets to combat cancer with minimal effects on normal tissues. Moreover, this implies that the Hippo pathway is kept normally activated in tissues to prevent YAP/TAZ activity.

## Upstream regulators

One open issue in the Hippo field is whether the Hippo kinase cascade is uniformly activated in cells and tissues, or whether upstream inputs exist that can pattern the activity of the Hippo pathway and/or of YAP/TAZ (Fig. 1B). Several reports point to cell-cell adhesions and establishment of apical-basal polarity in epithelia as regulators of the Hippo cascade (Piccolo, Dupont, and Cordenonsi, 2014). In this context, Merlin is particularly important: in confluent monolayers of mammalian epithelial cells, Merlin/NF2 is preferentially localized in close proximity to adherens and tight junctions (AJs and TJs). Reconstituting Merlin in NF2 deficient MDA-MB-231 breast cancer cells is sufficient to inhibit YAP/TAZ activity, and this inhibition is entirely dependent on LATS1/2 and YAP/TAZ phosphorylation, as knockdown of LATS1/2 is sufficient to completely rescue YAP/TAZ inhibition mediated by NF2 (Aragona et al. 2013). Multiple mechanisms have been proposed to explain how NF2/Merlin regulates the Hippo pathway, but the common idea is that Merlin serves as a scaffold to promote the assembly of multiprotein complexes, in turn promoting phosphorylation of YAP by LATS kinases. A recent report showed in *Drosophila* and mammalian cells that Merlin directly binds LATS, recruiting it to the cell membrane where it gets synergistically activated by the Hippo/Sav kinase complex (Yin et al. 2013). In this complex, the WW-domain containing protein Kibra may serve as a bridge between LATS and Merlin (Yu et al. 2010). NF2 also regulates recruitment of other proteins to cell-cell junctions, including Angiomotins, to regulate Rac1 activity, which was strongly linked to the ability of NF2 to suppress growth in shwannoma cells where it acts as tumour suppressor (Kissil et al. 2003). The observation that Angiomotins are capable of binding YAP led to several

hypotheses on the function of Angiomotins in the Hippo pathway, but the jury is still out on whether Angiomotin serve a pro- or anti-YAP function (Piccolo, Dupont, and Cordenonsi 2014).

Apart from NF2, other proteins related to cell-cell junctions and establishment of apico-basal polarity have been shown as potential upstream inputs in the Hippo pathway. For example, the membrane localization of the Scribble cell polarity determinant has been shown to modulate Hippo kinases in flies and mammalian cells. At the cell membrane, Scribble serves as adaptor for the Hippo kinases by assembling a complex containing MST and LATS, required for MST-mediated activation of LATS. Scribble localization to the plasma membrane is sufficient to oppose YAP/TAZ activity by reactivating the Hippo kinases (Cordenonsi et al. 2011). Loss of *Drosophila* Scribble, or of its partner LGL, causes dramatic tissue overgrowths that are genetically dependent on *yorkie* (Grzeschik et al. 2010). In addition to Scribble, other polarity proteins impact on YAP/TAZ function: the apical crumbs complex (CRB) binds to YAP/TAZ and plays a role in their cytoplasmic localization (Varelas et al. 2010), which has relevance for the correct differentiation of airway epithelial cells in mice (Szymaniak et al. 2015); alpha-catenin, the linker between cadherins and the actin cytoskeleton, is a potent inhibitor of YAP activity in keratinocytes, such that alpha-catenin mouse mutants develop skin hyperproliferation and stem cell expansion similar to YAP-overexpressing mice (Schlegelmilch et al. 2011; Silvis et al. 2011).

Altogether, this evidence indicates that normal epithelial architecture, characterized by specialized cell-cell junctions and apicobasal polarity, is a potent suppressor of YAP/TAZ activity, at least in part by Hippo core kinases activation. This might have relevance during the development of solid tumors, where loss-of-polarity is commonly observed, and where YAP/TAZ activity are instrumental for cancer cell growth and cancer stem-cell renewal (Cordenonsi et al. 2011). However, a completely opposite situation is found during early embryogenesis, where the antagonism between Hippo and YAP/TAZ is crucial to partition embryonic cells of the blastocyst into trophoblast and inner cell mass: in this system, Hippo is inactivated in cells with cell-cell junctions and apicobasal polarity (Sasaki 2015). This raises the question of what factor(s) account for this

divergent effect of tissue architecture on the Hippo pathway in the blastocyst compared to more differentiated epithelia.

## Mechanical regulation of Hippo pathway effectors YAP/TAZ

Mutation of Hippo pathway components in mammals leads to organ overgrowth, but not to unlimited growth; this indicates that alternative mechanisms exist to restrain tissue growth. One key property of such a postulated mechanism should be the ability to sense some organ- or tissue-level property, and to coordinate cell proliferation across several cell diameters. A possible candidate to fulfill these functions was proposed to be the mechanical properties of the cells themselves: mechanical signals (such as compression or stretching) can propagate from cell to cell very rapidly, and cells tend to accommodate these perturbations by uniformly reorganizing cell shape/architecture (Aegerter-Wilmsen et al. 2007). This hypothesis became real with the serendipitous finding that YAP/TAZ are regulated, in parallel to Hippo, by the mechanical properties of the cells/tissue: mechanical signals embedded in the extracellular matrix (ECM) or external mechanical stresses (such as stretching, fluid shear stress or mechanical loading and compression) impact on YAP/TAZ activity (Fig. 1C; Halder et al 2012). Dupont et al. 2011 showed that extrinsic physical forces delivered by the ECM modulate YAP/TAZ activity through actin cytoskeleton. Indeed, plating cells on a small adhesive area where cells are unable to develop forces against the substratum restrain YAP/TAZ activity, whereas allowing cells to spread trigger YAP/TAZ nuclear translocation. Similarly, a stiff and rigid ECM activates YAP/TAZ, while a more compliant matrix relocates YAP/TAZ into the cytoplasm, inhibiting their transcriptional activity. The mechanism by which extrinsic mechanical signals impact on YAP/TAZ activity implicates a role for filamentous-actin (F-actin): YAP/TAZ activity indeed depends on the activity of enzymes promoting the formation of F-actin filaments (Diaphanous-related), but not on F-actin networks promoted by Arp2/3 complexes (Aragona et al., 2013), and neither on the ratio between filamentous/globular actin (Dupont et al., 2011), which instead regulates the MRTF family of SRF cofactors (Miralles et al. 2003). Further evidence for the important role of F-actin as key regulator of YAP/TAZ came with the isolation of F-actin-capping and -severing proteins as YAP/TAZ

inhibitors in Drosophila and in mammalian cells (Aragona et al. 2013; Janody 2006; Sansores-Garcia et al. 2011) This function was shown by Aragona et al. to be key for the regulation of YAP/TAZ activity by mechanical cues, because knockdown of these F-actin and –severing proteins was sufficient to rescue YAP activity in conditions of low cytoskeletal tension.

Taken together these evidences demonstrated that mechanical signals play an important role in the regulation YAP /TAZ; however, whether or not the Hippo kinase cascade is involved in regulation of YAP/TAZ by mechanical cues is still debated. Data from our lab (Dupont et al. 2011, Aragona et al. 2013) exclude the involvement of Hippo pathway because knockdown of LATS1/2, or mutation of the Hippo-regulated serines on YAP and TAZ, does not block YAP/TAZ regulation by ECM stiffness. On the other hand, other studies clearly showed that the activity of LATS kinases is influenced by F-actin and RHO GTPases, indicating at least two parallel mechanisms by which mechanical cues regulate YAP/TAZ (Zhao et al. 2012; Sansores-Garcia et al. 2011). More importantly, it remains completely unknown how the F-actin cytoskeleton regulates YAP and LATS activity.

## Downstream effectors

The Hippo pathways act as tumour suppressor pathways as restrain the activity of YAP/TAZ. YAP and TAZ transcriptional co-activators are powerful inducer of cell proliferation and thus they are associated with tumor initiation, progression and metastasis by promoting cell proliferation, migration and survival (Harvey et al. 2013). Although the biological function of this pathway has been intensively characterized, the molecular mechanisms underlying this biological activity remain elusive. Genome-wide analyses of YAP /TAZ transcriptional targets have been carried out both in vitro and in vivo, leading to the identification of important target genes that are now widely used to monitor YAP/TAZ activity: CTGF, CYR61, ANKRD1, BIRC5 (Cordenonsi et al. 2011; Zanconato et al. 2015).

## The effects of manipulating Hippo pathway and YAP activity in

## the mouse liver

Unlike most other organs, the liver has the ability to tolerate substantial changes in size and high levels of stress, and possesses a distinct ability to regenerate after damage. For these reasons, the liver is the organ of choice for studying Hippo signaling in vivo. The transgenic overexpression of YAP in embryonic mouse liver is sufficient to induce a four-fold increase in liver/body weight ratio caused by proliferation of mature hepatocytes and the acquisition of biliary duct/liver progenitor cell traits (Dong et al. 2007; Camargo et al. 2007). Moreover restoring endogenous YAP levels after a period of overexpression leads to rapid reversal of the liver growth and normalization of the parenchymal architecture; suggesting that Hippo signalling and the effector YAP act as an important regulator of overall liver size. Phenotypes observed upon YAP overexpression are dependent on TEADs transcription factors: treatment of YAP transgenic mice with verteporfin, a small-molecule inhibitor of YAP/TEAD interaction, or co-expression of a dominant-negative version of TEAD2, rescue the growth phenotype (Liu-Chittenden et al. 2012).

Liver overgrowth was also observed upon tissue-specific genetic inactivation of Hippo pathways components MST1/2, Salvador/WW45, or NF2/merlin by using the hepatocytes specific albumin-Cre driver, which activity starts from embryonic stages. In these cases, increase in liver size and in cellular proliferation were interpreted mainly as an expansion of atypical ductal/oval cells, which are liver progenitors population able to differentiated both in hepatocytes and bile-duct epithelial cells after liver injury. However, recently lineage-tracing data revealed that expansion of atypical ductal/oval cells in Hippo mutant liver derived from dedifferentiated mature hepatocytes, reprogrammed upon YAP overexpression (Yimlamai et al. 2014). In all cases, hepatomegaly is followed by development of tumors of mixed characteristics, resembling both hepatocellular carcinomas and cholangiocarcinomas. Hepatocellular carcinoma develops from malignant transformation of hepatocytes whereas cholangiocarcinoma arise from the small intrahepatic bile duct epithelium, in line with aberrant expansion of atypical/ductal oval cells population. MST1/2 knockouts, but not NF2 and Sav mutants, display signs of liver inflammation, likely contributing to accelerated

tumor progression. Thus Hippo pathway components are suppressors of liver growth and liver tumor development. Importantly, the phenotypes due to NF2 inactivation were all rescued by the combined deletion YAP allele, establishing NF2 as a bone fide upstream regulator of YAP in mammals (N. Zhang et al. 2010). However, the requirement for YAP in MST1/2 or Sav/WW45 knockout livers has never supported by scientific data in animals model.

Less is known about the role of Hippo/YAP axis in adult mouse liver. Indeed, until now few Hippo mutants or YAP transgenic mice were develop in time-controlled manner. Data from Zhou et al. 2009 showed that tail-vein injection of adeno-cre virus in MST1 -/-; MST2 floxed/- 6-weeks-old mice lead to increase in liver/body weight ratio and Ki67 positive cells within 8 days, and decrease in survival rate around 4 months due to cancer development. Similar data obtained in Song et al. 2010 showed that CAGGCre-ER; MST1 -/-; MST2 floxed/- also developed hepatocellular carcinoma and cholangiocarcinoma, but only 6 months after tamoxifen-injection. A possible explanation for this discrepancy might be a different (mixed) genetic background, or the use in the first report of adeno-Cre virus injection, which might have caused an inflammatory condition facilitating cancer development.

As for liver-specific inactivation of YAP in the fetal liver, this causes an expected decrease in hepatocyte proliferation and increase in apoptosis, but it also induced impaired bile duct development, leading to steatosis and fibrosis (N. Zhang et al. 2010).

## Role of Hippo/YAP axis in tissue regeneration

Genetic experiments in mice indicate that YAP/TAZ are required during embryonic development (Varelas 2014; Pan 2010), while in adult tissues their activity appears constitutively repressed by the Hippo pathway. Indeed, loss of YAP/TAZ function in adult tissues failed to indicate a physiological function of YAP/TAZ in normal tissue proliferation and homeostasis. What are then YAP/TAZ required for? Several experiments suggest that YAP/TAZ are essential in response to injury for tissue regeneration in several tissues.

Some data indicate a role of Hippo signaling for intestinal regeneration (Hong, Meng, and Guan 2016). Using whole-body irradiation to induce intestinal damage, Gregorieff et al. 2015 found that YAP is activated in intestinal epithelial cells. In the DSS (dextran sulfate sodium)-induced model for intestinal regeneration, not only YAP is activated but knockout of YAP in intestinal epithelial cells impairs tissue regeneration (Cai et al. 2010).

The Hippo pathway has been also linked to heart regeneration. Inactivation of the Hippo pathway by genetic deletion of LATS1/2 or Salvador promotes heart regenerative response in adult mouse after cardiac apex resection, as well as after myocardial infarction (Leach et al. 2017; Matsuda et al. 2016; W.-F. Cai et al. 2016; Heallen et al. 2013). Moreover, transgenic mice that express an active form of YAP can regenerate more effectively than wild type mice after myocardial infarction (Xin et al. 2013). Mechanistically, activated YAP enables the proliferation of adult cardiomyocytes and opposes cardiac fibrosis. These data strongly indicate that inhibition of Hippo signalling might represent a significant approach to facilitate heart regeneration in patients suffering of ischemic heart damage, a primary cause of death in western countries for which very few pharmaceutical approaches are available.

The liver is known for the remarkable ability to regenerate following significant hepatocyte loss (Thorgeirsson SS, 1995; Fausto, 2004). The endpoint of regeneration consists in the restoration of the physiological liver-to-body weight ratio, and can be observed following partial liver resection (hepatectomy). A similar process is observed upon transplantation of small/partial livers; in contrast, transplantation of liver larger than the original organ tend to atrophy to reestablish the optimal liver/body weight ratio (Juan and Hong, 2016; Penzo-Méndez and Stanger, 2015). Even though numerous cytokines and growth factors play a role in liver regeneration, the precise mechanisms that are responsible for the initiation and termination of liver regeneration are not fully understood.

The role of YAP/TAZ in liver regeneration is surprisingly limited, and specific for selected types of damage. Alterations of the Hippo signaling pathway and YAP activity during the liver regeneration following partial hepatectomy suggested a

role for this pathway: Mst1/2 and Lats1/2 are inhibited during liver regeneration, leading to increase in YAP function (Grijalva et al. 2014). However, in this particular experimental model YAP/TAZ are genetically dispensable, perhaps due to redundant mechanisms ensuring reactivation of hepatocyte proliferation. Another model of adult liver injury is bile duct ligation, leading to cholestatic injury. In this model, YAP liver knockout mice display decreased duct cell proliferation and enhanced parenchymal damage, indicating a positive role for YAP in liver regeneration (Bai et al. 2012). In addition, activation of YAP also protects the liver from acetaminophen- and DCC-induced liver injury (Wu et al. 2013; Fan et al. 2016; K.-P. Lee et al. 2010). However, how Hippo mutant or YAP transgenics display protection from acetaminophen overdose remains controversial: Fan et al, 2016 attributed the protection of MST1/2 KO mice to the pro-apoptotic function of MST1/2 kinases reported earlier in mammalian cells (Matallanas et al. 2007); in Wu et al. 2013 the authors linked acetaminophen to inhibition of the Ets transcription factor GABP, in turn leading to inhibition of YAP transcription, thus disabling the tissue regenerative capacity mediated by YAP. According to these authors, MST1/2 KO mice would thus be protected because of unabated YAP expression rescuing hepatocytes from APAP toxicity. An alternative explanation could be the recent observation that hepatocyte-specific activation of YAP or inactivation of MST1/2 leads to defects in hepatocyte metabolic zonation, with a strong downregulation of pericentral gene-expression exemplified by GLUL (glutamine synthetase) (Fitamant et al. 2015): also the enzymes responsible for conversion of APAP into its toxic derivative, CYP1A2 and CYP2E1, are expressed around the central vein, and alteration of hepatocyte zonation is expected to alter their expression as well (Apte et al. 2009; Yang et al. 2014).

## Mechanobiology

A variety of mechanical forces function in living organisms, including fluid shear stress, pressure and tensional forces. Indeed, every single cell of our tissues is affected by physical forces: during early embryonic development, morphogenetic movements at gastrulation stage are key to place developing tissues in their correct spatial organization; during organogenesis, the forces exerted by blood

flow in the vascular system are required not only for the correct branching of the vascular tree, but also for the coordinated establishment of hematopoietic stem cells; in adult life, several tissues such as bone, muscle, and skin constantly remodel themselves in response to external forces (Mammoto and Ingberg 2010).

The general tenet in the field of developmental biology has been for a long time that chemical signals, also known as morphogens (chemokines, hormones and secreted signaling molecules), play a critical role in pattering cell behaviour, by controlling both cell differentiation and cell rearrangements (migration, expansion/contraction of epithelial sheets, intercalation, epiboly movements, invagination etc.). In this view, the ordered deployments of mechanical forces are part of a genetic program for tissue morphogenesis induced and regulated by chemical signals. However, growing evidence indicates that mechanical forces can act themselves as crucial signals controlling cell differentiation and cell behaviour in parallel or independently from chemical signals.

The first demonstration of the critical role of mechanical forces was from Albert K. Harris et al., 1984. When he seeded fibroblasts onto silicone substrata allowing the cells to exert traction forces and to deform the substratum, they formed regularly arranged clusters, instead of spreading homogeneously as they did when cultivated onto a non-deformable rigid surface such as glass or plastic. The authors concluded that mechanical inputs themselves can be sufficient to organize cell behaviour. Moreover, they implied that mechanical inputs, at difference with chemical signals, act on a short time-scale and at much longer spatial range than diffusing chemicals, and can generate a greater range of possible geometries than those possible using gradients of morphogens. The authors also described mathematically these results, using the idea of short-range activation and long-range inhibition from chemo-kinetics models adapted for mechanicals signals.

Following these seminal observations, multiple evidences accumulated indicating that mechanical forces are informational systems by which cells perceive their position, the architecture of the surrounding tissue, and perturbations in their environment, inducing them to respond until a proper mechanical balance is achieved. For example, the mechanical stiffness of the extracellular matrix

(ECM), in addition to its chemical composition, is a critical determinant of cell physiology, stem cell differentiation and tissue homeostasis. Conversely, abnormal changes in ECM stiffness contribute to the onset and progression of various diseases, such as cancer and fibrosis (Jaalouk and Lammerding 2009). This opened the way to studies aimed at understanding how cells translate extracellular mechanical and physical stimuli into intracellular biochemical signals enabling cells to respond to their physical environment, the so-called mechanotranduction mechanisms. Mechanotrasduction can be divided into three major steps: mechanosensing, mechanotrasmission, and mechanoresponse (Vogel and Sheetz, 2006).

## *Cellular mechanosensing*

Cells are able to sense multiple physical inputs. In principle, changes in the extracellular and intracellular cell organization and structure can alter the conformation of macromolecules and thus their function, enabling mechanosensing. Single-molecule measurements indeed indicate that the forces required to unwind proteins fall in the range of forces to which cells are actually exposed. The best-studied examples are stretch-activated ion channels (Lansman et al., 1987; Arnadottir and Chalfie, 2010), which directly sense plasma membrane tension. Other macromolecular sensors of forces are integrin receptors that physically connect ECM molecules to the cellular cytoskeleton. Integrins are transmembrane proteins with a head domain connecting to specific sites on ECM adhesive proteins (fibronectin, collagens, tenascin and others, in complex with proteoglycans and glycosaminoglycans such as hyaluronic acid) and with a cytoplasmic domain connecting integrins to cytoplasmic proteins.

When cells adhere to their substratum, binding of integrins to ECM molecules induce their local clustering into focal points, which then get connected to the actin cytoskeleton; contraction of the actin cytoskeleton, resisted by the ECM, enable tension developing, which triggers the maturation of focal points into more stable focal adhesion structures. The detailed structure and functioning of focal adhesions has been determined, and consists of several layers containing distinct proteins: a membrane-opposed layer containing integrin cytoplasmic tails, focal

adhesion kinase (FAK) and paxillin; and intermediate force-transduction layer containing talin and vinculin (whose recruitment to focal adhesions require force development across integrins, and involve a conformational switch), and an actin-regulatory layer consisting of zyxin, VASP (vasodilator stimulating phosphoprotein) and alpha-actinin, which links the focal adhesion (FA) to the actomyosin cytoskeleton.

The recognition of ECM macromolecules by integrin heads is itself responsive to physical ECM properties: atomic force microscopy (AFM) studies indeed showed that mechanical loading could directly influence the lifetime of integrin-ECM bonds. Some integrins, such as αIIβ3, exhibit a so-called slip-bond behaviour characterized by a decreased lifetime with increasing load, whereas others, such as α5β1, exhibit catch-bond behaviour characterized by an increased lifetime with increasing load (Kong et al. 2009). The lifetime of integrin-ECM bonds determine the ability of integrins to cluster and form stable adhesion complexes; thus, depending on the type of integrin receptors, focal adhesions will display different responses to extracellular mechanical forces.

Within the focal adhesion complex, talin is considered the main mechanosensing molecule, because applied forces can change its conformation and thus expose the vinculin binding sites (Ziegler et al. 2008). In fact, increasing the ECM mechanical loading promotes the assembly of larger integrin clusters, and of correspondingly increased intracellular focal adhesion plaques, allowing cells to counteract the mechanical loading. On the opposite, compliant matrices only support the assembly of focal point structures, if any at all (Paszek et al. 2009).

### *Transmission of mechanical stimuli: the actomyosin cytoskeleton*

The fundamental structure by which cells resist extracellular forces and develop internal ones is the cytoskeleton. The cytoskeleton is a highly dynamic structure composed of filamentous actin, intermediate filaments, and microtubules. Among these elements, the actin cytoskeleton is thought to play a prominent role in force development, while microtubules can be considered as force-resisting modules (E. Ingber, 2010). Globular actin monomers (G-actin) assemble into filamentous actin

(F-actin) polymers that serve as substrates for myosin motors. F-actin filaments are polarized: barbed ends are the fast-growing ends whereas the pointed ends correspond to the slow-growing ends. This intrinsic polarity enable the unidirectional action of myosin motors, that together with actin filaments represent the minimal contraction unit: a filament of myosins, bearing myosin molecules symmetrically arrayed at the two extremities, and two antiparallel actin filaments. If F-actin is not anchored, myosin activity will cause the sliding of the two F-actin filaments one relative to the other; if F-actin is anchored to other cellular structures, myosins will develop tension between these structures, eventually causing their displacement.

F-actin filaments in cells are mainly anchored to focal adhesions (and thus, to the ECM), to other passive resisting cytoskeletal structures (mainly, intermediate filaments), and to the nuclear envelope. When cells are in contact with the ECM, focal adhesions enable the direct transmission of forces between cells and the ECM. Contraction of filaments between two focal adhesions enable cells to deform the substrate; this inside-out signaling happens when cells actively pull on the ECM to remodel its organization, for example to align collagen fibers. Contraction of filaments anchored to a single focal adhesion on one side, and to other internal cytoskeletal structures on the other end, enable cells to migrate or to change their shape. Contraction of filaments anchored to the nuclear envelope contributes to keep the nucleus in place, and deforms the nuclear shape according to the degree of cell attachment to the ECM.

By this same system, cells can also measure extracellular forces and their variations. It is currently thought that a tensional equilibrium exists between cells and the ECM: when cells are subjected to external forces from the ECM, they immediately develop opposite internal forces through the actomyosin cytoskeleton (outside-in signalling) until a new equilibrium is reached. How this is then translated into intracellular biochemical signalling events (mechanotrasduction) remains poorly understood; clearly, the degree of focal adhesion maturation and the extent of F-actin reorganization in response to external forces play a fundamental role, impacting the activity/localization of mechanosensitive molecules.

*Mechanoresponses*

The intimate relationship between extracellular forces, focal adhesion maturation and intracellular actomyosin contractility represents the basic mechanism by which cells measure extracellular forces. In turn, the degree of cell-substrate adhesion and actomyosin tension development correlates with changes of cell shape: cells on a stiff ECM can spread and develop internal actomyosin tension, while cells on a soft substrate remain rounded and develop lower internal forces. Thus, changes in ECM elasticity, inhibition of internal actomyosin tension, or imposing cells a defined cell geometry enable experimental manipulation of mechanosensing, and the study of mechanostransduction mechanisms.

The instructive role of mechanical forces on cell behaviour in vitro was initially related to cell-substrate adhesiveness and changes in cell shape: experiments in which the availability of integrin binding sites was progressively reduced, leading to progressive reduction of cell shape, first suggested this was sufficient to regulate important cell phenotypes such as cell proliferation and death (D. E. Ingber et al. 1990; Mooney 1992). The introduction of micro-fabricated ECM patterns enabled a formal distinction between the effects of cell geometry and the amount of ECM (intended as potential chemical signal) on these phenotypes: seeding cells on patterns small enough to constrain cellular spreading confirmed that alterations of cell shape is sufficient to control the choice between proliferation and differentiation or apoptosis (Chen 1997; Watt et al., 1988; Mc Beath 2004). Moreover, others showed that the elasticity of the ECM controls both cell shape (spread on a stiff ECM, rounded on a soft ECM) and the differentiation of mesenchymal stem cell (MSC): compliant substrates (<1 kPa) leads MSC towards a neurogenic or adipogenic phenotype, myogenesis at medium stiffness (8-17 kPa), and osteogenesis at high stiffness (>25 kPa) (Engler et al., 2006; Fu et al., 2010). This concept has now been then extended to a number of other studies that showed that the balance between proliferation differentiation is modulated by extracellular environment rigidity in different cell types: skeletal muscle stem cell (Gilbert et al., 2010), hematopoietic stem cells (Holst et al, 2010) and embryonic stem cells (Chowdhury et al., 2010). Several of these phenotypes could be mimicked by treating cells plated on large / stiff ECM

substrata with inhibitors of actomyosin contractility, establishing a key role of intracellular contractility in driving these phenotypes. It was later confirmed, with the development of traction force microscopy techniques to measure the magnitude of the forces exerted by cells on their substrates, that a small area or a soft ECM indeed cause reduction of intracellular tension.

From these studied it became clear that cells respond to mechanical signals by changing their behaviour, which entails both immediate responses, such as durotaxis (i.e. the ability of some cells to migrate towards a stiffer substrate), and responses mediated by modulation of gene expression, including growth, differentiation and death. Several biochemical signalling pathways besides YAP/TAZ (see above) have been discovered by which actomyosin contractility and extracellular forces can regulate gene transcription and/or cell behavior. A stiff ECM microenvironment, for example, increases the binding between a crucial component of focal adhesions, Focal Adhesion Kinase (FAK), to Src and Mitogen-Activated Protein Kinases (MAPKs), enhancing activation of ERK1/2 (Provenzano et al., 2009). Cytoskeletal remodelling and focal adhesion maturation induced by mechanical stimuli also involve different members of the Rho GTPase family, such as Rho, Rac and Cdc42. Activation of Rho-GTPases promotes actin polymerization, shifting the balance between monomeric actin and polymerized filamentus-actin (F-actin); the decrease of monomeric actin, activates Myocardin-Related Transcription Factors MAL/MRTF, because G-actin directly binds MAL/MRTF blocking them into the cytoplasm. Once MRTF is free it translocate into the nucleus where, together with Serum Response Factor (SRF), it modulates gene transcription. Other transcription factors that respond to actin dynamics and forces in the context of epithelia are beta-catenin, SNAIL1, TWIST, Notch and KLF2a (Cha et al. 2016; Fernández-Sánchez et al. 2015; Brunet et al. 2013; K. Zhang et al. 2016; Wei et al. 2015; Desprat et al. 2008; Gordon et al. 2015; Wang et al. 2011).

In addition to these specific regulations, it has also been suggested that actin, and by association mechanical signals, might also regulate chromatin dynamics. The cytoplasmic actomyosin cytoskeleton structurally links ECM-focal adhesions with the nuclear envelope through a protein complex called the Linker of

Nucleoskeleton and Cytoskeleton (LINC). This complex crosses the nuclear envelope and is anchored to the nuclear lamina, forming the nuclear cytoskeleton; by this direct link, the LINC complex mediates force transmission from focal adhesions to the nuclear envelope, resulting in nuclear deformation and possibly to alterations of chromatin structure and organization. A number of diseases of muscle tissue (some forms of muscular dystrophies and cardiomyopathies) result from mutations in LINC complex components and/or nuclear lamina (Gundersen et al. 2013). Finally, a more obscure role for F-actin has been postulated in the regulation of chromatin, owing to the observation that the SWI/SNF nucleosome remodelling complex contains actin and actin-related subunits (Schubert et al. 2013), and possibly also of transcription through DNA PolII and of protein translation by association with initiation factors (Miyamoto and Gurdon 2013)

## Role of mechanobiology in physiology and disease

It is known from a long time that tissues and organs are able to respond to external forces: this goes from the ability of muscles, tendons and bones to remodel in response to intensive use to the capacity of the circulatory system to sense variations in blood pressure and activate feedback homeostatic mechanisms. Evidence also exist for a specific role of ECM mechanical stiffness in vivo: the capacity of mesenchymal stem cells to remodel collagenous ECM by secreting metalloproteases, for example, is key for their balanced differentiation into osteoblasts and adipocytes (McBeath et al. 2004). A number of studies indicate that connective tissue fibrosis is a process initiated by extrinsic stimuli (damage, inflammation, etc.) leading to conversion of fibroblasts into collagen-secreting and -contracting myofiborblasts, which stiffen the ECM, in turn recruiting new myofibroblasts and thus creating a self-sustaining loop. In the disease known as scleroderma this loop is not interrupted or mitigated, leading to propagation of dermal fibrosis from scars to the adjacent healthy skin. A very similar situation is found in some cancers, where cancer cells themselves, or cancer-associated fibroblasts, stiffen the ECM surrounding the tumor and thus contribute to cancer cell growth and dissemination (Butcher, Alliston, and Weaver 2009; Calvo et al. 2013). Furthermore, in some types of cancers such as hepatocellular carcinoma

and lung cancer, tissue fibrosis is considered an important risk factor (Li et al. 2014; Cox and Erler 2014; Dănilă and Sporea 2014). Further reflecting the importance of mechano-environment in the physiology of the tissue, changes in ECM composition that compromise the mechanical properties of tissues are causative for disease in genetic diseases including osteochondrodysplasia, mucopolysaccharidoses and collagenopathies (Bateman, Boot-Handford, and Lcamandé 2009).

# Part I

# Rationale

Here we aimed to develop and characterize a novel genetic tool to probe the role of actomyosin mechanical signals in mammalian tissues. Given that YAP/TAZ are seemingly not active in normal tissue conditions, we designed an approach leading to YAP/TAZ hyperactivation to test whether or not mechanical signals play a role to keep YAP/TAZ inhibited. Recent evidence showed that in vitro depletion of F-actin remodeling factor CAPZB, Cofilin and Gelsolin in one human epithelial cell line is sufficient to reactivate YAP/TAZ also when ECM mechanical cues would inhibit them (Aragona et al. 2013). Moreover, genetic studies done in *Drosophila* suggest that CAPZ subunits (*Cpa and Cpb*) and Cofilin (*Twinstar*) inactivation in imaginal disc cells can activate *Yki* (YAP homologous), leading to enhance proliferation phenotypes (Garcia-Fernandez et al. 2011; Janody 2006). Among these F-actin regulatory factors, we chose to inactivate CAPZB for simplicity: CAPZ is a dimeric protein complex composed of one α and one β subunit, and the β subunit is encoded in the mammalian genome by a unique gene. Thus, at difference with CAPZA1/2, ADF/COFILIN1/2, and Gelsolin family members for which extensive redundancies are expected (Pollard & Copper 2009), knockout of CAPZB should be sufficient to completely inhibit CAPZ function, without the need of complicated genetic crossings.

Much of what is known on CAPZ derives from in vitro biochemical studies with reconstituted actin microfilaments (Pollard et al. 2009). Actin filament growth, stability and disassembly are controlled by a plethora of actin-binding proteins; among these, CAPZ restricts the accessibility of the filament barbed end, inhibiting addition or loss of actin monomers, thus locally stabilizing filaments, and preventing their growth and/or treadmilling (Cooper, 2009). CAPZ has also been postulated to influence actin monomer dynamics, because inhibition of monomer exchange at one F-actin filament (capped by CAPZ) would favor the

availability of monomers for other non-capped filaments. This has been shown to be important in the regulation of bacterial actin-based motility, because CAPZ promotes filament nucleation by the ARP2/3 complex (Akin and Mullins 2008; Loisel et al. 1999), the balance between lamellipodia and filopodia (Mejillano et al. 2004; Iwasa and Mullins 2007) and Dictyostelium amoeboid migration (Jung et al. 2016; Hug et al. 1995). This might also relate to the defective migration of CAPZ-null neural crest cells in the zebrafish embryo, which may explain the observation that CAPZB deletion is found in human patients affected by micrognathia / cleft palate (Mukherjee et al. 2016). In mammalian cells CAPZ also plays a role in the maturation of oocytes (Jo et al. 2015) and in formation of the autophagosome (Mi et al. 2015), phenotypes relating to a structural role of CAPZ in the assembly of specific actin networks. However, what is the functional requirement for CAPZ in mammalian tissues, and whether it may perform signaling functions related to YAP/TAZ activity, remains unknown. Thus, tissue-specific genetic inactivation of CAPZB could represent a tool to elucidate the physiological relevance of mechanical forces in mammalian tissue, and to unveil a signalling function of F-actin previously unrecognized.

# Results

## Derivation and validation of *CAPZB* floxed allele

CAPZ is a dimeric protein composed of an alpha and a beta subunit; the alpha subunit is encoded by two independent loci in mammals (CAPZA1 and CAPZA2), while the beta subunit is encoded by the CAPZB locus. We thus chose to knockout the CAPZB locus for simplicity. We obtained frozen embryos of the Capzbtm1a (EUCOMM) Wtsi line from the EUCOMM/EMMA repository, revived the line, and then crossed it to a constitutive FRT deleter transgenic strain to get rid of the selection cassette. We thus obtained mice bearing a CAPZB floxed allele, with two loxP sites flanking the third exon of the gene for conditional recombination, which we then crossed to obtain homozygous CAPZB floxed mice, fl/fl (Fig 2A).

To test the effectiveness of this genetic design in knocking out the gene we derived primary tail-tip fibroblasts from an adult CAPZB fl/fl mouse and recombined them in vitro with adenoviral-Cre infection, which led to an efficient depletion of the endogenous CAPZB protein after few days in culture (Fig. 2B). This also indicated that absence of CAPZB is compatible with cell survival, at least in vitro. To better validate the new allele we then evaluated the role of CAPZB in the formation of muscle sarcomeres. Indeed, CAPZ was identified as one major component of the Z-line of sarcomere, and is expected to significantly contribute to sarcomere structure stability. We isolated primary foetal cardiomyocytes from CAPZB fl/fl mice and infected them in vitro with adenoviral-Cre; this led to a rapid disassembly of contractile actomyosin structures in Cre-expressing cells (Fig. 2C), validating that our allele leads to functional inactivation of CAPZ, and that CAPZ is required for sarcomere stability.

## Inactivation of CAPZB changes the cell's perception of ECM mechanical inputs in vitro

Previous data from our lab indicate that CAPZ regulates the sensitivity of immortalized mammary epithelia cells to ECM stiffness (Aragona et al. 2013). We thus sought to explore whether genetic inactivation of CAPZB also regulates mechanical signalling in cells isolated from our knockout mice. For this, we used primary adult fibroblasts from CAPZB floxed/floxed mice, since fibroblasts are known to faithfully respond to mechanical cues by regulating YAP/TAZ (Wada et al. 2011; Calvo et al. 2013). As shown in Fig. 3A, WT fibroblasts indeed respond to a small cell geometry by inactivating YAP/TAZ (as assayed by exclusion from the nucleus) and by decreasing proliferation (as assayed by EdU incorporation). In contrast, CAPZB KO fibroblasts retain nuclear YAP and keep proliferating, at least to a certain extent (Fig. 3A). We also tested the effects of CAPZB KO on the regulation of YAP/TAZ by ECM rigidity. For this we plated primary fibroblasts on ECM-coated hydrogels showing a gradient of stiffness ranging from 0,5kPa (very soft) to 25kPa (stiff), and measured expression of two established YAP/TAZ target genes, *Ankrd1* and *Ctgf*. We found that CAPZB KO fibroblasts exhibited enhanced *Ankrd1* and *Ctgf* expression, compared to WT fibroblasts, over a range of stiffnesses (Fig 3B). Of note, CAPZB KO fibroblasts did not exhibit basal induction of target genes in the stiffer context (25kPa), i.e. when actin tension is already above the threshold to keep YAP/TAZ active (Dupont et al. 2011; Roca-Cusachs et al. 2013; Elosegui-Artola et al. 2016). These data indicate that genetic loss of CAPZB with our allele can be used as a tool to change the cell's perception of extracellular mechanical inputs, at least in vitro.

## Control of organ size by conditional inactivation of CAPZB

Prompted by these results, we set-up crossings to probe the function of CAPZB as negative regulator of YAP/TAZ activity in vivo. We focused on the liver, as this is the best-characterized model system for YAP in mammals (Piccolo, Dupont, and Cordenonsi 2014). We thus obtained Albumin-CreERT2; CAPZB fl/fl; ROSA26-LSL-beta-gal mice (hereafter named CAPZB LKO), enabling the deletion of CAPZB in adult hepatocytes in a time-controlled manner by administration of tamoxifen. The ROSA26-LSL-beta-gal allele drives stable expression of beta-galactosidase only after Cre-mediated excision of the LSL (lox- STOP-lox) cassette, and was included as a reporter of tamoxifen-induced

recombination efficiency. The presence of this allele also helped distinguish at single-cell level KO from non-recombined cells, in absence of an antibody faithfully detecting endogenous CAPZB by immunofluorescence or IHC in tissues (among the many antibodies that we tried, none worked). As controls, we used mice from the same litter, but not carrying the Albumin-CreERT2 transgene (hereafter named WT), or mice carrying the transgene but not injected with tamoxifen. CAPZB LKO and WT mice received five consecutive daily intraperitoneal injections of tamoxifen diluted in corn oil, starting at 4 weeks of age. After four weeks, mice were sacrificed for phenotypic analyses. Inactivation of CAPZB caused hepatomegaly, readily visible upon inspection, and quantified in a two-fold increase liver/body weight ratio (Fig. 4A). This was accompanied by a powerful increase in hepatocyte cell proliferation, as measured by EdU incorporation (Fig. 4B), suggesting hepatocyte cell hyperplasia as the basis for hepatomegaly. qPCR and RNA-seq experiments further confirmed a general increase in proliferation and survival genes such as *Top2a, Birc5, Cdc6, Cdk1* (GO-cell cycle progression) and anti-apoptotic genes such as *Bcl2* and *Bcl2l1* (Jourdan et al. 2009), in keeping with liver overgrowth (Fig. 4C). Thus, loss of CAPZB causes rapid and fully penetrant liver over-growth phenotypes also observed in Hippo-mutant and YAP-overexpressing livers. Further in line with observations in Hippo-mutants, CAPZB KO mice showed a mild increase in serum ALT levels (Fig. 4D) suggesting alterations of liver functions, which was not accompanied by sign of inflammation, as assayed by recruitment of inflammatory leukocytes (CD-45 positive cells - Fig. 4E).

## CAPZB regulates YAP/TAZ in adult hepatocytes

We then tested whether deletion of CAPZB was accompanied by increased YAP/TAZ activity by monitoring a series of established targets of YAP/TAZ, including general targets such as *Ankrd1, Cyr61, Ctgf*, and more liver-specific targets such as *Tagln2 and Bicc1*. Figure 5A shows that all YAP/TAZ target genes tested are strongly induced in CAPZB LKO mice. Analyses are underway to quantitatively determine, at a genome-wide level, the similarity of gene expression in CAPZB LKO with that observed in LATS DKO, MST1/2 DKO and YAP transgenic livers. Encouraged by these results, we monitored endogenous

YAP protein localization in hepatocytes by immunofluorescence, and found a significant increase in hepatocytes displaying YAP nuclear localization (Fig 5B). In line, we observed accumulation of TAZ levels by western blotting on whole liver protein extracts (Fig. 5C), in keeping with its regulation by proteasomal degradation upon variation of mechanical cues (Dupont et al., 2011). As expected, we did not observe robust changes in total YAP protein levels by western blotting on whole liver protein extracts (Fig. 5C); however we observed overall increase of YAP immunofluorescent signal in CAPZB KO cells, as compared with patches of non-recombined hepatocytes occasionally found in CAPZB LKO mice, identified by the absence of beta-galactosidase staining from the co-deleted ROSA26 allele (Fig. 5D). Thus, deletion of CAPZB leads to powerful YAP and TAZ activation in adult hepatocytes, indicating that CAPZ is a functionally relevant inhibitor of YAP/TAZ activity in the mammalian liver tissue.

## CAPZB controls liver cell fate

One of the most established YAP-dependent phenotypes in the liver, besides hepatomegaly and increased proliferation shown above, consists in the expansion of atypical ductal/oval cells positive for A6 and CK19 staining, and similar in gene expression to cholangiocytes (Tanimizu and Mitaka 2014; Takiya et al. 2013; Lu et al. 2015). A similar oval cell response is commonly observed in the liver following hepatocyte damage, and is thought to represent expansion of stem cells with bi-potent progenitor ability (i.e. differentiating into both hepatocytes and bile-duct cells) for liver regeneration. As shown in Figure 6A, CAPZB LKO livers displayed a remarkable expansion of CK19- and A6-positive cells forming disorganized strands in the liver parenchyma, mainly distributed around the portal area (i.e. portal vein, hepatic artery and bile duct), and occasionally forming ectopic ductular structures. To further investigate this phenotype, we analyzed RNA-seq data and found a clear increase in genes related to the cholangiocyte lineage, including *Sox9* and *Sstr2* (Fig. 6B) and many others.

Atypical ductal/oval cells have been recently shown to derive from differentiated hepatocytes reprogrammed by YAP activation (Yimlamai et al., 2015); this can be followed by the appearance of cells double-positive for CK19 and HNF4alpha,

markers of oval/ductal cells and hepatocytes, respectively. We indeed found that a portion of the CK19-positive cells in the CAPZB LKO livers also stained positive for HNF4alpha, whereas this was never observed in WT livers (Fig. 6C). This was paralleled by a general decreased expression of HNF4a target genes associated with a mature hepatocyte phenotype and by re-expression of foetal hepatocyte genes, as previously observed upon YAP activation in hepatocytes (Yimlamai et al. 2014, data not shown). Thus CAPZB deletion not only induces liver overgrowth, but also induces dedifferentiation of hepatocytes typically observed upon YAP activation.

## CAPZB controls hepatocyte zonation and reprograms xenobiotic liver metabolism

In addition to the classical proliferation and oval cell phenotypes above described, YAP has been more recently implicated in regulating liver zonation (Fitamant et al. 2015). Metabolic zonation of the liver is characterized by localized patterns of expression of metabolic genes along the periportal to pericentral axis of the liver lobules, exemplified by preferential expression of glycogen synthase 2 in the periportal area, corresponding to a preferential involvement of these hepatocytes in glucose/glycogen metabolism, and of glutamine synthetase around the pericentral vein, corresponding to hepatocytes mainly involved in glutamine synthesis (Benhamouche et al. 2006; Gougelet 2014; Torre, Perret, and Colnot 2011). Liver zonation is governed by the interplay of several signalling cues, including HNF4alpha, oxygen, nutrient concentrations and the WNT/beta-catenin pathway (Gebhardt 2014; Kietzmann 2017; Birchmeier 2016). Recent evidence indicates that YAP activity is also patterned along this axis and contributes to liver zonation by restraining pericentral gene expression; we thus compared glutamine synthase (GS, also called GLUL) in WT and CAPZB LKO mice, and found a marked loss of pericentral staining (Fig. 7A). This was accompanied by strongly reduced expression of several other pericentral-specific genes by RNA-seq, and by the concomitant increased expression of some periportal-specific genes such as *Hes1* and *Aldh1b1* (Fig. 7B). These data indicate that CAPZB activity is relevant to pattern physiological liver functions, and suggest that F-

actin and mechanical cues can act as zonation gatekeepers in vivo.

Among the pericentral genes downregulated in CAPZB LKO mice, we also found several genes belonging to the cytochrome P450 family (CYP) known to display zoned gene-expression (Fig. 7B, Lindros 1997; Buhler et al. 1992; Braeuning et al. 2006). Cytochromes are mainly involved in xenobiotic metabolism, i.e. the ability of liver cells to toxify or detoxify exogenous chemical substances; among these, we focused our attention on *Cyp2e1* and *Cyp1a2*, the main genetic determinants of acetaminophen toxicity in the mouse (Zaher et al. 1998), which we found to be among the most profoundly downregulated genes in CAPZB LKO livers (Fig 7C). To test the functional relevance of this regulation, we induced acute acetaminophen liver injury and visualized tissue damage by histology, TUNEL staining (Fig. 7D) and ALT levels in the serum (Fig. 7E). At sub-lethal doses (350mg/Kg) sufficient to cause extensive cell death in the pericentral zone of WT mice, CAPZB LKO mice remained completely insensitive (Fig. 7D and 7E). Thus, CAPZ deficiency efficiently reprograms not only glutamine synthetase expression, as previously shown, but also xenobiotic liver metabolism.

*Cyp2e1* and *Cyp1a2* expression is specifically and highly upregulated in hepatocellular carcinomas and hepatoblastomas bearing activating mutations of the WNT/beta-catenin pathway, and has thus been proposed to expose these cancer cells to increased sensitivity to acetaminophen toxicity (Singh et al. 2013). A limitation to the use of acetaminophen to kill cancer cells is the limited therapeutic index due to endogenous toxicity on pericentral hepatocytes. Our data may imply that treatments interfering with CAPZ activity, or promoting F-actin activity, might be beneficial to decrease acetaminophen toxicity in normal hepatocytes, thus enabling the use of higher doses of acetaminophen to kill beta-catenin-addicted tumor cells.

## Liver phenotypes caused by CAPZB inactivation genetically depend on YAP

Data shown so far indicate that inactivation of CAPZB in hepatocytes leads to a remarkable phenocopy of YAP activation; however, similar phenotypes can be

also obtained by regulation of other signaling pathways, which led us to ask whether YAP inactivation is sufficient to normalize these phenotypes. For this we obtained mice with compound deletion of CAPZB and YAP in the liver (Albumin-CreERT2; CAPZB fl/fl; YAP fl/fl mice, hereafter named CAPZB+YAP LKO). As control we checked for the efficient of allele recombination looking at RNA-seq data (Fig. 8A). Analysis of double knockout livers indicates that liver overgrowth (Fig.8B), proliferation (Fig. 8C), oval cell response and liver zonation phenotypes (Fig. 8D) all genetically depend on YAP. Residual phenotypes observed in some cases (e.g. liver size is not entirely normalized) might be attributed to the hyperactivation of TAZ, which we found stabilized in CAPZB LKO mice.

## CAPZB regulates YAP/TAZ in parallel to Hippo

The molecular mechanisms by which mechanical signals regulates YAP/TAZ remain elusive. Several data indicate that mechanical regulation of YAP/TAZ includes the regulation of LATS1/2 kinases; other data indicate that LATS1/2 regulation is not sufficient to account for the regulation of YAP/TAZ by mechanical signals, because LATS1/2 knockdown was not sufficient to rescue YAP transcriptional activity in a soft ECM or small geometry microenvironment (Aragona et al., Cell 2013), and because loss of LATS1/2 cooperated with loss of capping/severing proteins (Aragona et al. 2013; Sansores-Garcia et al. 2011). We thus set-up an experiment to explore how Hippo-mediated regulation of YAP interacted with CAPZB inactivation in vivo. For this, we took advantage of hydrodynamic tail vein injection techniques to target liver cells with PiggyBac transposon plasmids expressing a Hippo-insensitive FLAG-TAZ4SA cDNA, enabling stable expression in the targeted hepatocytes. By using cell proliferation (EdU staining) as read-out of YAP/TAZ activity in liver cells, we found that TAZ4SA and CAPZB KO display additive effects (Fig. 8E). This data suggest that mechanical cues can enhance the activity of YAP/TAZ in a synergistic manner with their phosphorylation status.

## CAPZB is a genetic determinant of cell mechanosensing

CAPZB is the prototypical F-actin barbed-end capping protein. In vitro this activity restrains F-actin filament growth, which is thought to locally stabilize F-actin filaments, but also to free G-actin monomers to enable polymerization of other uncapped filaments. However, what are the effects of CAPZB inactivation in cells and tissues has never been thoroughly explored. This is particularly relevant in light of the observation that CAPZB inactivation has the ability to reactivate YAP/TAZ in conditions of low forces/tensions imposed by ECM mechanical cues, raising a number of questions: what is the mechanoresponsive F-actin structure regulated by CAPZB? How inactivation of a capping protein, in principle regulating F-actin growth, impact on F-actin contractility? Do CAPZB knockout cells display a stiffer cytoskeleton and/or a higher degree of contractility?

To tackle these issues, we decided to compare liver stiffness in WT and CAPZB LKO mice using atomic force microscopy (AFM), in collaboration with Kristian Franze's group. A simple hypothesis is that loss of CAPZB in hepatocytes would lead to increased cellular contractility and stiffness, reflecting in YAP activation but also in whole tissue stiffness. Indeed we found that CAPZB KO liver show increase in overall tissue stiffness (Fig 9A), indicating that loss of CAPZB in hepatocytes is sufficient to change whole liver mechanical properties.

We also better characterized the cytoskeleton in CAPZB KO fibroblasts. Staining of mouse primary fibroblast plated on fibronectin-coated glass with phalloidin, a tool to visualize F-actin bundles, indicates that CAPZB inactivation does not lead to a simple increase in F-actin content / thickening of F-actin stress fibers. Actually, actin appears more disorganized, with shorter/thinner stress-fibers, or less stable stress fibers, and increased peripheral F-actin filaments (Fig. 9B). The accumulation of peripheral F-actin structures is similar to results previously observed with CAPZ siRNAs, and that were linked to reduced turnover/dynamics of filopodia (Sinnar et al. 2014). To further deepen the functional effects of these changes we stained cells for vinculin, a component of focal adhesion complexes that is recruited to in response to cytoskeletal tension. KO fibroblasts display an overall increase of vinculin staining at the adhesion plaques and/or an increased size of focal adhesions at the cell edges (Fig. 9C). The increased of vinculin

recruitment likely suggest an increase in contractility of CAPZB KO fibroblast in respect to WT, leading to exacerbated focal adhesion maturation. In collaboration with Franze's lab at the University of Cambridge, we are now performing traction force microscopy (TFM) measurements on fibroblasts, in order to test if CAPZB KO fibroblast shows an increase in net force in respect to WT, and whether this is also conserved in conditions of decreased ECM stiffness. If so, in the future we should also observe residual actomyosin contractility in fibroblasts seeded on a soft ECM, as visualized by phospho-MLC staining, and this should be paralleled by increased phospho-MLC and vinculin stainings in CAPZB LKO hepatocytes.

# Discussion

## Liver specific inactivation of CAPZB recapitulates phenotypes observed upon YAP activation

Recent works in cultured cells showed that mechanical signals impacts on YAP/TAZ activity by remodeling filamentous-actin (F-actin) and by modulating cells contractility: plating cells on small ECM micropatterns or on a soft ECM substratum, leading to inhibition of cell contractility, or similar inhibition obtained by inhibition of RHO, ROCK, and non-muscle myosin II (NMII) lead to YAP/TAZ inhibition (Dupont et al. 2011). In keeping with the idea that F-actin bundles are the preferential substrate for NMII to contract the cytoskeleton, also inhibition of formin/diaphanous F-actin polymerizing factors, but not inhibition of ARP2/3 branched actin networks, lead to YAP/TAZ inhibition (Aragona et al. 2013). Among F-actin inhibitory proteins, F-actin capping/severing protein such as CAPZB, Cofilin and Gelsolin play a more specific or important role in setting the responsiveness of cells to experimental manipulation of the cells' mechanical properties, because their depletion was sufficient to rescue YAP/TAZ activation on a soft ECM or in a confluent monolayer, i.e. where YAP/TAZ are inhibited by low actomyosin tension (Aragona et al. 2013).

Prompted by similar observations carried out in Drosophila, here we decided to use CAPZB inactivation as a genetic tool to probe the functional relevance of actomyosin mechanical signals for mammalian tissue homeostasis. Our results indicate that loss of CAPZB in the adult mouse liver modulates intracellular actin cytoskeleton in a way that is sufficient to induce YAP/TAZ activation and to recapitulate all the YAP-dependent phenotypes described so far in this organ. Indeed, CAPZB LKO mice display increased nuclear localization of YAP/TAZ and activation of their target genes, followed by: 1) liver overgrowth, quantified in a two-fold increase liver/body weight ratio and associated with a potent increase in adult hepatocytes proliferation; 2) expansion of atypical/ductal cells and transdifferentiation of hepatocytes into cells with mixed

hepatocyte/cholagiocyte identity, which together suggest that absence of CAPZB leads to conversion of a significant number of hepatocytes into bipotent progenitors as previously observed for YAP activation (Yimlamai et al. 2014); 3) restriction of hepatocytes with liver pericentral identity and concomitant extension/increase in periportal zone gene expression, sufficient to reprogram xenobiotic liver metabolism and to profoundly alter sensitivity of CAPZB KO mice to acetaminophen (APAP) toxicity. Importantly, we also obtained experimental evidence that all these phenotypes are genetically dependent on YAP.

## A new genetic system to probe mechanosignalling in vivo

Overall, and considering that phenotypes of similar strength have been observed by inactivation of classical Hippo pathway components, these data validate CAPZB as an important endogenous YAP/TAZ limiting factor in mammals, at least in the liver tissue. Moreover, and perhaps more importantly, the data also indicate that, among the multiple possible functions described for CAPZB and, by extension, for F-actin, regulation of YAP/TAZ represents a primary target, with relevance for regulation of organ homeostasis. This is to our knowledge one of the first examples for a signaling role of F-actin in mammalian living tissues, and will open now the way to using CAPZB knockout as a tool to query in which other tissues this happens. Indeed one clear advantage of this system, compared to other strategies used in the past to modulate tissue mechanics such as fibrosis induction, collagen or lysyl-oxidase (LOX) overexpression, is that it alters F-actin dynamics directly, in a cell-autonomous manner, and without interfering effects on other cells and on other ECM parameters (including chemical composition, ECM architecture and ligand density) that can in principle affect cell signaling pathways independently of ECM mechanical cues.

In the future, we plan to explore the effects of CAPZB inactivation in other epithelial tissues. Epithelia are constantly exposed to mechanical forces as they cover the body surface and line a body cavity. In addition, a body of evidences show that overexpression of YAP as well as mutation in the Hippo pathway components can lead to alteration of basal epidermal cells (H. Zhang, Pasolli, and

Fuchs, 2010; J.-H. Lee et al. 2008; Schlegelmilch et al. 2011). These data indicates that the embryonic skin is a tissue particularly sensitive to alteration of YAP/TAZ levels. Interestingly, Camargo et al. 2007, Vasioukhin et al. 2001 and Kobielak and Fuchs, 2006 identified a role for alpha-catenin as suppressor of YAP/TAZ in the embryonic skin, because genetic ablation of a-catenin in the murine epidermis leads to keratinocyte hyper-proliferation and squamous cell carcinoma development, through hyperactivation of YAP. Alpha-catenin has been shown to act as a mechanosensitive molecule, able to recruit vinculin depending on external forces in a similar manner to talin in focal adhesions (Yonemura et al. 2010); given that alpha-catenin is a YAP/TAZ inhibitor and integrins are required for YAP activity (Bouvard et al. 2001), this may indicate that the relative tension development at adherens junctions (through alpha-catenin) and at focal adhesions (through talin) oppositely regulate each other and/or YAP activity. It will be thus interesting investigates the role of CAPZB in epithelia tissues such as the epidermis, as well as other epithelial tissues (esophagus, intestine, mammary gland, prostate, cervix).

## What are the molecular mechanisms underlying mechanoregulation of YAP/TAZ in vivo?

There is still strong debate regarding how mechanical signals regulate YAP/TAZ activity. Collective evidences from different studies and data (Sansores-Garcia et al. 2011; Wada et al. 2011; Fernandez et al. 2011; Aragona et al. 2013) on one hand suggest an involvement of LATS in mechanotransduction but on the other hand also strongly indicate that this is only a fragment of a more complex picture that we are struggling to understand. We now have a mammalian mouse model that could be interrogated to elucidate the underling molecular mechanism in a physiological setting, or at least to validate in vivo hypotheses from more biochemical / in vitro studies.

We performed an experiment to explore if and how Hippo-mediated regulation of YAP interacts with mechanical regulation mediated by CAPZB in mammalian liver, and found that inactivation of CAPZB can function additively with expression of a Hippo-insensitive TAZ4SA mutant, at least for regulating cell

proliferation. This supports the idea that mechanical cues can enhance the activity of YAP/TAZ even in absence of TAZ phosphorylation mediated by Hippo. In the future, we plan to perform additional experiments to clarify the contribution of the Hippo pathway in the mechanical regulation of YAP/TAZ in vivo, by obtaining compound CAPZB and LATS1/2 mutant cells, and to verify whether or not inactivation of these two YAP-regulatory branches cooperate.

## How does CAPZB regulate mechanoresponsiveness?

As part of the characterization of CAPZB knockout mice, we isolated primary adult fibroblast and found that genetic inactivation of CAPZB is sufficient to rescue YAP/TAZ activity inhibited by plating cells on a soft ECM substratum, similarly to previous data in mammary epithelial cells (Aragona et al. 2013), and extended this observation to small adhesive ECM islands, where only forced YAP expression was shown so far to be able to rescue cell proliferation (Dupont et al. 2011). Thus, and at difference with LATS1/2 knockdown, CAPZB inactivation is sufficient to rescue YAP activity in any mechano-regulated set-up, let this be a soft ECM, a small island or a confluent monolayer (Aragona et al. 2013 and this thesis). The finding that CAPZB inactivation increases YAP/TAZ activity in the liver thus implicitly demonstrates that also the liver tissue corresponds to a low-actin-tension situation. In support of this idea, we also found by AFM on freshly isolated tissue that CAPZB LKO liver is significantly stiffer than the normal organ.

The importance of CAPZB for regulation of cell and tissue mechanoresponsiveness opens two very interesting questions that our knockout could help address in the future:

A) How inactivation of a capping protein, in principle regulating F-actin filament elongation, impact on F-actin contractility?

B) Since CAPZB has a physiological role in restraining F-actin dynamics and YAP/TAZ activity, when/how is CAPZB activity or levels regulated?

To start addressing the first question we initiated a better characterization of the

actin and mechano-related phenotypes of CAPZB KO fibroblasts. These cells display a more disorganized actin, with thinner/shorter stress-fibers, and an increase of peripheral F-actin filaments; in addition, CAPZB KO fibroblasts display an overall increase of vinculin staining at focal adhesions, and a corresponding increase in focal adhesion area. Experiments are now on the way to characterize the KO fibroblast in a soft ECM context, where CAPZB loss more significantly impact on YAP/TAZ activity, to see whether CAPZB inactivation is sufficient to rescue F-actin bundles, formation of mature focal adhesions, and/or contractility. This will be correlated by traction force microscopy (TFM) measurements of fibroblasts on different substrate stiffness to determine the net force applied by WT and KO fibroblasts, in the hypothesis that KO cells retain the ability to develop actomyosin contractility even of soft substrates. If so, this would indicate that CAPZB plays a key role in setting the stiffness threshold above which cells are able to mature focal adhesions, develop contractility, and activate YAP/TAZ (Elosegui-Artola et al. 2016).

## Cues to new mechano-regulated pathways?

Our data indicate that CAPZB regulates the YAP/TAZ mechanosignaling pathway in the liver tissue. In principle, ECM mechanical cues, CAPZB and F-actin dynamics can also regulate a variety of other cellular processes and signaling pathways, including NFKB, MAL/MRTF, beta-catenin, SNAL/TWIST EMT regulators, and KLF2a (see above), but it remains unknown which of these mechanotransduction mechanisms is at work in the liver. Moreover, other mechanotransduction pathways might exist that we haven't yet identified. We thus plan in the future to develop on this idea by closely analyzing gene expression in CAPZB LKO mice. We will compare genes regulated in CAPZB LKO livers, genes regulated in CAPZB+YAP LKO livers, and genes regulated by transgenic YAP activation to highlight YAP-dependent and YAP-independent mechano-regulated genes. GSEA analyses of these genes might suggest new molecular pathways sensible to mechanical alterations in vivo.

# Part II

# Rationale

Recent genetic evidences indicate a key role of the Hippo pathway in the regulation of cardiomyocytes proliferation, and inactivation of the Hippo pathway or activation of its downstream effectors YAP/TAZ improves cardiac regeneration in the adult heart (Fig. 10A, Leach et al. 2017; Matsuda et al. 2016; W.-F. Cai et al. 2016; Heallen et al. 2013; Xin et al. 2013). The identification of Hippo inhibitory (and YAP activatory) small-molecule compounds represent a so far unexplored possibility to pharmacologically sustain heart tissue regeneration in patients suffering from heart attack. Aragona et al. 2013 developed a robust bioassay specifically dependent on LATS1/2 activity to distinguish between LATS-dependent and LATS-independent YAP regulatory mechanisms: reconstituting MDA-MB-231 breast cancer cells, that are genetically null for NF2, with an expression plasmid encoding for NF2 is sufficient to strongly inhibit YAP/TAZ transcriptional activity (followed by 8xGTCII-Lux, a YAP/TAZ luciferase reporter), and this entirely depends on LATS1/2 kinases because knockdown of LATS1/2 makes YAP/TAZ activity insensitive to NF2 expression (Fig. 10B). We thus decided to take advantage of this robust LATS-dependent bioassay to perform a screening aimed at identifying small-molecule compounds able to overcome YAP/TAZ inhibition mediated by the Hippo pathway.

# Results

## FDA-approved chemical-library screening

To identify negative modulators of the Hippo pathway, we performed a luciferase-based screening on FDA-approved chemical library compounds (See Materials and Methods) based on activation of Hippo signaling by NF2. Targeting the Hippo pathway has several advantages: 1) available genetic evidence already indicates that Hippo is a desirable target in the heart tissue, compared to other YAP/TAZ regulatory pathways; 2) kinases are more easily targetable with small molecules than protein-protein interactions; 3) the Hippo pathway consists of a series of well-known players, enabling an easier identification of the molecular targets of the isolated compounds, and thus an easier assessment of their efficacy in preclinical and potentially in clinical settings (by looking for example at YAP phosphorylation, or at MST1/2 and LATS1/2 autophosphorylation sites); 3) the screening is performed in a re-gain-of-activity setting, which usually leads to much less false positive hits compared to a simple gain-of-activity screening.

To activate the Hippo pathway specifically, we transiently expressed NF2/Merlin in cells that bear a null mutation in the NF2 locus, leading to a strong and reliable inhibition of YAP activity. The inhibition of YAP mediated by NF2 overexpression is completely dependent on LATS1/2 activity and on YAP phosphorylation (Aragona et al. 2013). We optimized the assay for 96-well and 384-well plate screening, and checked in this set-up if LATS1/2 siRNA transfection was able to rescue YAP/TAZ activity, as previously seen in similar experiments performed in larger vessels (not shown). This was important also to test whether this assay would enable us to capture small-molecules able to rescue YAP/TAZ activity after it had been inhibited by NF2, at difference with previous experiments in which cells were transfected with siRNA for LATS1/2 the day before receiving NF2 DNA transfection: transfection of LATS1/2 siRNAs after NF2 DNA transfection, and for as little as 24 hours, was sufficient to see a significant rescue of YAP activity (Fig. 10B).

We then searched for compounds able to rescue YAP/TAZ activity by using a drug-repurposing library of FDA-approved chemicals (in collaboration with Miguel Mano and Giannino Del Sal at the ICGEB of Trieste), with the idea of obtaining compounds already tested in phase I trials and with acceptable pharmacokinetics, and thus potentially available for a more rapid application to treat heart disease. Out of this screening, we isolated two molecules that can prevent inhibition of YAP/TAZ by NF2 (Fig. 10C).

## Hits validation

To initially revalidate the screening's hits we re-ordered the compounds and repeated the experiment by using our classical protocol for luciferase assay (See Materials and Methods). Also in this set-up, both the compounds efficiently rescue YAP/TAZ activity at different doses of NF2-overexpression (Fig. 10D). Moreover, looking at basal effects of the compounds on YAP/TAZ activity, i.e. in parental MDA-MB-231 that did not experience NF2-mediated YAP/TAZ inhibition, we found that both the small molecules alone had no effect. We thus confirmed a specific role of these small-molecules compounds in regulating YAP/TAZ through the Hippo pathway.

In order to exclude trivial non-specific effects of the compounds in the screening set-up, we checked for equal expression of exogenously-expressed NF2 protein in MDA-MB-231 cells. Transfection of 10ng of NF2/Merlin is sufficient to inhibit YAP/TAZ activity, as measure by luciferase activity of the 8xGTIIC YAP/TAZ luciferase reporter; we found that both the compounds did not affect NF2 mRNA expression and NF2 protein production or stability (Fig. 11A).

We then better validated the compounds by performing different assays to monitor YAP/TAZ activity at different levels. A read-out of YAP activity regulated by NF2 is its nuclear exclusion and cytoplasmic accumulation (Zhao et al. 2012). We compared YAP localization by immunofluorescent staining of control vs. NF2-expressing cells (i.e. positive for co-transfected NF2 and Cherry plasmids), and found that one compound efficiently rescues NF2-induced YAP nuclear exclusion (Fig. 11B). We thus continued the characterization of this

compound further.

NF2 function as activator of LATS1/2 kinases, the core component of the Hippo pathway, whose target phosphorylation sites on YAP are well known (Zhao et al. 2010). We monitored phosphorylation of the two most relevant LATS1/2-dependent phosphorylation sites (S127 and S381) of YAP, and found that the compound decreases phosphorylation of YAP at both sites (Fig. 11C). This result suggests that one of the identified compounds efficiently modulate YAP/TAZ activity and phosphorylation, indicating that it works at the level or upstream of LATS1/2.

Future work will be required to determine whether these compounds have specific effects, and if they can be used for regenerative medicine applications.

# Discussion

Several evidences suggest that YAP/TAZ are not necessary for the physiological maintenance of adult tissues, but are absolutely required to heal damaged tissues (Reviewd in Juan and Hong 2016; Piccolo, Dupont, and Cordenonsi 2014; Y. Wang, Yu, and Yu 2017). This has been linked to the ability of YAP and TAZ to promote stem-cell self- renewal and cell proliferation in several cellular systems, and perhaps to the recently described ability of YAP/TAZ to reprogram terminally differentiated cells to their specific stem cell progenitors (Panciera et al. 2016). A point in case is the recent finding that inactivation of the Hippo pathway by genetic deletion of LATS1/2 or Salvador (Sav/WW45), or overexpression of activated YAP, permit a regenerative response in adult mouse models of myocardial infarction (See Introduction). These data indicate that a transient inhibition of Hippo, and thus a transient upregulation of YAP/TAZ activity, might represent a novel approach to facilitate heart regeneration in patients suffering of ischemic heart damage, and maybe for regenerative medicine approaches more in general.

We here describe a new pipeline to isolate and characterize new compounds able to selectively inhibit the Hippo pathway that could be used to transiently reactivate YAP activity and foster endogenous tissue regeneration ability during the appropriate therapeutic time window. By using a library of 640 FDA-approved molecules (Sorrentino et al. 2017) with a strong bias for drugs aimed at cancer treatment and reduced chemical variability, we could isolate two different compounds able to rescue YAP/TAZ activity in cells: this indicates a wide potential for isolating new small-molecule inhibitors by using alternative small-molecule libraries in the future.

Moreover, this proof-of-principle screening already provides at two initial lead compounds to further develop our assays, and to possibly obtain modified compounds of increased potency/specificity to be tested in vivo. It will be important to understand how these molecules work, and whether they have some

specificity towards Hippo signaling (for example, in case we isolate a potential LATS1/2 inhibitor, how much gene expression regulated by the compound matches gene expression regulated by genetic ablation of LATS1/2? How many other kinases can be targeted by the same compound?). Ideally, these compounds should be then tested for the ability to foster cardiomyocytes proliferation in vitro, in vivo in the normal heart, and then in mouse models of cardiac injury such as surgical resection of left ventricle (apex resection) or coronary artery occlusion (myocardial infarction), to test whether these compounds are beneficial in this context.

# Materials and methods

- Mice

We obtained the Capzbtm1a (EUCOMM) Wtsi line from the EUCOMM/EMMA repository. Yap fl/fl and R26-LSL-LacZ mice were from Stefano Piccolo Lab. Animal experiments were performed adhering to our institutional guidelines as approved by OPBA and authorized by the Ministry of Health.

For genotyping, mice were anesthetized using isoflurane and tail tip was removed and isolated. The genomic DNA from tip tail was extracted with NaOH at 95°C for 30 minutes. Tris-base pH8 was then used to neutralize the alkaline lysis. The stock DNA was diluted in clean water and directly used for PCR or stored a +4°C. Genotyping were performed using the recommended set of primers.

- Isolation of mouse primary fibroblasts.

For Mouse Adult Fibroblast (MAF) isolation, mice were anesthetized with intraperitoneal injection of Avertin and tail tip was carefully sterilized with ethanol and cutted. After sampling, tail was cauterized and tissue biopsy transferred in a clean petri dish. Under biological hood, the biopsy was minced with scalpel and washed three times with HBSS plus 2x Pen/Strep. Chopped tissues were then digested with 2000 U/ml of collagenase (C9891 Sigma) in HBSS for 30 minute at 37°C. Digested chopped tissue was centrifuged and a second step of digestion in 0,05 % of trypsin for 20 minutes a 37°C was performed. Finally completely digested chopped tissue was plated in T25 with DMEM (Gibco-Life Technologies) 20% FBS, 1% Gln and Pen/Strep. The day after, culture medium was refreshed in order to eliminate cells and debris that didn't attach to flask.

- Mouse liver collection

For liver sampling, mice were euthanized and abdominal contents exposed by

performing an excision at the midline of mice body. In order to reduce blood contaminants, trans cardiac perfusion with cold PBS was performed. The excision was extended toward sternum to expose the heart inside the rib cage. A 29-gauge needle connected to a syringe primed with cold PBS 1x was insert to heart and perfusion started. Around 10-20 ml of cold PBS 1x were used for perfusion and then liver tissue was removed and placed in a clean petri dish with PBS on ice. Immediately the liver was divided in different parts and snap-frozen in liquid nitrogen or embedded in OCT and then stored at -80°.

- Antibody, western blotting and immunofluorescence

Antibodies were CPbeta (sc-81804), YAP/TAZ (sc101199), phospho S127 YAP (CST4911), phospho S381 YAP (#13619), B-catenin (sc-7973), GAPDH (Millipore MAB374).

For immunofluorescence, cells were fixed in 4% PFA for 10 minutes. After wash, cells were permeabilized with PBS-Triton 0,5% for 20 minutes following by blocking buffer (PBS- Triton 0,1%, 2% goat serum) incubation for 1 hour. The primary antibody was incubated overnight at +4° in humid chamber. The following day cells were wash in PBS-Triton 0,1% and incubated with Alexa fluor - conjugated secondary antibody for 1 hour at room temperature. Finally, cells were washed and mounted with ProLong™ Gold Antifade Mountant with DAPI (P36935 Thermofisher). Images were acquired with a Leica SP5 confocal microscope equipped with CCD camera or with Leica DM5000B microscope. Images were analysed using Fiji free software.

For immunofluorescences of liver slices, tissues were dissect, embedded in OCT and frozen at -20 to -80 °C. Embedded tissue was sectioned at 5-7 µm thick with Leica CM1950 cryostat and slices were put on glass coverslip (VWR), dried at room temperature for 30 minutes and stored at -80°C. Tissue slices were hydrated in PBS, fixed in 4% PFA for 15 minutes and washed three times in PBS. Permeabilization of tissue slice was performed in PBS-Triton 1% for 20 minutes. After three washes in PBS-Triton 0,5%, tissue slices were incubated in blocking buffer (10% goat serum in PBS-Triton 0,5%) at room temperature for 1 hour.

Incubation with primary antibody were performed over night at +4°C in humid chamber. Secondary antibody incubation were performed at room temperature for 1 hour and mounted with ProLong™ Gold Antifade Mountant with DAPI (P36935 Thermofisher). For EdU labelling, mice were injected with 12,5 mg/kg of EdU, 15 hours before sacrifice and staining was performed according to Click-iT™ EdU Alexa Fluor™ 488 Imaging Kit instruction protocol. TUNEL staining was performed according to DeadEnd™ Fluorometric TUNEL System, Promega.

- Cells, plasmid and reagents

Primary Mouse Adult Fibroblast (MAF) were cultured in DMEM (GIBCO, Life Technologies) supplemented with 20% FBS, 1% glutamine and antibiotics (Pen/Strep). Micropatterned glass slides were from Cytoo and for fibronectin-coated hydrogels see Dupont et al. 2011. For hydrogels, 5.000 cells/cm2 were seeded in drop on top of for fibronectin-coated hydrogels; after attachment, the wells containing the hydrogels were filled with medium. For assays on large square (Cytoo), 600.000 cells were plated in a 35mm dish containing a single Cytoo glass slide, and non-adherent cells were washed with medium after 2 hr. The FDA-approved library (Screen-Well FDA-Approved Drug Library) used for the screening was composed of 640 chemical compounds dissolved at 10 mM in DMSO, as in Sorrentino et al. 2017.

- Luciferase assays

For transient transfections, cells were plated in 24-well format and luciferase reporter plasmids were transfected with CMV-lacZ to normalize for transfection efficiency based on CPRG (Merck) colorimetric assay, together with pGl3-CMV-renilla, the YAP/TAZ luciferase reporter 8XGTIIC-lux plasmid (Addgene 34615) and with plasmids encoding for the indicated proteins. Transfected DNA content was kept uniform by using pKS Bluescript. Cells were harvested in luc lysis buffer (25 mM Tris pH 7.8, 2.5 mM EDTA, 10% glycerol, 1% NP-40). Luciferase activity was determined in a Tecan plate luminometer with freshly reconstituted assay reagent (0.5 mM D-Luciferin, 20 mM tricine, 1 mM $(MgCO_3)_4Mg(OH)_2$, 2.7 mM $MgSO_4$, 0.1 mM EDTA, 33 mM DTT, 0.27 mM CoA, 0.53 mM ATP).

Each sample was transfected at least in two biological duplicates in each experiment to determine the experimental variability; each experiment was repeated independently with consistent results.

- Serum ALT measurement

Alanine Aminotransferase enzymatic activity was measured in mice serum. Mice were anesthetized with Avertin, mixture of tribromoethyl alcohol #(T48402 Sigma) and 2-methyl-2-butanol (#240486 Sigma), and bloods were collected from retro-orbital sinus. Blood were allowed to clot at room temperature for at least 1 hour and then centrifugated for 10 minutes in a cold centrifuge in order to separate the two phases of the blood. The supernatant (serum) was immediately transferred in a clean tube and stored at -80° until measure. Serum ALT measurements were performed using ALT Activity Assay (MAK052 Sigma).

- Hydrodynamic tail vein

Hydrodynamic tail-vein technics was used to delivery exogenous DNAs into hepatocytes. Plasmid DNA were suspended in sterile Ringer's solution in a volume equal to 10% of the body weight and injected in 8-10 seconds via the tail vein of 4-6-wk-old mice (mice weight around 18-22g). The amount of injected DNA was 50 μg of total transposon plasmids together with 10 μg of PB transposase plasmids. No statistical method was used to predetermine sample size.

- APAP-induced hepatotoxicity

Acute liver hepatotoxicity was induced in mice by intraperitoneal injection of 350mg/kg of acetaminophen (#A7085 Sigma-Aldrich) in sterile PBS. Serum for ALT activity assay was collected 8 hours after APAP-injection and 24 hours after APAP-injection liver was collected for histopatological analysis. The number of animals per group used in this study was 5 animals for each genotype.

- RNA extraction and Real-time PCR

Total liver RNA extractions were performed using guanidinium thiocyanate

/phenol/chloroform protocol extraction, starting from 5-10 mg of liver tissue snap-freezing in liquid nitrogen. Contaminant DNA was removed by RNase free-DNase (Ambion-Lifetechnologies) digestion. For cells, total RNA extraction was performed using RNeasy kit (Quiagen) and contaminant DNA was removed by RNase-Free DNase Set (Qiagen). Retro-transcription of total RNA to cDNA was carried out with dT-primed M-MLV Reverse Trascriptase (LifeTechnologies).

Real-time qPCR analyses were carried out with triplicate samplings of each sample cDNA on a on QuantStudio 6 Flex Real-Time PCR System (Lifetechnologies) with FastStart SYBR Green Master Mix (Roche). Expression levels are calculated relative to GAPDH

- RNA sequencing protocol

Liver RNA-sequencing was carried out in CRIBI facility at University of Padova. Library preparation was performed starting from liver RNAs using TruSeq Stranded mRNA Library Prep Kit (Illumina) according to the manufacturer's protocol. The pooled libraries were sequenced with NextSeq 500 platform (Illumina).

- Statistical analysis

Statistical analyses were performed using Prism software (GraphPad software). Mean values and standard deviations (SD) are shown in graphs that were generated from several repeats of biological experiments, unless otherwise indicated. In some graph, resulted value from each mouse replicates are shown.

# Figures

## Figure 1. Regulation of YAP/TAZ and overgrowth phenotype caused by their activation

**A.** Hippo mutant phenotype in flies and mice showing over-growth phenotypes. From Halder and Johnson, 2011.

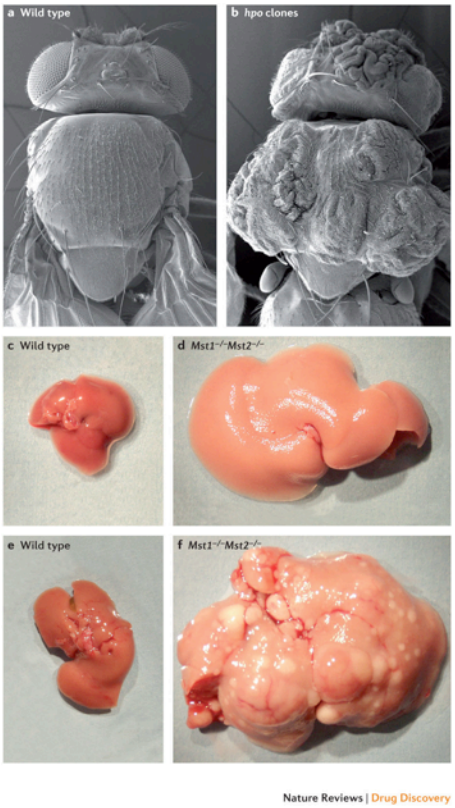
**B.** YAP/TAZ activity is regulated by multiple inputs. YAP/TAZ can shuttle between the nucleus, where they bind TEAD transcription factors on DNA to regulate gene expression, and the cytoplasm. Several different pathways and cellular features can modulate the activity of YAP/TAZ. These include the Hippo pathway, that regulates YAP/TAZ through phosphorylation, baso-lateral polarity complexes and cell–cell adhesion molecules in epithelia, G-protein coupled receptor (GPCR) signalling throught small G proteins (Gs and Gq/11), WNT signalling through the APC/Axin destruction complex, but also autophagy, metabolic pathways and energy stress (here depicted by one glucose and one mevalonate molecule). Inhibitory inputs and proteins are indicated in red; activatory inputs in green. From Dupont 2016.

**B.** The scheme indicates a cell in which mechanical cues sustain the increase of actin polymerization and thus the development of high levels of actomyosin contractility, which in turn sustain YAP/ TAZ nuclear transcription. Cells probe external resisting forces by pulling on focal adhesions, thanks to the contractile activity of non-muscle myosins (NMII). Formation of focal adhesions and of contractile actomyosin bun- dles mutually sustain each other, either by direct effects on focal adhesion com- ponents (recruitment of vinculin and talin), or through RHO/ROCK (RHO kinase)/MLCK (myosin light chain kinase) signalling. F-actin capping/severing proteins (CAPZB, but also Cofilin and Gelsolin) are key elements opposing the formation of actomyosin bundles. Development of high levels of F-actin contractility then promotes the activity of YAP/TAZ: available data indicate that this control possibly entails LATS1/ 2 activation, promotion of YAP/TAZ activity in a LATS-independent manner (left), and promotion of YAP/TAZ activity through RHO signalling, which is also a

LATS- independent event (right). The molecular mediators of these regulatory interactions are still unknown (factor X, kinase X). This is the proposed mechanism connecting YAP/TAZ activity to ECM stiffness and cell geometry sensing, from Dupont, 2015.

# FIGURE 1

A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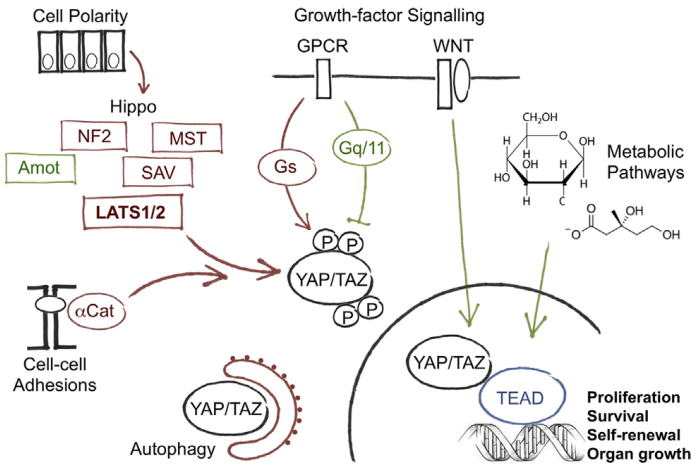
Nature Reviews | Drug Discovery

B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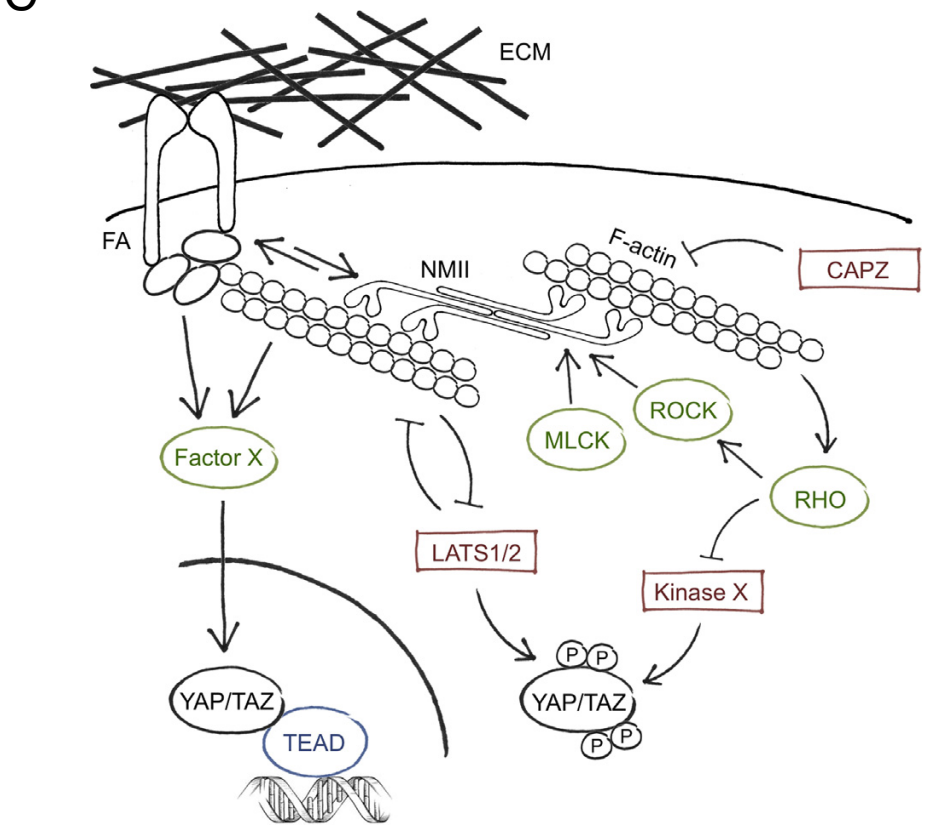
Self-renewal
Organ growth

C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FA
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F-actin
CAPZ
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MLCK
ROCK
RHO
LATS1/2
Kinase X
YAP/TAZ
TEAD
YAP/TAZ

## Figure 2. Derivation and validation of CAPZB floxed allele

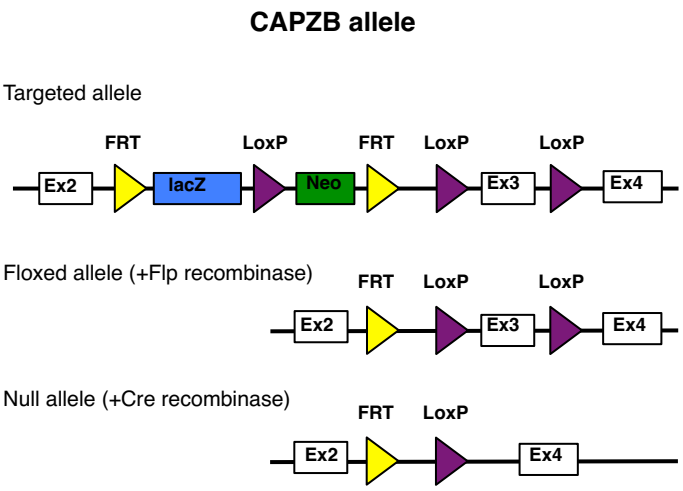
**A.** Schematic representation of strategy to generate CAPZB floxed mice. The targeted allele was crossed to CMV-Flp mice to delete the lacZ and Neo cassettes, originating the Floxed allele. Crossing of the floxed allele with tissue-specific Cre transgenic cause a frame shift mutation with premature STOP codon at the level of exon 3 (Null allele).

**B.** Immunoblot of wild-type (WT) and infected mouse fibroblast with adeno-control (Adeno-Co) and adeno-CRE virus. CRE recombinase expression efficiently recombined CAPZB allele as demonstrated by complete depletion of CAPZB protein. Laminin-b was used as loading control.

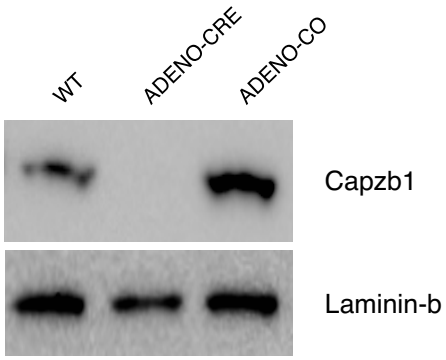
**C.** Schematic representation of sarcomere structure highlighting the localization of CAPZB on Z-disc. Representative picture of primary mouse cardiomyocytes infected with adeno-CRE virus (positive for CRE staining), displaying a disruption of sarcomeric a-actinin staining.

# FIGURE 2

A

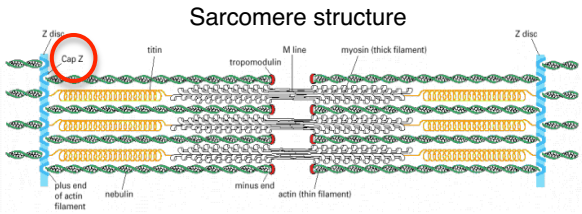


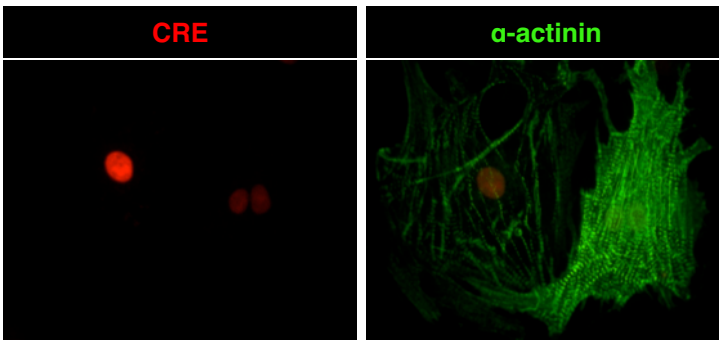

B




Primary mouse adult fibroblasts

C




Primary mouse adult cardiomyocytes

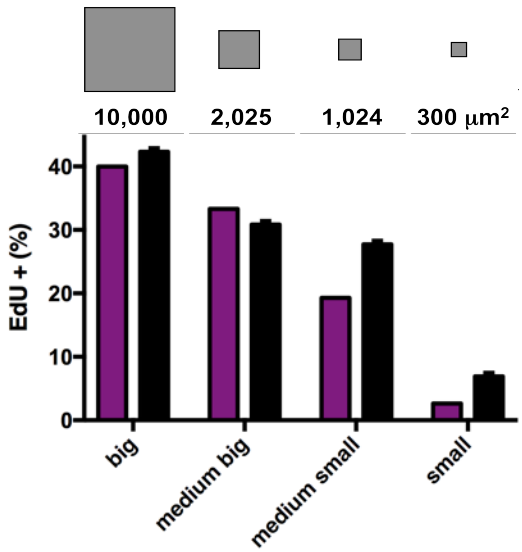
## Figure 3. CAPZB KO changes the cell's perception of ECM in fibroblasts.

**A.** Percentage of EdU positive cells and YAP localization (nuclear localization is a read-out of YAP activation) in fibroblasts isolated from mouse carrying homozygous floxed CAPZB allele and infected with adeno-co (WT) and adeno-Cre (KO). Fibroblasts were plated on microprinted ECM-coated islands of variable adhesive area (big = 10000um$^2$, medium big = 2025 um$^2$, medium small = 1024 um$^2$ small = 300 um$^2$). Representative images of WT and KO fibroblasts stained for EdU and YAP are shown.

**B.** Real Time PCR in primary mouse fibroblasts. WT are non-infected control cells, Adeno-co fibroblasts were infected with an empty adenovirus, Adeno-CRE fibroblasts were infected with a CRE expression adenovirus. CRE recombinase production efficiently recombined the CAPZB floxed alleles in primary mouse fibroblasts (showed in Fig. 2B). Fibroblasts were cultured on ECM-coated acrylamide hydrogels of variable stiffness (expressed as Young's Modulus, in KPascal).

# FIGURE 3

A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1,024
300 μm²
EdU + (%)
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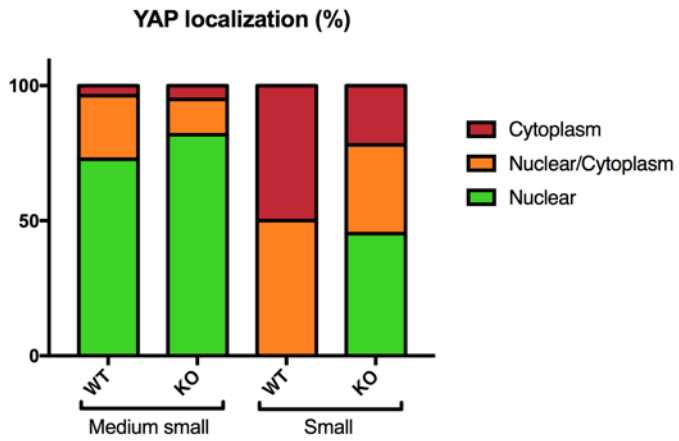
30
20
10
0
big
medium big
medium small
small
WT
KO


YAP localization (%)
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0
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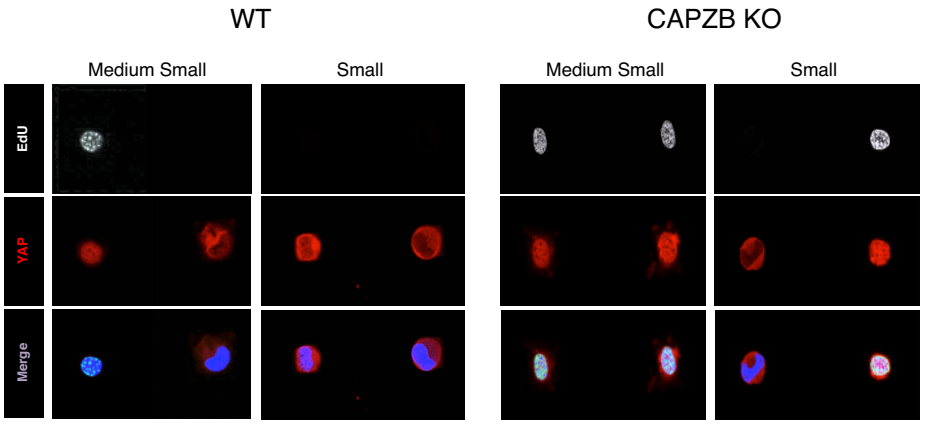
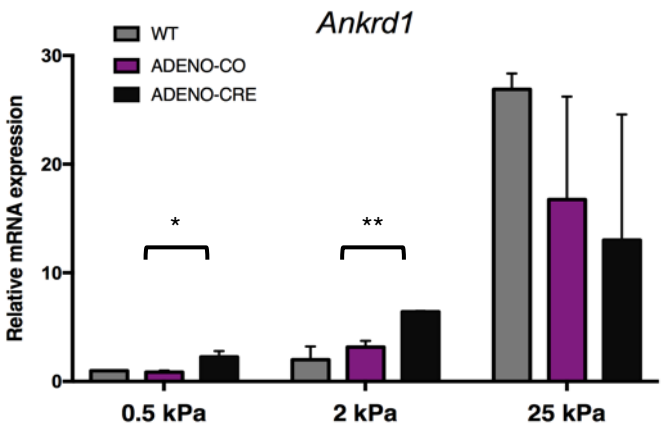
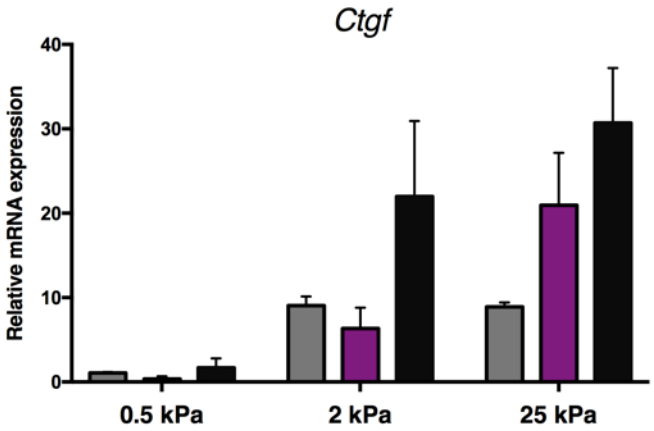
KO
WT
KO
Medium small
Small
Cytoplasm
Nuclear/Cytoplasm
Nuclear


WT
CAPZB KO
Medium Small
Small
Medium Small
Small
EdU
YAP
Merge

B


Ankrd1
WT
ADENO-CO
ADENO-CRE
Relative mRNA expression
30
20
10
0
*
**
0.5 kPa
2 kPa
25 kPa


Ctgf
Relative mRNA expression
40
30
20
10
0
0.5 kPa
2 kPa
25 kPa

## Figure 4. Liver-specific inactivation of CAPZB causes liver overgrowth and increases of hepatocytes proliferation

**A.** Gross appearance of mouse liver 1 month after tamoxifen-mediated recombination. On the right, quantification of liver/body weight ratio in WT and KO mice. n = 9, **** $p < 0,0001$.

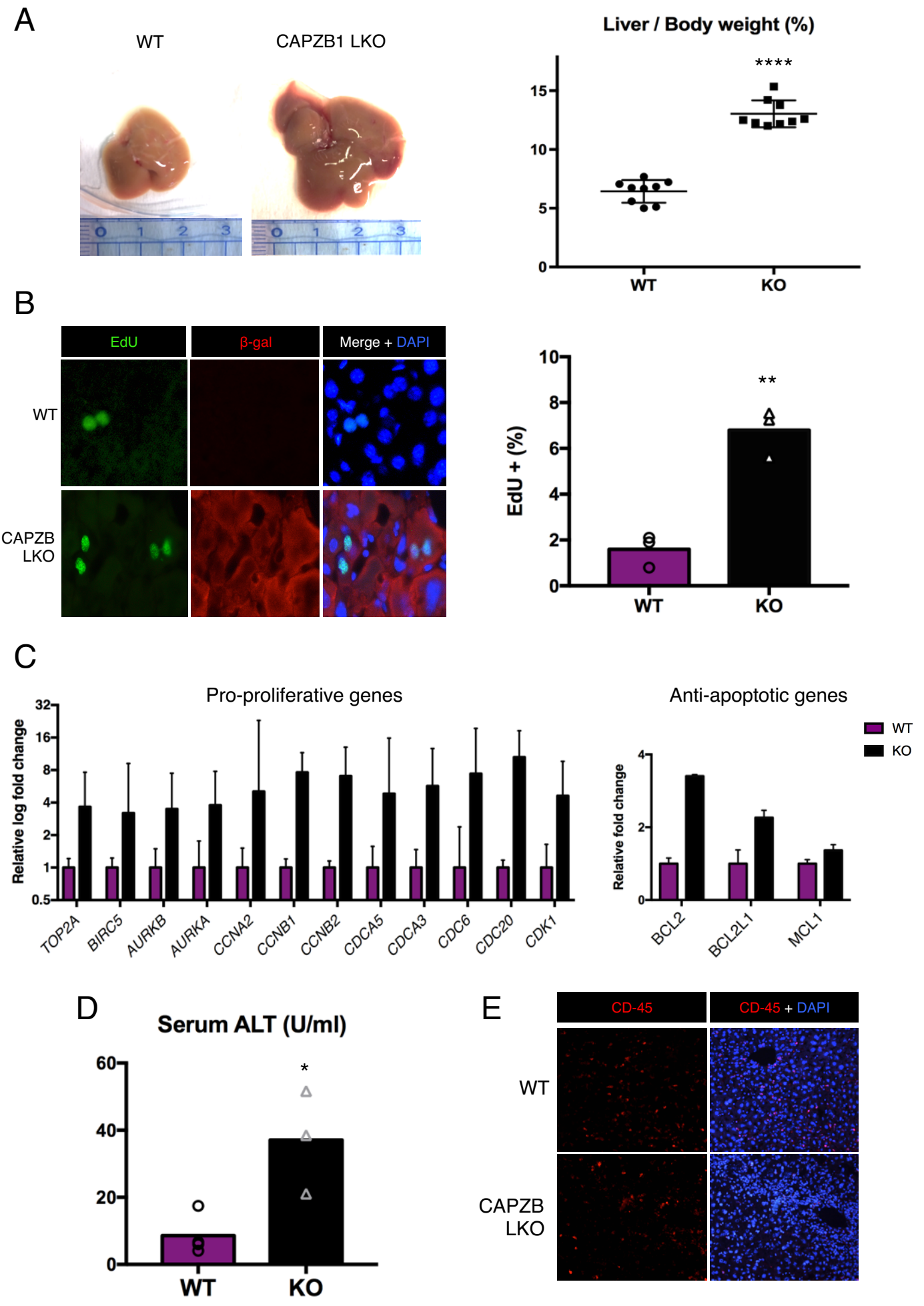
**B.** Representative EdU staining and quantification. β-galactosidase staining was used to follow recombined hepatocytes thanks to the co-recombined ROSA26 allele. Single mouse results are plotted. ** $p < 0.01$

**C.** Relative mRNA expression from liver RNAseq data of a group of pro-proliferative genes (GO-cell cycle progression) and anti-apoptotic genes (Jourdan et al. 2009). Data are plotted as absolute fold change; y-axis is logarithmic (n=4), $p < 0,05$.

**D.** Serum ALT measure expressed in units / ml. Serum was isolated from mouse blood collected from retro-orbital sinus, 1 month after tamoxifen-induced recombination (just before sacrifice). Single mouse results are plotted. * $p < 0,05$.

**E.** Representative immunofluorescence staining for CD-45 in liver parenchyma of WT and KO mice. CD-45 is used as marker of leucocytes cells.

# FIGURE 4


A
WT
CAPZB1 LKO
Liver / Body weight (%)
****
15
10
5
0
WT
KO
B
EdU
β-gal
Merge + DAPI
WT
CAPZB LKO
EdU + (%)
10
8
6
4
2
0
**
WT
KO
C
Pro-proliferative genes
Anti-apoptotic genes
WT
KO
Relative log fold change
32
16
8
4
2
1
0.5
TOP2A
BIRC5
AURKB
AURKA
CCNA2
CCNB1
CCNB2
CDCA5
CDCA3
CDC6
CDC20
CDK1
Relative fold change
4
2
0
BCL2
BCL2L1
MCL1
D
Serum ALT (U/ml)
60
40
20
0
*
WT
KO
E
CD-45
CD-45 + DAPI
WT
CAPZB LKO

## Figure 5. Deletion of CAPZB leads to activation of YAP/TAZ in adult hepatocytes

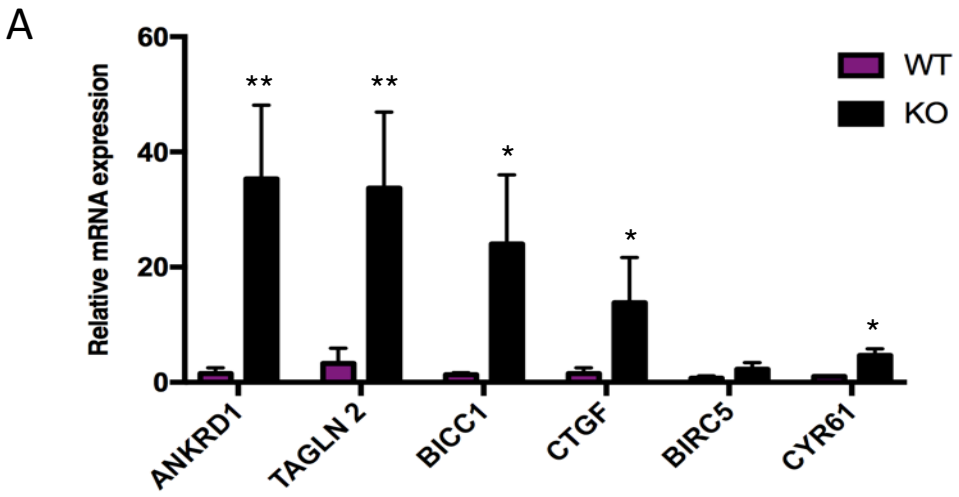
**A.** Real-time PCR in WT and KO livers of YAP/TAZ target genes plotted as relative mRNA expression to GAPDH (n=6) mean, SD. * $p < 0.05$; ** $p < 0.01$.

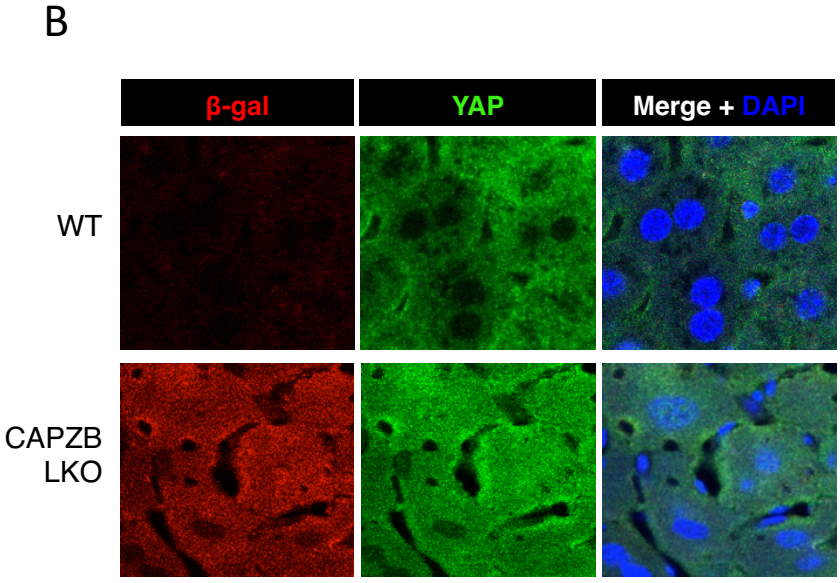
**B.** Immunofluorence of WT and KO livers stained for β-galactosidase (β-gal) to follow recombined hepatocytes and for YAP (nuclear localization is a read-out of YAP activation). On the right, number of hepatocytes with nuclear YAP (expressed as percentage relative to the total of counted cells) in WT and KO liver. n = 3; mean, SD. * $p < 0.05$

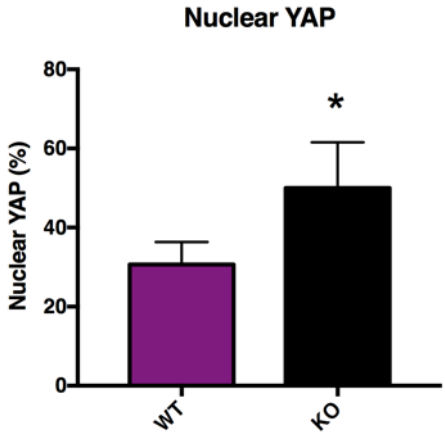
**C.** Immunoblot of WT and KO whole liver protein extracts for CAPZB, YAP and TAZ. B-CATENIN was used as loading control. KO mice showed reduction of CAPZB protein, confirming efficiently recombination of floxed allele (a little amount of CAPZB protein is still visible because of not-recombined cell types present in whole liver protein extracts such as fibroblast, Kupffer cells; with no active albumin-cre promoter). On the bottom, quantification of protein bands, using ImageJ software, highlights accumulation of total TAZ protein level in CAPZB LKO.

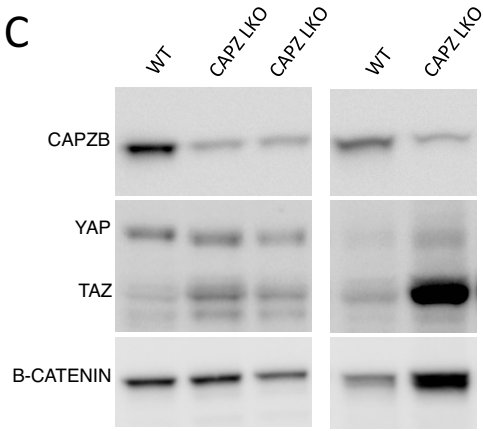
**D.** Representative immunofluorescence of liver stained for β-galactosidase (β-gal), showing an example of a not recombined patch in KO liver parenchyma and YAP. Overall YAP signal decreases in not-recombined area (β-gal negative).

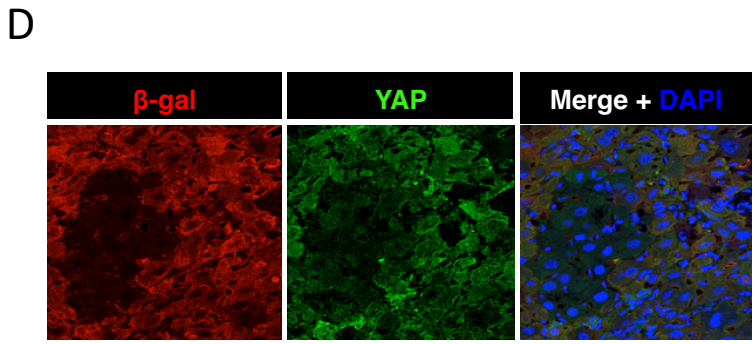
# FIGURE 5

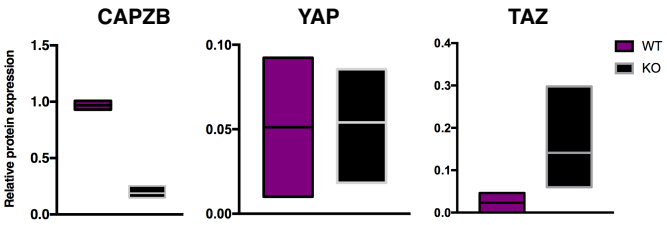

A
Relative mRNA expression
60
40
20
0
**
**
*
*
*
WT
KO
ANKRD1
TAGLN 2
BICC1
CTGF
BIRC5
CYR61


B
β-gal
YAP
Merge + DAPI
WT
CAPZB
LKO


Nuclear YAP
Nuclear YAP (%)
80
60
40
20
0
*
WT
KO


C
WT
CAPZ LKO
CAPZ LKO
WT
CAPZ LKO
CAPZB
YAP
TAZ
B-CATENIN


D
β-gal
YAP
Merge + DAPI


CAPZB
YAP
TAZ
Relative protein expression
1.5
1.0
0.5
0.0
0.10
0.05
0.00
0.4
0.3
0.2
0.1
0.0
WT
KO

## Figure 6. CAPZB loss induces expansion of oval/atypical cells, partially derived from mature hepatocytes

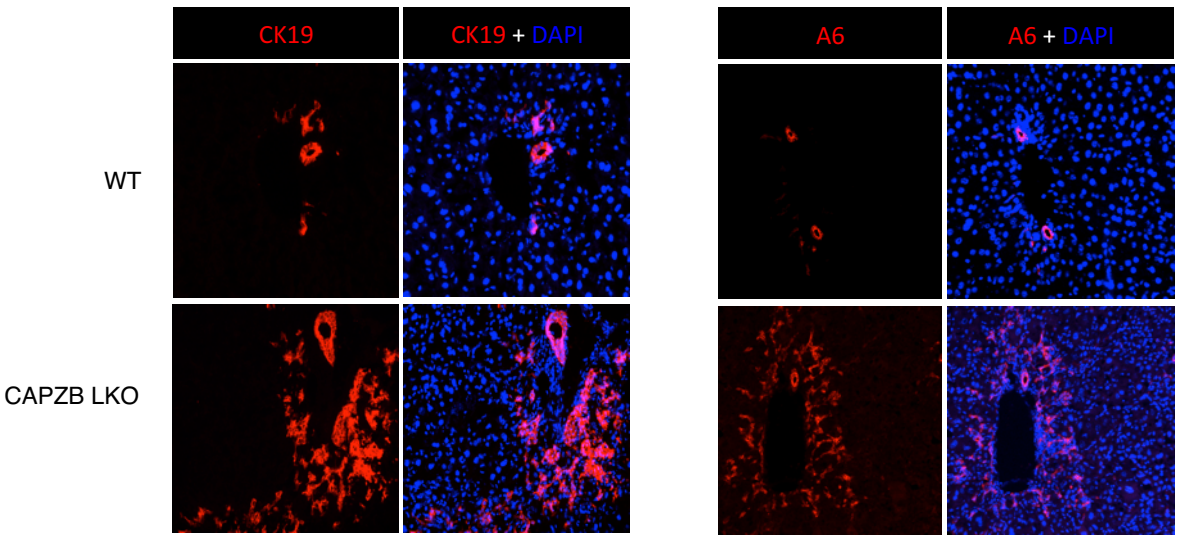
**A.** Immunofluorescence of WT and KO liver stained for CK-19 and A6, established marker of oval cells. Pictures were taken by selecting periportal areas of the liver lobules.

**B.** Relative mRNA expression from liver RNAseq data of cholangiocyte / oval cell marker genes expressed in fold change relative to WT expression n=4, p <0,005.

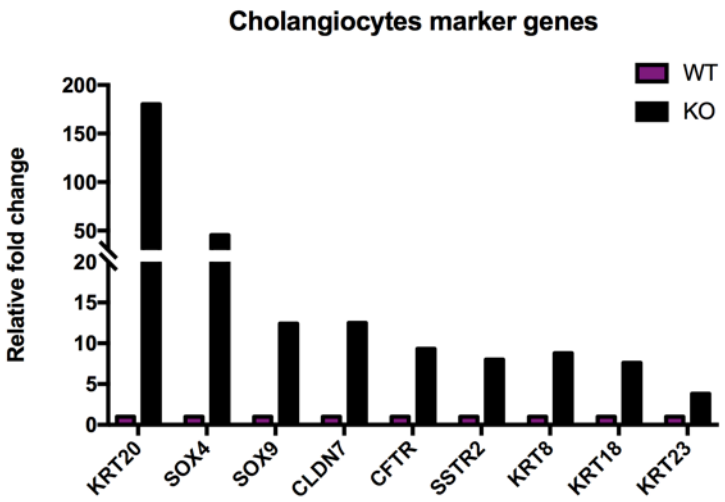
**C.** Representative immunofluorescence of KO liver stained for CK-19 and HNF4alpha to highlight double positive cells (arrowed), which are never found in WT liver. Count of cells stained for the respective markers expressed in percentage of total; pictures were taken by selecting periportal areas of the liver lobules.

# FIGURE 6

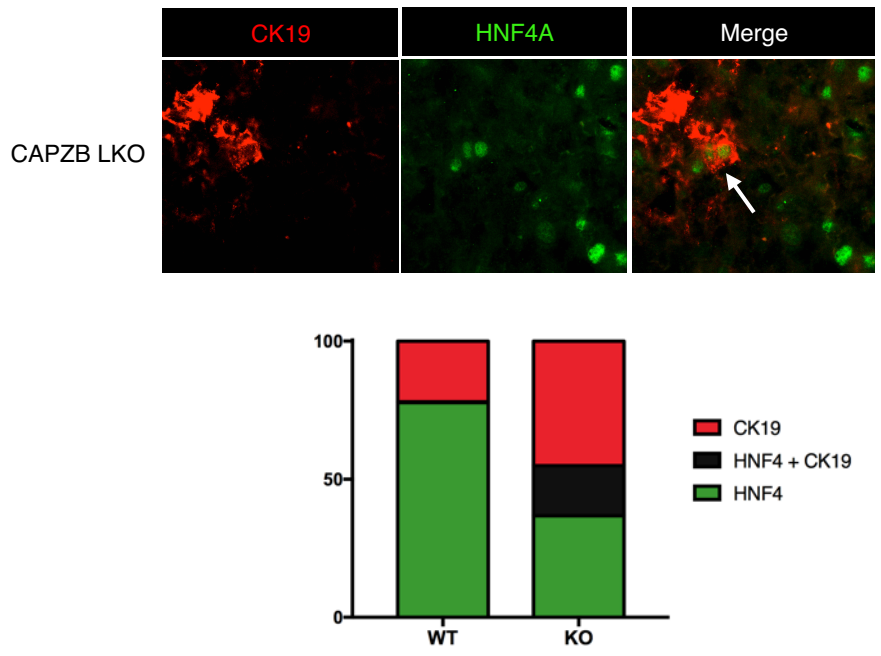
A


CK19
CK19 + DAPI
A6
A6 + DAPI
WT
CAPZB LKO

B


Cholangiocytes marker genes
WT
KO
Relative fold change
200
150
100
50
20
15
10
5
0
KRT20
SOX4
SOX9
CLDN7
CFTR
SSTR2
KRT8
KRT18
KRT23

C


CK19
HNF4A
Merge
CAPZB LKO
100
50
0
WT
KO
CK19
HNF4 + CK19
HNF4

## Figure 7. CAPZB is a gatekeeper of hepatocytes zonation and its inactivation reprogram xenobiotic liver metabolism

**A.** Immunofluorescence for GS (glutamine synthase) of WT and CAPZB LKO mice liver tissue. GS showed a specific staining around central vein (CV) of liver parenchyma.

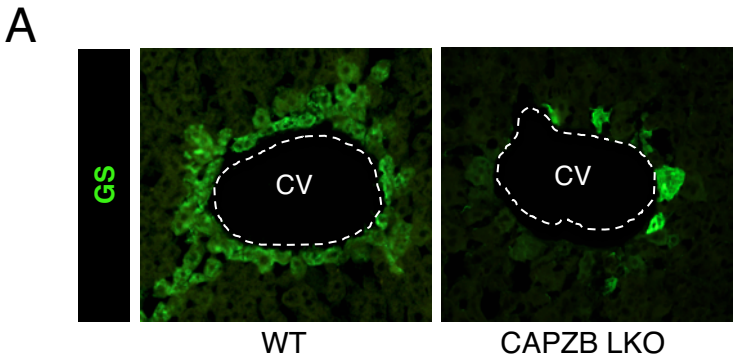
**B.** Heat-map of RNA-seq data; unsupervised clustering was applied with ClustVis web tool (n=4). Schematic representation of liver parenchyma highlights periportal (PP) and pericentral (CV) area and relative marker genes differentially expressed between WT and KO liver (Braeuning et al. 2006).

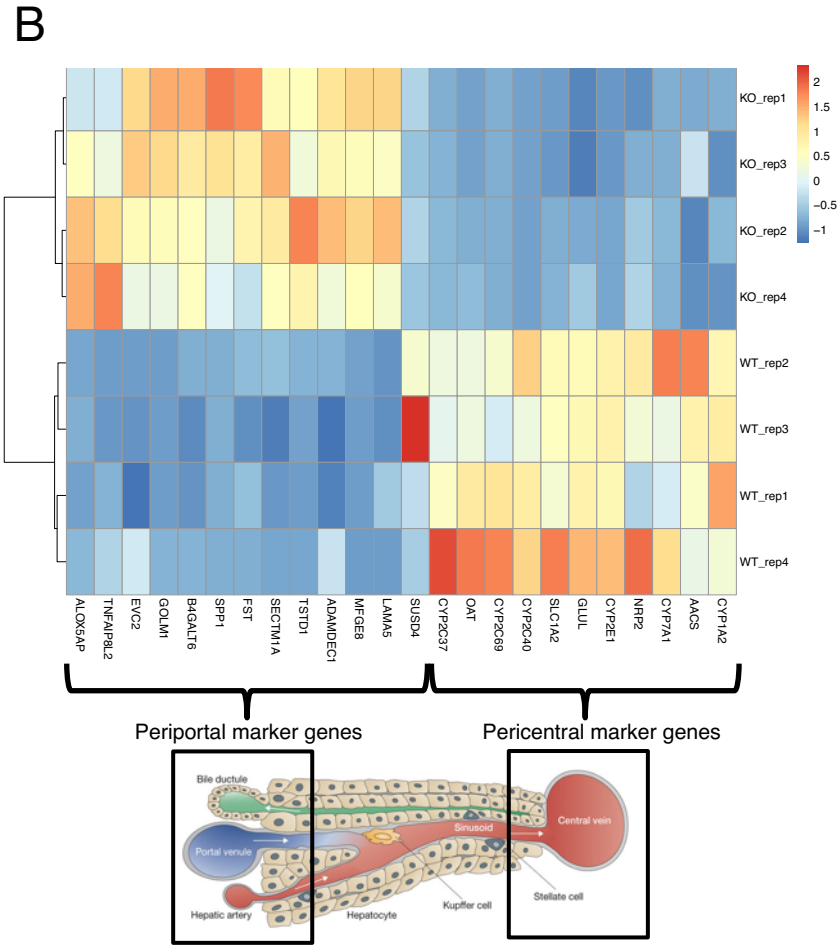
**C.** Relative liver mRNA expression of *Cyp1a2* and *Cyp2e1* expressed in fold change relative to WT expression. n=4, p <0,005.

**D.** TUNEL staining of WT and KO liver in the area around central vein. 24 hours after APAP injection, WT mice display an evident apoptotic area around central vein. While, CAPZ LKO mice show almost no TUNEL positivity (i.e. apoptotic cells).

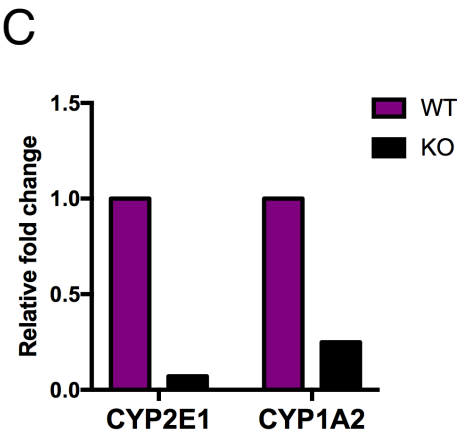
**E.** Serum alanine aminotransferase (ALT) levels, expressed in units/ml, 8 hours after acetaminophen (APAP) intraperitoneal injection. Single mouse measure are plotted (circle its WT mice and triangle its KO mice) * p < 0,05.

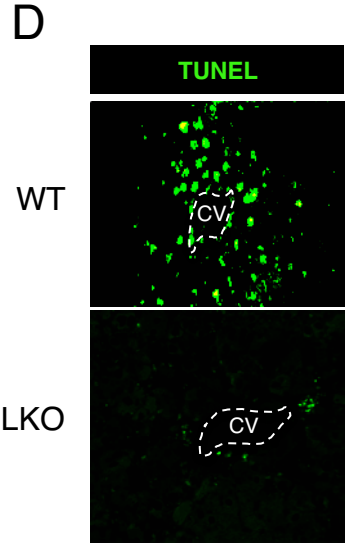
# FIGURE 7

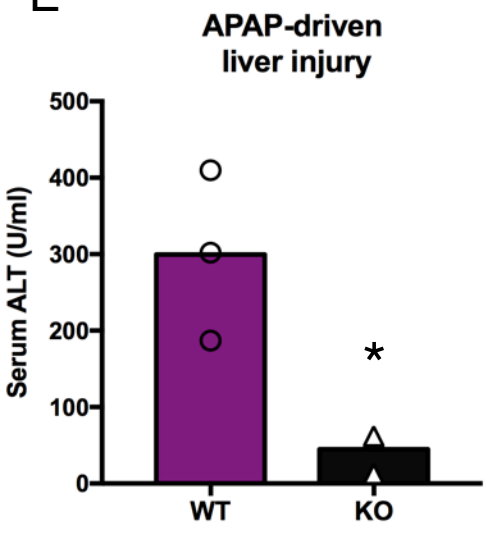

A
GS
CV
CV
WT
CAPZB LKO


B
KO_rep1
KO_rep3
KO_rep2
KO_rep4
WT_rep2
WT_rep3
WT_rep1
WT_rep4
2
1.5
1
0.5
0
−0.5
−1
ALOX5AP
TNFAIP8L2
EVC2
GOLM1
B4GALT6
SPP1
FST
SECTM1A
TSTD1
ADAMDEC1
MFGE8
LAMA5
SUSD4
CYP2C37
OAT
CYP2C69
CYP2C40
SLC1A2
GLUL
CYP2E1
NRP2
CYP7A1
AACS
CYP1A2
Periportal marker genes
Pericentral marker genes


Bile ductule
Portal venule
Hepatic artery
Sinusoid
Central vein
Kupffer cell
Hepatocyte
Stellate cell


C
Relative fold change
1.5
1.0
0.5
0.0
CYP2E1
CYP1A2
WT
KO


D
TUNEL
WT
CV
CAPZB LKO
CV


E
APAP-driven
liver injury
Serum ALT (U/ml)
500
400
300
200
100
0
*
WT
KO

## Figure 8. CAPZB regulates organ size and hepatocytes proliferation through YAP.

**A.** Liver mRNA expression of *Capzb*, *Yap1* and *Taz/WWTR1* in WT, CAPZ LKO and CAPZB+YAP LKO expressed in fold change relative to WT expression. n=4, p <0,005.

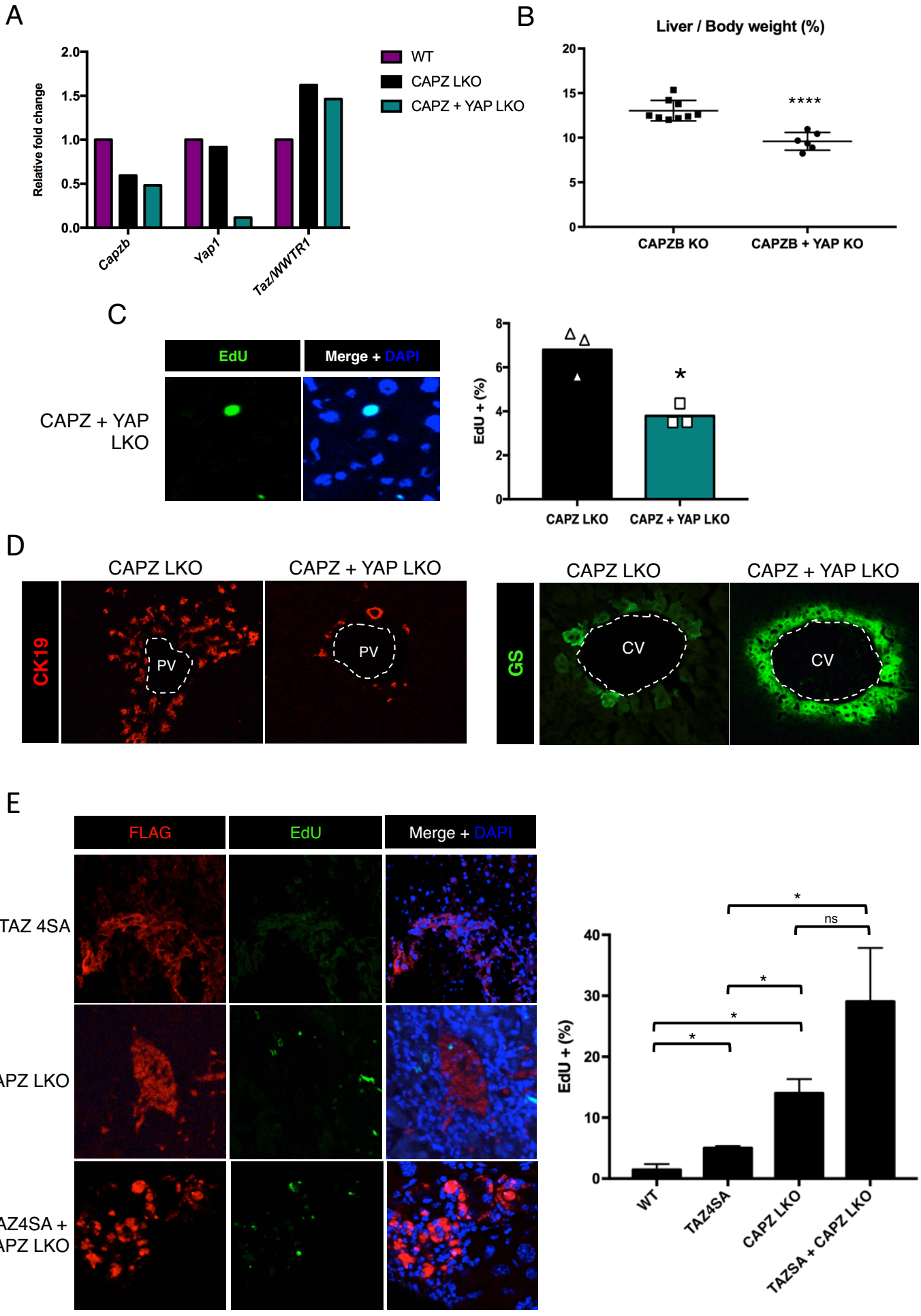
**B.** Liver/body weight ratio in CAPZB LKO and CAPZB+YAP LKO mice expressed in percentage. Combined knockout of CAPZB and YAP partially rescue the tissue over-growth phenotype. n > 6, **** p < 0,0001

**C.** Representative immunofluorescence of EdU staining in CAPZB + YAP LKO and quantification. YAP knockout efficiently rescue hepatocytes proliferation driven by CAPZB loss. Each value represents single mouse result (triangle its WT mice and square its KO mice). n = 3, * p < 0,05.

**D.** Immunofluorescence of CK-19 and GS in CAPZB LKO and in CAPZB+YAP LKO. YAP knockout efficiently rescue oval cells expansion and glutamine synthase loss driven by CAZPB deletion.

**E.** Representative immunofluorescence of liver tissue staining for EdU and FLAG in FLAG-TAZ4SA-injected mice, FLAG-injected CAPZB LKO mice and FLAG-TAZ4SA-injected CAPZB LKO mice. Quantification of EdU positive cells is expressed in percentage. n = 3, * p < 0,05, ns = not significant.

# FIGURE 8


A
Relative fold change
2.0
1.5
1.0
0.5
0.0
Capzb
Yap1
Taz/WWTR1
WT
CAPZ LKO
CAPZ + YAP LKO
B
Liver / Body weight (%)
20
15
10
5
0
****
CAPZB KO
CAPZB + YAP KO
C
EdU
Merge + DAPI
CAPZ + YAP LKO
EdU + (%)
8
6
4
2
0
CAPZ LKO
CAPZ + YAP LKO
D
CAPZ LKO
CAPZ + YAP LKO
CK19
PV
PV
GS
CV
CV
E
FLAG
EdU
Merge + DAPI
TAZ 4SA
CAPZ LKO
TAZ4SA + CAPZ LKO
EdU + (%)
40
30
20
10
0
*
ns
*
*
*
WT
TAZ4SA
CAPZ LKO
TAZSA + CAPZ LKO

## Figure 9. Mechano-characterization of WT and CAPZB KO cells and tissue

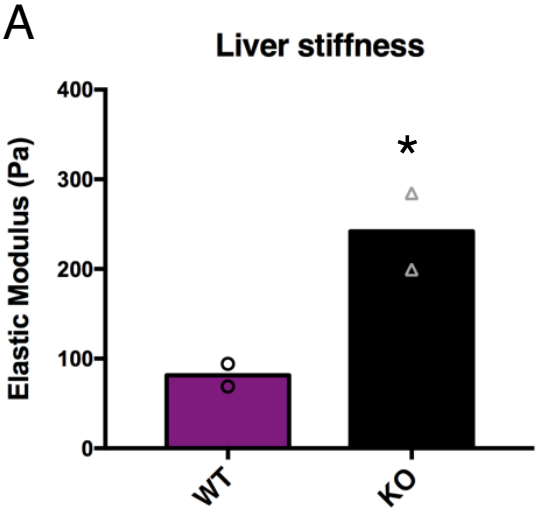
**A.** Measure of liver stiffness in WT and KO livers. Average of AFM maps (n = 2) are plotted. Two different mice for each genotype were analyzed. * $p < 0,05$.

**B.** Representative phalloidin staining of WT (infected with adeno-co) and CAPZB KO (infected with adeno-Cre) fibroblasts.

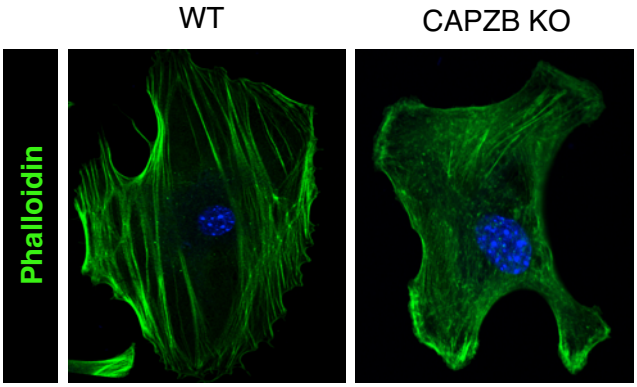
**C.** Representative vinculin, marker of focal adhesion, and phalloidin, marker of filamentous actin, stainings in WT and CAPZB KO fibroblasts. On the right, zoomed area highlights the focal adhesion plaque.

# FIGURE 9

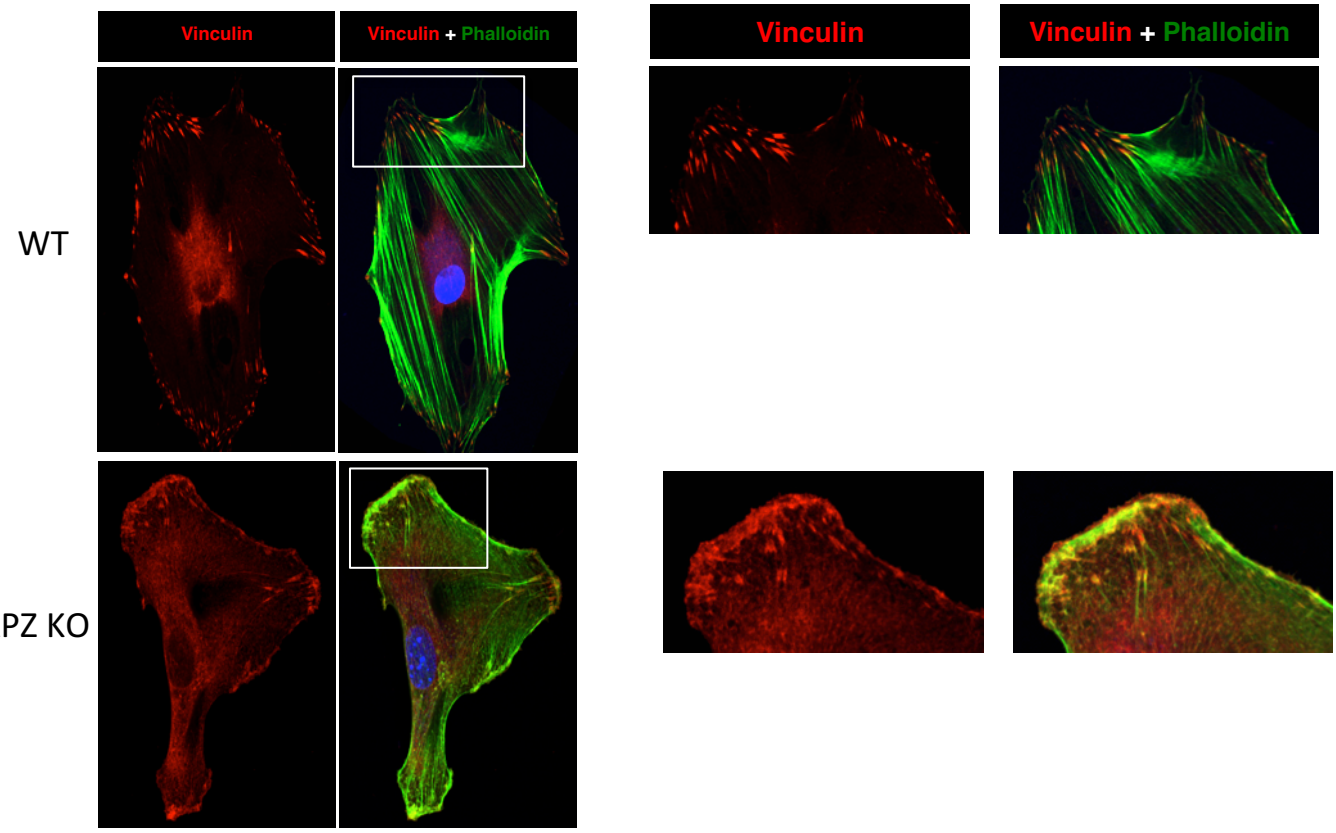
A


Liver stiffness
Elastic Modulus (Pa)
400
300
200
100
0
*
WT
KO

B


WT
CAPZB KO
Phalloidin

C


Vinculin
Vinculin + Phalloidin
Vinculin
Vinculin + Phalloidin
WT
CAPZ KO

## Figure 10. Hippo-dependent luciferase-based chemical-library screening

**A.** Inhibition of Hippo pathway or stimulation of YAP has proven to be an effective strategy to stimulate cardiomyocytes proliferation and enhance cardiac regeneration after a cardiac injury. From Papizzan and Olson, 2014.

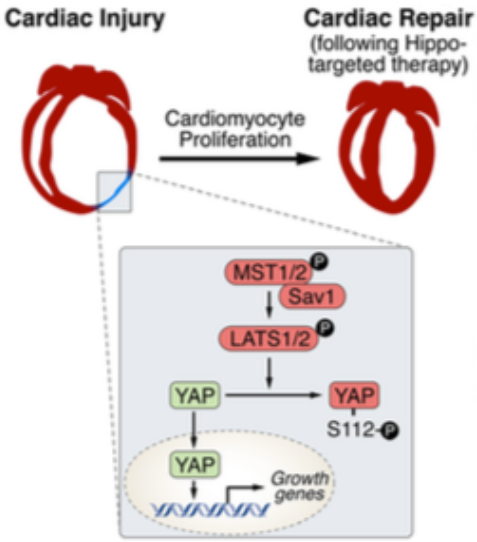
**B.** Relative luciferase activity in MDA-MB-231 transfected with the indicated siRNAs and with control DNA (untreated) or with NF2 expression plasmid. siRNAs transfection were performed after DNA transection and for 24-hours, as for optimized screening protocol. LATS1/2 knockdown rescues YAP/TAZ inhibition by NF2/Merlin.

**C.** Schematic representation of optimized protocol for 96-well library screening. At day one cells were plated in 10cm-dish and the day after transfection of YAP/TAZ reporter (8xGTIIC-Lux) and NF2 expression plasmid were performed. Six hours later cells were replated in 96-well plates. 24 hours after cells were treated with small-molecules library. 96-well plates were harvest in lysis buffer 24 hours after small-molecules treatments. On the bottom, results of small-molecules library luciferase-based screening. Black dots indicate small-molecules library compounds and red dots indicate control treatments. Compound 1 and Compound 2 efficiently rescue YAP/TAZ activity, overcoming NF2-dependent inhibition.

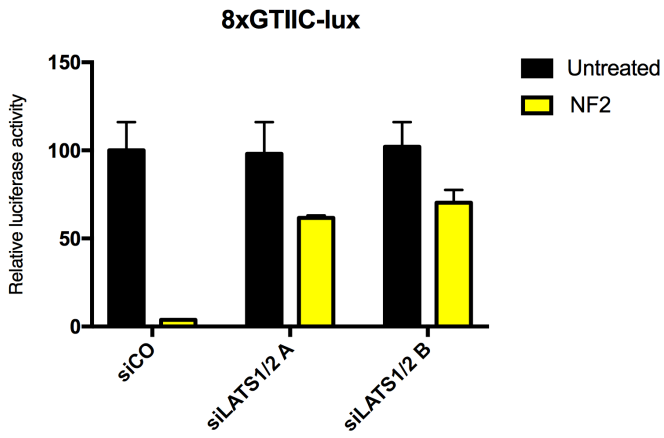
**D.** Validation of screening's hits using classical home-made protocol for luciferase assay (See Materials and Methods) and freshly prepare and resuspend small-molecules. Cells were transfected with different doses of NF2.

# FIGURE 10

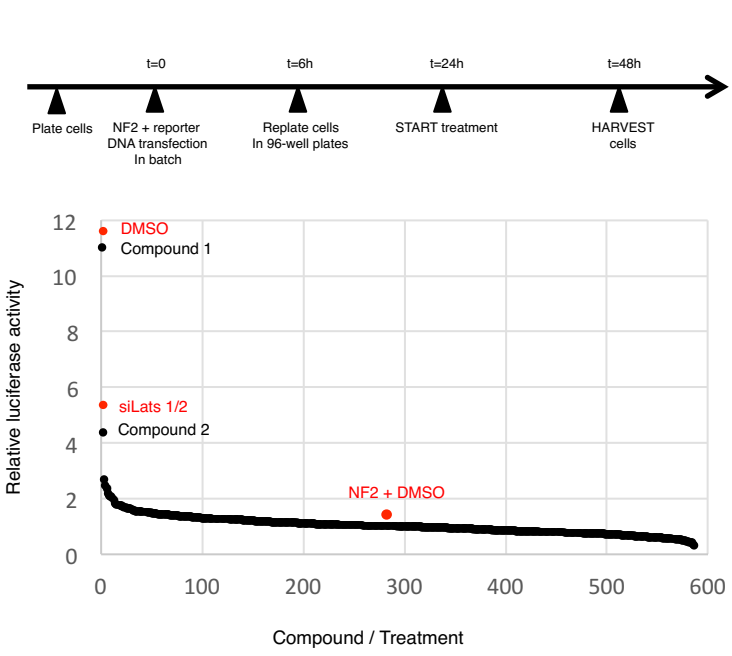
A



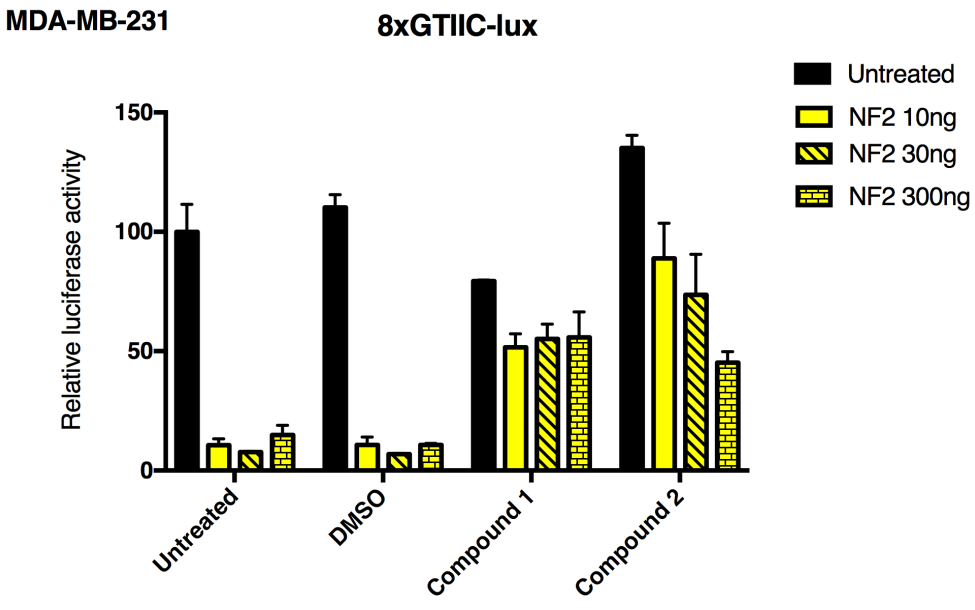

B




C




D



## Figure 11. The lead compounds efficiently rescue YAP/TAZ activity

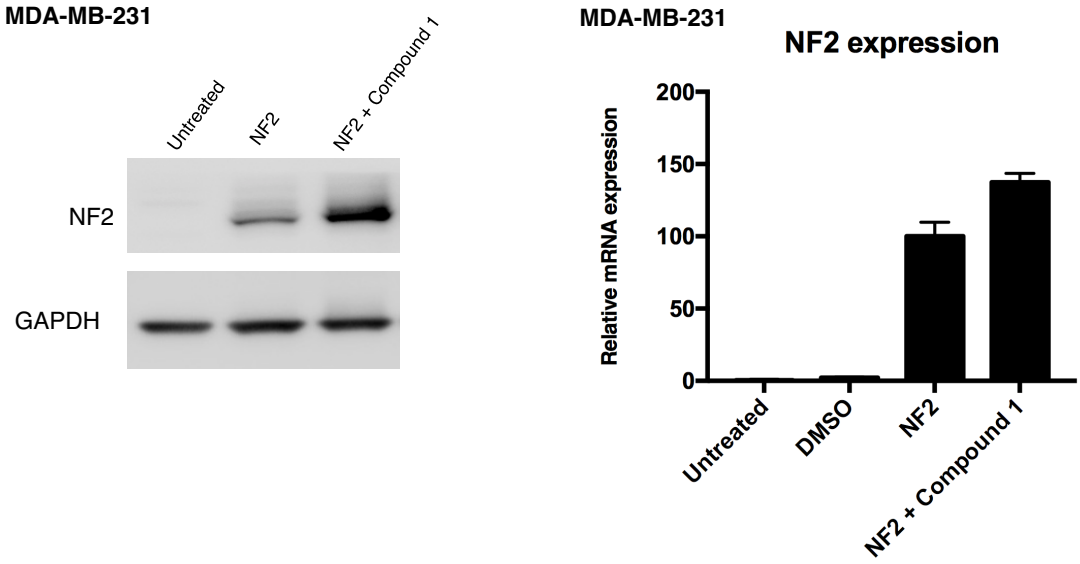
**A.** Levels of NF2 in MDA-MB-231. On the left, western blotting for NF2, GAPDH was used ad loading control. Compound 1 did not influence exogenous NF2 protein production. On the right, mRNA expression of NF2 in MDA-MB-231 transfected or not with NF2 and treated with compound 1 or DMSO as control. NF2 mRNA expression is relative to GAPDH.

**B.** Representative immunofluorescence of YAP and Cherry in MDA-MB-231 cells transfected with NF2 expression plasmid and treated with Compound 1 or DMSO as control. On the right, count of YAP localization, expressed in percentage of total cells ($n > 100$).

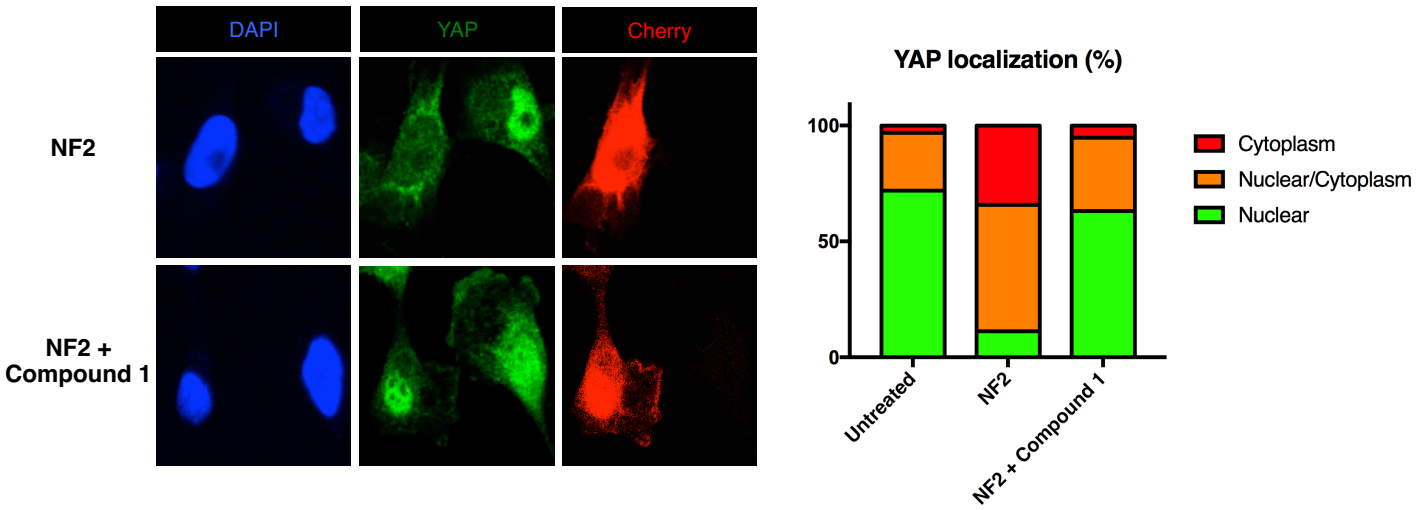
**C.** Western blotting for YAP, TAZ and two phosphorylated form of YAP, serine-127 (p-YAP S-127) and serine-381 (p-YAP S-381) in MDA-MB-231 cell. GAPDH was used as loading control. Two independent replicates for each condition are shown.

# FIGURE 11

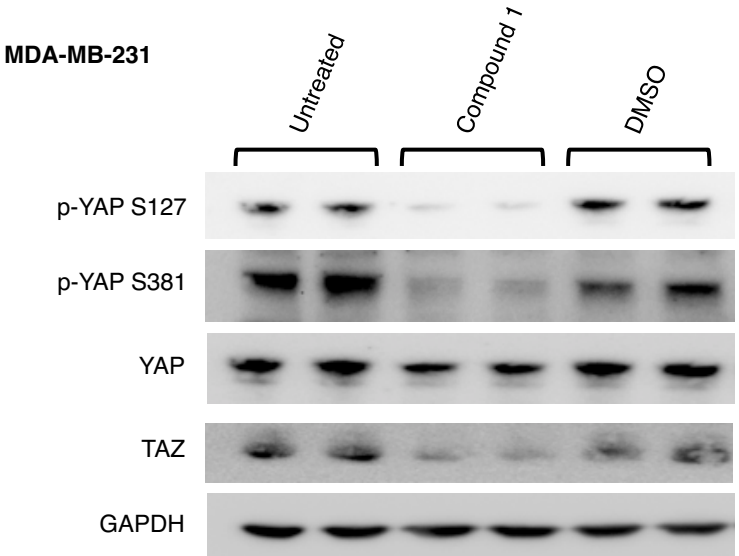
A




B




C



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