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PhD program in "Genetics and Human Biology" – XXXV Cycle

"Extracellular Signal-Regulated Kinase 5 (ERK5) is required for the Yes-associated protein (YAP) co-transcriptional activity"

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SUMMARY

ABSTRACT

Yes-associated protein (YAP) is a transcriptional cofactor involved in the regulation of various physio-pathological cellular processes. YAP is the main downstream effector of the tumor-suppressive Hippo pathway. When Hippo signaling is activated, it induces YAP phosphorylation and cytoplasmic sequestration, thus inhibiting its co-transcriptional activity. On the contrary, when Hippo signaling is inhibited, YAP translocates into the nucleus where it drives several transcriptional programs in a cell- and context-dependent manner. However, recent observations indicate that YAP activity can be also modulated by Hippo independent/integrating pathways, still largely unexplored. In this study, we demonstrated the role of the extracellular signal-regulated kinase 5 (ERK5)/mitogen-activated protein kinase in the regulation of YAP activity. By means of ERK5 inhibition/silencing and overexpression experiments, and by using as model liver stem cells, hepatocytes, and hepatocellular carcinoma (HCC) cell lines, we provided evidence that ERK5 is required for YAP transcriptional activity. Mechanistically, ERK5 is required for the recruitment of YAP on the promoters of target genes and for its physical interaction with the transcriptional partner TEAD4. Moreover, we observed that ERK5 modulates YAP activation in cell adhesion dynamics, TGFβ-induced EMT of liver cells and cell migration. Furthermore, we

demonstrated that ERK5 modulates the activity also of a YAP mutant non-phosphorylatable by LATS1/2, thus providing evidence of its, at least in part, independence from the Hippo pathway. Finally, preliminary results obtained in mice overexpressing YAP in the liver confirmed the relevance of ERK5/YAP axis *in vivo* and suggested the ERK5 involvement in the YAP-induced liver fibrosis. Therefore, our observations identify ERK5 as a novel upstream regulator of YAP activity, thus unveiling a new target for therapeutic approaches aimed at interfering with its function.

INTRODUCTION

1. YES-ASSOCIATED-PROTEIN 1

Yes-associated protein 1 (**YAP**), Yki orthologue, encoded by the *YAP* gene in the human chromosome 11q22, is a transcriptional cofactor playing an essential role in the regulation of several cellular processes, including proliferation, stemness maintenance, differentiation, apoptosis, organ and tissue homeostasis (*Varelas X 2014*). Moreover, YAP dysregulation has been well documented in several human cancers, where it contributes to the development and progression of the disease (*Zanconato F et al. 2016*).

YAP, in a complex with its paralog Transcriptional co-activator with PDZ-binding motif (**TAZ**), integrates several environmental cues converting them in cell- and context-specific gene expression programs and, eventually, in multiple cell behaviors (*Varelas X. 2014*).

YAP, although lacking a DNA-binding motif, can be recruited on the DNA by the interaction with transcription factors, mainly those belonging to the **TEAD/TEF** family. However, increasing evidence of additional transcriptional partners of YAP and of their role in

triggering cell type- and context- specific cellular responses has been collected. In particular, p73, RUNXs, EGR-1, TBX5, SMADs have been identified as transcriptional partners of YAP able to drive specific gene expression (*Varelas X. 2014; Mo JS et al. 2015*).

Once on the DNA, YAP/TAZ regulates gene expression by the recruitment of chromatin remodeling complexes and epigenetic modifiers. In particular, YAP/TAZ recruits the chromatin remodeling complex **SWI/SNF** and the H3K4 methyl transferase **NcoA6**, inducing the transcription of many target genes, such as Ctgf, Cyr61, Ankrd1 (*Oh H et al. 2013; Oh H et al. 2014; Qing Y et al. 2014; Skibinski A et al. 2014*). By recruiting the complex with deacetylase activity **NuRD**, instead, YAP/TAZ can inhibit the transcription of target genes, such as DDIT4 and Trail (Fig. 1) (*Kim M et al. 2015*).

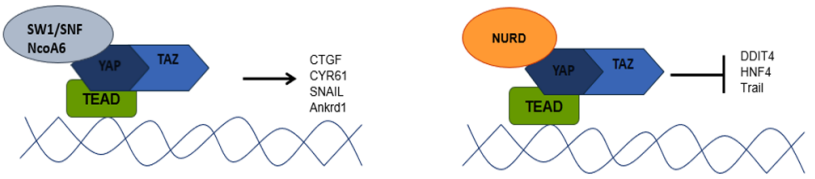

Fig. 1 Co-transcriptional activity of YAP/TAZ.

1.1 YAP REGULATION

Hippo pathway and Upstream Regulators of the Hippo Cascade

YAP is the downstream effector of **Hippo signaling**, an important tumor suppressive pathway involved in the transduction of several micro-environmental signals (*Zhao B et al. 2011*). This pathway, highly conserved during the evolution from *Drosophila Melanogaster* to mammals (*Mo JS et al. 2014*), involves different proteins, with a well-characterized regulative core constituted by the kinases **MST1/2** and **LATS1/2**.

When Hippo signaling is on, MST1/2 phosphorylate and activate the serine/threonine kinases LATS1/2 that, in turn, phosphorylate YAP on S127 and S381, leading to its proteasome-mediated degradation or its cytoplasmic sequestration by interaction with 14-3-3 protein (*Varelas X. 2014; Pan D. 2010*). On the contrary, when the Hippo pathway is off the un-phosphorylated YAP protein translocates into the nucleus where controls several transcriptional programs (Fig. 2).

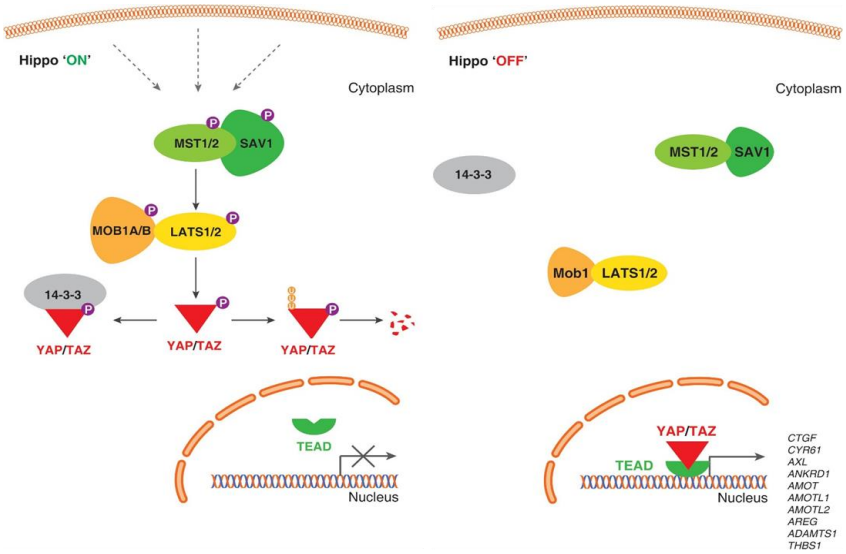

Fig. 2 Hippo pathway regulation of YAP activity *(Boopathy and Hong, 2019).*

Alternative regulatory pathways, activating LATS1/2 independently from MST1/2, have been recently identified (*Harvey KF et al. 2013; Yin F et al. 2013*). Many regulative elements, most of them acting on LATS1/2, belong to membrane complexes. Moroishi et al. unveiled the role of cell-cell junction components in Hippo pathway activity in epithelial cells (*Moroishi T et al. 2015*). Scribble (SCRIB), as component of basolateral cell junctions, positively controls the activated state of MST and LATS kinases, leading to YAP phosphorylation/inactivation in adherent cells.

When cell-cell contacts are lost, SCRIB is delocalized with consequent lower activity of the kinases, YAP/TAZ nuclear translocation and activation can be observed (*Cordenonsi M et al. 2011; Zhao B et al. 2007*). Another membrane protein with a role in Hippo pathway modulation is the tumor suppressor Neurofibromin 2 (NF2). When localized to the membrane, it is able to interact with a large set of proteins (such as α-catenin and Angiomotin AMOT) and to recruit and activate LATS1/2 kinases (*Yin F et al. 2013*). Moreover, NF2 can inhibit YAP also through the binding to the ubiquitin ligase CRL4-DCAF1, thus interfering with the ubiquitin-dependent and proteasome-mediated LATS degradation (*Wilson KE et al. 2014*). Furthermore, YAP/TAZ activity can be modulated by other proteins, including AMOT, the tyrosine phosphatase PTPN14 and α- Catenin, acting downstream of Hippo and preventing YAP/TAZ nuclear translocation (Fig. 3) (*Liu X et al. 2013; Wang W et al. 2012*). AMOT binds to F-actin and multiple tight junction components and can inhibit YAP both by binding and sequestering it in the cytoplasm in a Hippo–independent manner and by promoting LATS phosphorylation. In addition, LATS kinases can act indirectly on YAP by phosphorylating the binding domain of actin filaments and thus releasing AMOT, favoring the AMOT-YAP binding (*Adler JJ et al. 2013; Dai X et al. 2013; Mana-Capelli S et al. 2014*). PTPN14 interacts directly with the YAP WW domain, leading to its sequestration in the cytoplasm (*Liu X et al. 2013; Wang W et al.*

2012). Alfa-Catenin stabilizes in the cytoplasm the interaction between phosphorylated-YAP and 14-3-3 protein (*Schlegelmilch K et al. 2011*).

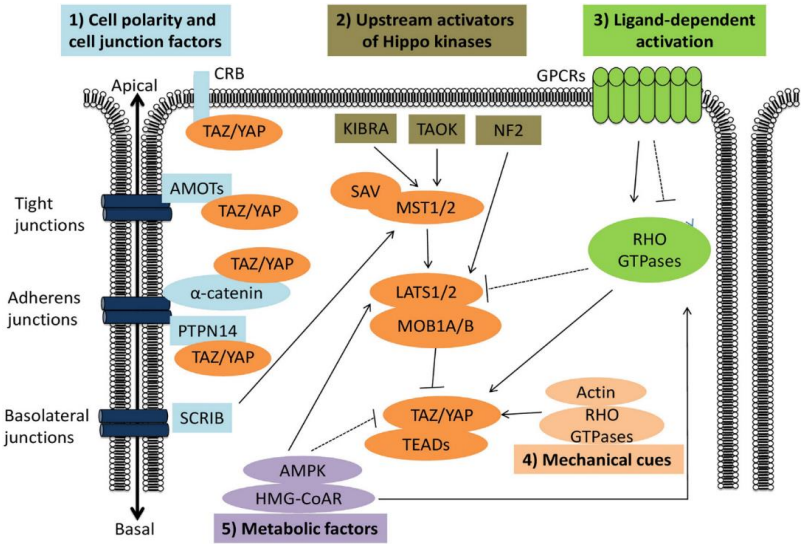

Fig. 3 Upstream regulation of the Hippo-YAP/TAZ pathway *(Maugeri-Saccà and De Maria, 2018).*

Hippo-Lats1/2 independent YAP regulation

In recent years, the wide complexity of the YAP activity regulation has been unveiled and several elements acting independently from the

kinase core of the Hippo pathway have been revealed (*Guo Y et al. 2022*).

Various **stress signals**, including hypoxia, osmotic stress and endoplasmic reticulum (ER) stress play a role in this Hippo-independent regulation. In particular, the osmotic stress induces YAP nuclear localization and activation through Nemo-like kinase (NLK)-mediated phosphorylation at Ser128, overriding the effect of Ser127 phosphorylation induced by LATS1/2 (*Hong AW et al. 2017*).

In **cells stretched** by a substrate with a high elasticity module, YAP/TAZ are nuclear and transcriptionally active. In this condition a key role is played by the Rho GTPase and the tension of the cytoskeletal elements. The depletion of LATS1/2 has no effect on this cellular response indicating an independence of the process by the enzymatic core of the Hippo pathway (*Dupont S et al. 2011*).

G protein-coupled receptors (GPCRs), the largest family of cell surface receptors, recently were found to regulate either positively or negatively the YAP/TAZ activity, depending on the type of their ligands, the downstream G proteins activated, the activity of protein kinases PKA or PKC and the induced actin cytoskeleton rearrangements (*Guo Y et al. 2022*). GPCRs can be activated by several elements leading to the inhibition of LATS1/2 (*Guo Y et al.*

2022) but also to act directly on YAP protein favoring its sequestration in the cytoplasm (*Yu FX et al. 2012*) or, on the contrary, its nuclear retention and the consequent activation of its transcriptional program (*Fang L et al. 2018*).

Moreover, YAP/TAZ are essential components of the **β-catenin** destruction complex, and regulated by canonical **Wnt** pathway: in the absence of Wntf, YAP/TAZ is recruited in the complex by a direct binding with Axin, APC and beta-catenin (*Azzolin L et al. 2014)*; in the presence of Wnt, YAP/TAZ is free to translocate into the nucleus, and to regulate the transcription of target genes (*Guo Y et al. 2022*).

Notably, it has been recently described a significant Hippo-independent role of some cytoplasmic and nuclear serine/threonine kinases, mainly accomplished by the direct phosphorylation of YAP or of its functional cofactors (*Zhang L et al. 2015; Deng Y et al. 2018; Lamar JM et al. 2019; Seo J et al. 2019; An L et al. 2020*). A role of some **MAPKs** (i.e. p38, JNK and ERK1/2) in the regulation of YAP activity has been also reported (*Liu Z et al. 2016; Lin KC et al. 2017; Yuo B et al. 2015*). In particular, Liu Z et al. observed that JNK and p38 MAPK signaling mediates actin cytoskeleton remodeling-induced nuclear YAP expression in alveolar stem cells (*Liu Z et al. 2016*). In stress conditions, p38 MAPK promotes TEAD cytoplasmic translocation in a Hippo-independent manner, thus suppressing YAP-

driven cancer cell growth (*Lin KC et al. 2017)*. Inhibition of ERK1/2 by siRNA or small-molecular inhibitors in non-small cell lung cancer cells decreases YAP protein level and target gene expression, reducing cell migration and invasion (*Yuo B et al. 2015*).

1.2 YAP BIOLOGICAL FUNCTIONS

YAP as effector of cellular responses to mechanical cues

YAP is a main effector of mechanical stimuli, such as extracellular matrix (**ECM**) stiffness or stretching, shear stress and cell shape (Fig. 4) (*Karaman R & Halder G 2018*; *Meng Z et al. 2016; Panciera T et al. 2017; Piccolo S et al. 2014)*. In particular, YAP response to extracellular matrix stiffness has been well characterized: in high stiffness conditions, YAP is nuclear and active on its target genes, while in low stiffness conditions it undergoes cytoplasmic retention and proteasome-mediated degradation (*Dupont S et al. 2011*). RhoA GTPase is a key player of the relaying of ECM elasticity to the cell. It can receive ECM mechanical stimuli through focal adhesion, regulate the stress fiber formation and ultimately regulate YAP and TAZ in both Hippo-dependent and -independent manner *(Dupont S et al. 2011*). Moreover, it has been shown that, in high stiffness conditions, a connection between stress fibers and the nuclear membrane is

established, with consequent stretch of nuclear pores and increased YAP nuclear import (*Elosegui-Artola A et al. 2017*). Moreover, Meng et al. observed that low stiffness conditions could activate GTPase RAP2, leading to LATS1/2 activation and YAP/TAZ inhibition *(Meng Z et al. 2018)*.

In our laboratory it has been demonstrated that hepatic stem cells, characterized by high level of YAP protein localized in the nucleus and transcriptionally active, when grown on a low stiffness module rapidly differentiate into mature hepatocytes showing low level of YAP protein, prevalently localized in the cytoplasm. Interestingly, and coherently with these observations, it has been identified the master gene of hepatocyte differentiation HNF4α as a new negative transcriptional target of YAP (*Cozzolino AM et al. 2016; Noce V et al. 2019*).

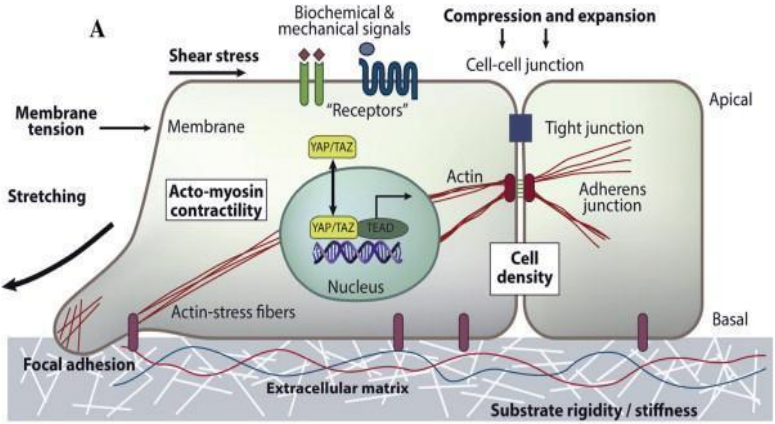

Fig. 4 YAP/TAZ as effectors of mechanical stimuli *(Low et al. 2014).*

YAP and Epithelial-to-Mesenchymal Transition

Epithelial-to-Mesenchymal Transition (EMT) is a cellular process which allows epithelial cells to transdifferentiate into mesenchymal cells, in response to various physio-pathologic signals. Epithelial cells that undergo EMT lose cell-cell contacts and acquire fibroblast-like morphology. EMT cells reprogram their gene expression, with downregulation of epithelial markers and upregulation of mesenchymal ones. The EMT in cancer cells induces invasive and migration ability favoring the metastasization process. EMT is transcriptionally driven by a series of master factors, including the

transcription factors Snail, Zeb, Twist. Several studies demonstrated the critical role played by YAP in the promotion of EMT/metastasization of epithelia tumor cells. Cheng et al., observed that YAP overexpression in CRC (Colorectal Cancer) causes E-Cadherin downregulation and Slug upregulation, which in turn promotes EMT; by means of overexpression and inhibition experiments, they demonstrated that YAP is a driver of EMT, by directly activating Slug transcription with consequent repression of its target gene, E-Cadherin (*Cheng D et al. 2020)*.

Interestingly, my research group has previously identified, together with **HNF4**α as a new negative target of YAP, the EMT master gene **Snail** as a new YAP positive target. Snail is a well-characterized transcriptional inhibitor, involved in the EMT and in liver stemness maintenance; HNF4α is a transcriptional factor which promotes the transcription of several epithelial markers and confers to fibroblast an epithelial morphology and it is capable to re-establish a differentiated state in cancer cells, driving a mesenchymal-to-epithelial transition (**MET**). Snail and HNF4α are part of an epistatic liver-specific mini-circuitry (Fig. 5), being able to reciprocally inhibit each other, that plays a key role in the regulation of various cell outcomes. Notably, a direct involvement of YAP in controlling liver cell differentiation has been unveiled. In fact, YAP upregulates Snail

and inhibits HNF4α transcription levels. Interestingly, Snail and HNF4α, in turn, are able to modulate YAP expression (in a positive or negative manner, respectively) (*Noce V et al. 2019)*.

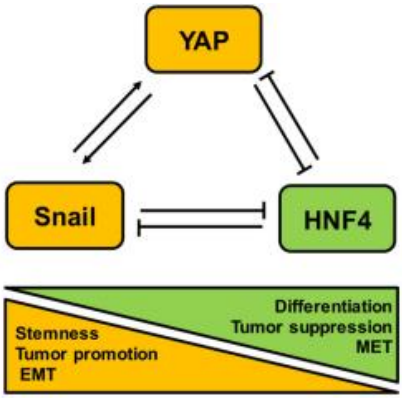

Fig. 5 Molecular circuitry of reciprocal regulation among YAP, Snail, and HNF4α proteins *(Noce et al. 2019).*

YAP and fibrosis

Fibrosis is a pathological condition characterized by impaired epithelial tissue repair and excessive production of ECM elements as a consequence of chronic injury. YAP/TAZ has been recently indicated as important players in the onset and progression of fibrosis in different soft tissues and organs (lung, liver, kidney, skin, and cardiovascular system) (*He X et al. 2022*).

In the **liver**, chronic injury leads to continued activation of hepatic stellate cells (HSC) causing fibrosis and ultimately cirrhosis. ECM accumulation activates fibroblast, thus inducing in turn more and more accumulation of the matrix, describing a feed-forward loop. Sustained HSC activation is essential for this pathological process and several signaling pathways involved in their activation have been identified. In particular, Mannaerts et al. demonstrated the role of YAP in controlling HSC activation *(Mannaerts et al. 2015*). As said before, YAP/TAZ are regulated by matrix stiffness. In particular, they are activated in a high stiffness condition and mediate fibroblast activation and matrix synthesis. Thus, the YAP/TAZ activation in response to an increased stiffness, leads to persistent activation of fibroblast and to fibrosis *(Liu F et al. 2015*).

Moreover, in vitro and in vivo experiments show that YAP- induced activation of Cyr61 is essential for the recruitment of macrophages and for the inflammation-mediated liver fibrosis (*Mooring M et al. 2020*).

Unhealthy tissues accumulating fibrotic lesions have a higher risk of developing cancer. As seen before, in addition to their role in fibrosis, YAP/TAZ are involved in the EMT and their activation is pivotal for tumor progression and stemness maintenance. Thus, YAP/TAZ

activation can be considered as a functional link between fibrosis and cancer.

YAP and cancer

Aberrant YAP/TAZ hyperactivation is associated with poor clinical outcomes in several human **cancers** (*Zanconato F et al. 2016; Cao J & Huang W 2017)* including lung, liver, colon and pancreas cancer and brain tumors; in fact, their activity is essential for tumor growth and for the acquisition of hallmarks of cancer progression, such as uncontrolled proliferation, resistance to apoptosis, stemness and metastatic properties. YAP promotes cancer onset and progression by several mechanisms. Firstly, it increases cell proliferation rate activating the transcription of several genes involved in DNA synthesis and cell cycle progression and inducing the transcription of relevant proto-oncogenes, such as c-Myc (*Zanconato et al., 2016*). Moreover, YAP/TAZ overexpression leads to apoptosis resistance inducing several negative regulators of this process, such as the IAP family members BIRC5/survivin and BIRC2/cIAP1, and the BCL2 family gene MCL1 (*Dong et al. 2007*). Furthermore, YAP/TAZ activation in tumor-associated fibroblasts promotes the expression of ECM proteins such as laminin and fibronectin, leading to an increase in the stiffness and in the maintenance of stability of cancer stem cells (*Calvo F et al. 2013*). Finally, high levels of YAP in cancer

cells stimulates EMT with a reprogramming of gene expression and loss of cell-cell adhesion, cell polarity and acquisition of cell motility and metastatic properties.

2. EXTRACELLULAR SIGNAL-REGULATED KINASE 5

Extracellular signal-regulated kinase 5 is a member of conventional **MAPK** (mitogen-activated protein kinase) family, involved in the transduction of different pathophysiological stimuli, driven by hormones, mitogens (EGF, NGF, FGF), neurotransmitters, cytokines (LIF, IL-6, TGFβ), oxidative and osmotic stresses and mechanical stimuli (shear stress and cell stretching), and able to induce a multitude of cell responses (such as proliferation, differentiation, migration and escape from apoptosis) (Fig. 6) (*Abe J et al. 1996; Drew BA et al. 2012; Marchetti A et al. 2008*).

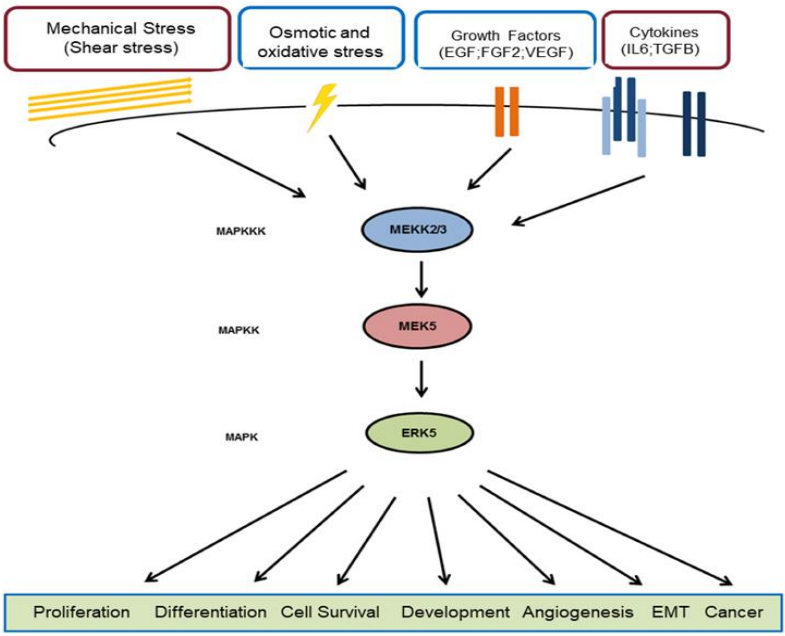

Fig. 6 ERK5 pathway: from extracellular signals to physio-pathological outcomes.

MAPKs are evolutionary conserved enzymes which predominantly phosphorylate serine/threonine residues preceded by a proline residue. As well as other MAPKs, ERK5 is activated by a three-tiered hierarchical kinase module, which begin with the phosphorylation of the apical MEKK2/MEKK3, a mitogen-activated kinase kinase kinase (MAPKKK) that phosphorylates MEK5, a mitogen-activated kinase kinase (MAPKK) which subsequently phosphorylates ERK5. MEK5-

mediated phosphorylation of ERK5 on its Thr219 and Tyr221 residues on the TEY sequence allows ERK5 auto-phosphorylation and therefore its activation. Notably, while MEKK2/MEKK3 can be involved in the modulation of other MAPKs, MEK5/ERK5 interaction is specific (*Drew BA et al. 2012*).

Discovered in 1995, ERK5 has been named **Big Map Kinase 1 (BMK-1)** for its larger size compared to other MAPKs, due to a unique **non-kinase C-terminus domain** (Fig. 7). The C-terminus contains a nuclear localization signal (**NLS**) essential for ERK5 entry into the nucleus, a transcriptional activation domain (**TAD**) which interacts and activates many transcription factors, and a myocyte enhancer factor 2 (**MEF-2**) interacting region. It has been shown that ERK5 can take two different conformations: a closed conformation, characterized by an interaction between the N- and C- terminal domains and the association with chaperons HSP90 and CDC37 and retaining the kinase in the cytosol, and an open conformation following the MEK5-induced phosphorylation of its C-terminus domain, that promotes HSP90 release and the NLS exposition. After the nuclear translocation, ERK5 regulates the transcription of specific genes by phosphorylating other transcription factors (such as Sap1, c-FOS, c-Myc and MEF2) *(Kamakura S et al. 1999; Kato Y et al. 1997; Terasawa K et al. 2003)*, and/or improving their transactivation activity (*Kato Y*

et al. 1997; Morimoto H et al. 2007; Kasler HG et al. 2000). Once the closed conformation is re-established, the nuclear export protein CRM1 recognizes a nuclear export signal (NES) and shuttles the kinase in the cytosol (*Tubita et al., 2020*).

MEK5-independent mechanisms responsible for ERK5 phosphorylation and nuclear translocation have been also described (*Tubita A. et al. 2020*). During mitosis ERK5 can be phosphorylated by CDK1 at S753 and/or T732, and by other kinases such as CDK5 (at T732) enhancing its nuclear level.

ERK5 nuclear translocation could be induced in response to stimuli such as FGF, EGF, neuregulin, CSF-1 or PDGF, depending on the specific cell line. Oncogenic signals such as an active RAS or a mutated form of BRAF (BRAFV600E), could stimulate ERK5 phosphorylation via ERK1/2 and/or CDK1 with subsequent activation and nuclear translocation. Moreover, some phosphatases, such as Ser/Thr PP1/PP2A and tyrosine PTP, are responsible for the ERK5 inactivation, also preventing its nuclear translocation (*Tubita A. et al. 2020*).

Other types of post translational modifications, such as ubiquitination or sumoylation, can be involved in ERK5 nuclear localization and activity. In particular, Woo et al. reported for the first time that ERK5 may be SUMOylated at K6 and K22 in its N-terminus,

resulting in the inhibition of its transcriptional activity (*Woo, C.H. et al. 2008*). More recently, it has been observed that ERK5 SUMOylation can stimulate ERK5 nuclear localization and transcriptional activity (*Erazo T et al. 2020*).

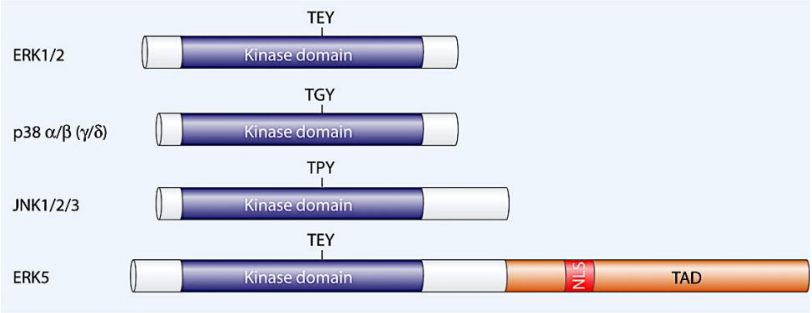

Fig. 7 Schematic representation of the structure of ERK5 compared to that of other conventional MAPKs *(Cargnello and Roux, 2011).*

One of the main indicators of ERK5 activity is the activation of its principal targets, the transcription factors belonging to the MEF2 family. Myocyte enhancer-binding factors family is composed by four transcription factors, MEF2A/B/C/D, which have been first identified as regulators of several muscle-specific genes but then shown to have important roles also in other physiologic functions (myogenesis, embryonic development, angiogenesis). ERK5 activates MEF2 proteins not only by phosphorylation, but also by increasing its

transcriptional activity thanks to its TAD domain (*Kato Y et al. 1997; Kasler HG et al. 2000*). For this reason, one of the most reliable and sensitive methods to measure ERK5 activity in cells is based on the transactivation activity of a MEF2-responsive construct in luciferase assays (*Bliss SP et al. 2012*). Of note, among MEF-2 family members MEF2D has been shown to be specifically controlled by ERK5 (*Kato Y et al. 2000; Yang CC et al. 1998*) while MEF2A and C can be also phosphorylated and activated by p38 MAPK (*Han J et al. 1996, Ornatsky OI et al., 1999*).

2.1 ERK5 FUNCTIONS

ERK5, is present in a large variety of cell types and although it shares some substrates with other MAPKs, its role is not redundant. To unveil ERK5 functions in vivo, a deletion of its gene in mice has been performed, resulting in defective blood-vessel, neuronal and cardiac development, with consequent embryonic death around days 9.5-10.5 *(Regan CP et al. 2002)*. In particular, it has been observed a delayed and abnormal myocardium development due to a disorganization and abnormal morphology of endothelial cells and an angiogenesis failure characterized by disorganized and not mature

blood vessels (*Regan CP et al. 2002; Sohn SJ et al. 2002; Wang X et al. 2005*).

In the adult, ERK5 is required for muscular differentiation, endothelial cells maintenance and for a good function of the immune system (*Rovida E et al. 2008; Dinev D et al. 2001; Pi X et al. 2004; Roberts OL et al. 2010; Sohn SJ et al. 2002*). The kinase plays an essential role in cell proliferation and in cell cycle regulation. In particular, ERK5 is involved in G1/S transition by modulating cyclin D1 transcription; moreover, it drives G2/M transition and mitotic entry, through the transcriptional upregulation of NF-kB, which in turn upregulates the cyclins B1 and B2, thus promoting mitosis (*Li Z et al. 2012; Cude K et al. 2007*).

Furthermore, ERK5 exerts a role in the **epithelial-mesenchymal transition**, in particular by upregulating at the transcriptional and post-transcriptional level the EMT master genes, Slug and Snail (*Parent AE et al. 2004; Marchetti A et al. 2008; Arnoux V et al. 2008*).

2.2 ERK5 AND CANCER

Recent evidence points to a key role of ERK5 in pathological conditions, in particular in **cancer** onset and progression. As said

before, the kinase is in fact essential for the regulation of many processes correlated to cancer. ERK5 contributes to sustain cell proliferation and survival, promote angiogenesis and inflammation, support cell invasion and metastasis (*Stecca B & Rovida E 2019*). An overexpression of ERK5 has been observed in many types of human cancers, and is associated with bad prognosis: metastatic phenotype, apoptosis and drug treatment resistance (*Esparís-Ogando A et al. 2002; Ramsay AK et al. 2011; Simões AE et al. 2015; Weldon CB et al. 2002; Zen K et al. 2009*). Furthermore, accumulating evidence in preclinical models indicates the benefits of using MEK5-ERK5 inhibitory strategies for the treatment of human cancers and the contribution of MEK5-ERK5 signaling to therapy resistance (*Yang Q and Lee J-D, 2011*).

Recently, many studies highlighted the key role of MEK5-ERK5 signaling in proliferation of cancer cells and tumor onset and progression.

In prostate cancer (PC), high levels of expression of ERK5 have been found and correlated to an enhanced cell proliferation (*Kasavan K et al. 2004*). ERK5 results to be highly expressed also in triple-negative breast cancer (TNBC) cells, where it contributes to drug resistance and metastatic progression by regulating EMT, ECM integrity and angiogenesis (*Hoang VT et al. 2020*). Moreover, targeting the

MEK5/ERK5 pathway in chronic myeloid leukemia cells leads to a reduction of their proliferation rate and stem-progenitor potential, both in culture and in vivo models *(Tusa I et al. 2018 a*).

ERK5 is also involved in the onset of human melanoma. In particular, combining the targeting of ERK5 to the mutated BRAF or ERK1/2 inhibition improves the effectiveness of single treatments and impaired melanoma cell proliferation and drug resistance (*Tusa I et al. 2018 b; Song C et al. 2017*). Recently, it has been unveiled a role of MEK5-ERK5 signaling in lung cancer where high levels of these proteins are associated with poor survival of the patients (*S Sánchez-Fdez A et al. 2019*).

In particular, a direct involvement of ERK5 in **hepatocellular carcinoma (HCC)** has been established, where the aberrant activation of MEK5/ERK5 signaling and ERK5/MAPK7 gene amplification have been reported and correlated with tumor progression and poor prognosis (*Zen K et al. 2009; Rovida E et al. 2015; Zamani A et al. 2017*). HCC is one of the most frequent typologies of liver cancer. A chronically injured liver could undergo cirrhosis and then develop hepatocellular carcinoma, due to the microenvironment characterized by inflammation, oxidative stress and hypoxia. Inhibition of ERK5 affects HCC cell proliferation, migration and

invasion, and modulates tumor development and growth in vivo (*Rovida E et al. 2015*).

Altogether, these data indicate a pivotal role of ERK5 upregulation in cancer, due to its function in the regulation of many cell processes. For this reason, its targeting represents a promising anti-cancer strategy.

3. FUNCTIONAL CORRELATIONS BETWEEN ERK5 AND YAP

Recently, a body of correlative evidence suggests a functional link between ERK5 and YAP. Firstly, ERK5 can mediate the signal transduction from mechanical stress (i.e. fluid shear stress and stretching), where the YAP involvement is well known (*Low BC et al. 2014*). In particular, laminar flow activates ERK5 in endothelial cells, by means a molecular pathway starting from a mechanosensitive Ca+/channel and involving a Ca+/calmodulin dependent kinase, MEKK3 and MEKK5 (*Zheng Q et al. 2022*).

Secondly, both ERK5 and YAP have a role in EMT. In fact, ERK5 upregulates EMT transcription factors, increases the release of extracellular matrix metalloproteinases, thus favoring the extracellular matrix breakdown and local tumor invasion, promotes

the expression of several mesenchymal genes and decreases E-cadherin level (*reviewed in Bhatt AB et al. 2021*). As said above, YAP too is a further crucial player of the EMT, during which it is significantly upregulated. In particular, recent reports showed that YAP acts as a primary mediator of the EMT triggered by TFGβ (*Liu Y et al. 2017; Gao C et al 2021*) and is required for the SMAD2/3 nuclear translocation (*Labibi B et al. 2020*). Moreover, we demonstrated that both ERK5 and YAP are positive regulators of the EMT master transcription factor Snail. In particular, ERK5 is involved in the TGFβ-induced Snail protein regulation being required for its stabilization (*Marchetti A et al. 2008*), while YAP is involved in Snail transcription regulation (*Noce et al. 2019*).

Interestingly, recent evidence demonstrated a key role of both YAP and ERK5 in the regulation of the embryonic stem cell (ESC) identity: overexpression of YAP in human embryonic stem cells promotes the generation of naive pluripotent stem cells (*Qin H et al. 2016*); ERK5 is required for the maintenance of ESC in the naïve state and for the inhibition of specific differentiation programs (*Williams CA et al. 2016*). Furthermore, the MEK5/ERK5 pathway activation by YAP in promoting muscle cell differentiation has been recently reported (*Chen TH et al. 2017*).

Finally, as said above, both ERK5 and YAP were shown to play crucial roles in tumor growth and progression of several kinds of human cancers, including hepatocellular carcinoma (*Zanconato F et al. 2016; Stecca B & Rovida E 2019).*

All these links indicate ERK5 as a good candidate to represent a new regulator of YAP activity.

PREVIOUS DATA AND AIM OF THE WORK

In the hypothesis of a regulation of YAP protein by the MEK5/ERK5 signaling pathways, preliminary studies by my research group were conducted in different liver cell models.

Firstly, a significant correlation between YAP and ERK5 subcellular localization and activity has been observed in a cell line of undifferentiated liver precursor cells named RLSCs, from Resident Liver Stem Cells, and in a cell line of terminally differentiated hepatocytes named Hep14 (both cell lines have been obtained and largely characterized in our laboratory). As shown in figure 8, while **RLSCs** show high levels of nuclear YAP and ERK5, **HepE14** cells have only residual and predominantly cytoplasmic presence of YAP protein, which correlates with a more diffuse localization of ERK5. The activities of both proteins have been investigated by means of two constructs in luciferase assays: the YAP-responsive **8XGTIIC-luc** construct, where the reporter gene is under the control of a promoter containing eight binding sites for TEAD, the main transcriptional cofactor of YAP; the ERK5-responsive **MEF2-luc** construct, where the reporter gene is under the control of a promoter responsive to MEF2D, the main transcription factor induced by ERK5 activity. As expected, YAP transcriptional activity was found higher in RLSCs than in HepE14 (Fig. 8B, right panel), as

confirmed also by the higher expression of its positive target gene, Ctgf, assessed by RT-qPCR (Fig. 8C). Importantly, ERK5 activity on its responsive construct resulted to be correlated with that of YAP (Fig. 8B, left panel).

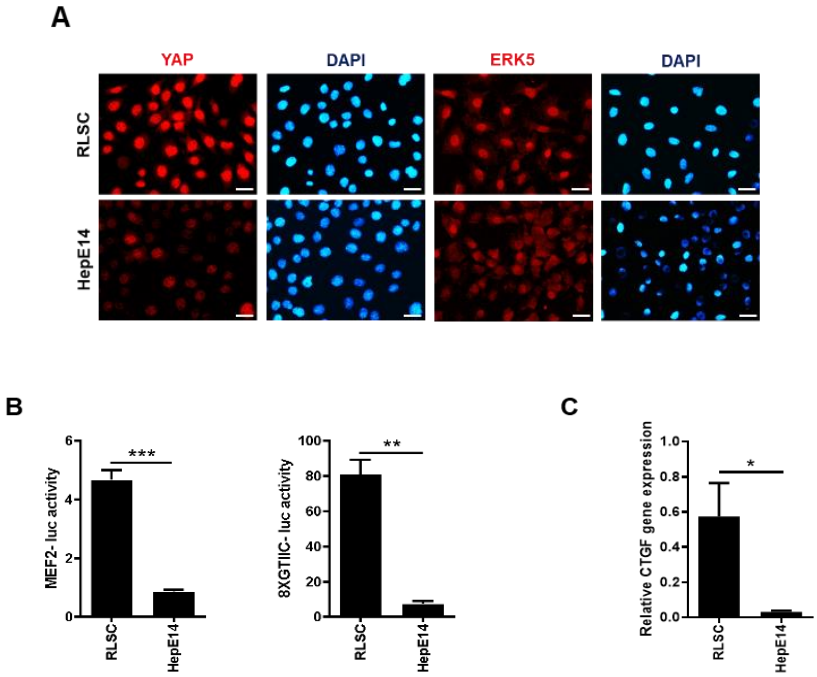

Fig. 8 ERK5 and YAP activity are directly correlated in liver cell differentiation.
A Immunofluorescence analysis of RLSC and HepE14 cell lines. Cells were stained with anti-YAP or anti-ERK5 antibodies (red) and DAPI (nuclei, blue). Scale bar: 50 µm.
B Luciferase assay. MEF2-luc or 8xGTIIC-luc reporters were transiently co-transfected in RLSC and HepE14 cell lines, together with a Renilla expression vector. Luciferase activities were normalized for Renilla luciferase activity and expressed as arbitrary units. Statistically significant differences are reported (**$p < 0.01$; ***$p < 0.001$).
C RT-qPCR analysis of Ctgf gene expression in RLSC and HepE14 cell lines. The values are calculated by the $2(-\Delta Ct)$ method and shown as means ± S.E.M. of at least three independent experiments. Statistically significant differences are reported (*$p < 0.05$).

Starting from the knowledge that YAP is modulated by mechanical cues, in particular by ECM stiffness signals (*Dupont S et al. 2011*), the correlation between YAP and ERK5 activity has been assessed in RLSCs grown on substrates of different rigidity. Interestingly, while cells grown on a high stiffness substrate showed high levels of nuclear YAP (Fig. 9A, upper panels) with a significant activity of both proteins (Fig. 9B and C), RLSCs cultured on a low stiffness substrate showed a strong reduction of nuclear localization of YAP (Fig. 9A lower panels) with a corresponding decrease of their activity (Fig. 9B and C).

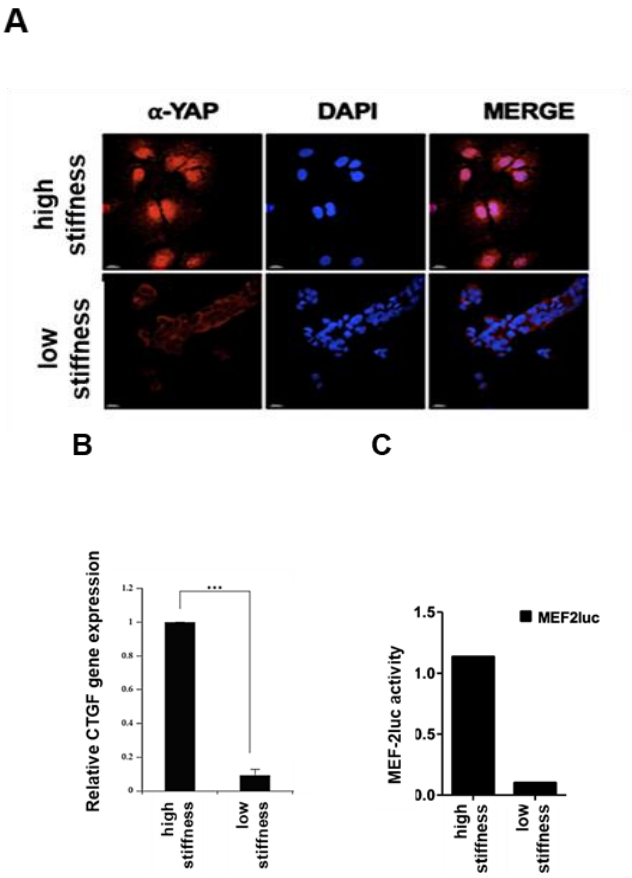

Fig. 9 ERK5/YAP correlation is maintained in ECM stiffness dynamics.
A Immunofluorescence analysis of RLSC cell lines. Cells were stained with an anti-YAP antibody (red) and DAPI (nuclei, blue). Scale bar: 50 µm.
B RT-qPCR analysis of Ctgf gene expression in RLSC cells grown in high (plastic) or low (hydrogel 0,4 kPa) stiffness conditions. The values are calculated by the 2(−ΔCt) method and shown as means ± S.E.M. of at least three independent experiments. Statistically significant differences are reported (**p<0,01; ***p<0,001).
C Luciferase assay. MEF2-luc reporter was transiently co-transfected in RLSC cells in the two different stiffness conditions, together with a Renilla expression vector. Luciferase activities were normalized for Renilla luciferase activity and expressed as arbitrary units.

Finally, the correlation between YAP and ERK5 was found in HepE14 cells induced in EMT by the treatment with the cytokine TGFβ, which exerts its trans-differentiating function by activating ERK5 (*Marchetti A et al. 2008*). In EMT-induced cells, indeed, in addition to the expected increase of MEF2-luc vector activity (Fig.10A, left panel), a significant increase of 8XGTIIC-luc vector activity (Fig. 10A, right panel) and a consistent increase in the expression of the two positive target genes of YAP, CTGF and Cyr61 (Fig. 10B), were observed.

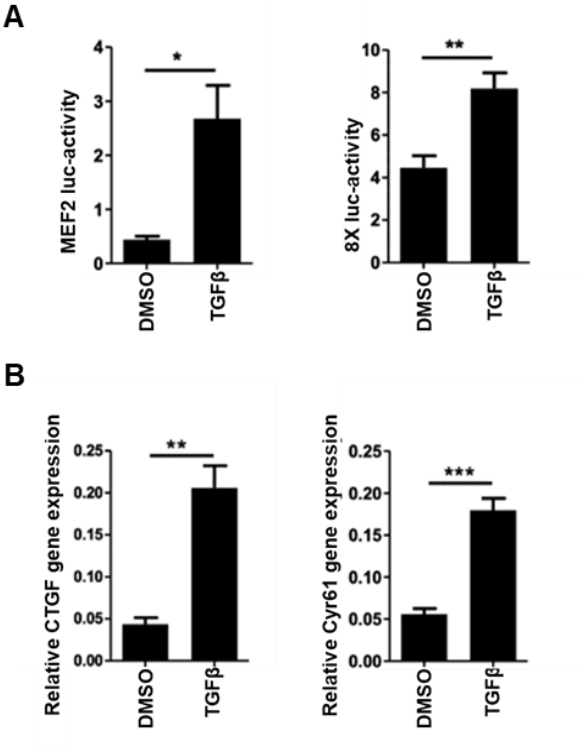

Fig. 10 ERK5 and YAP are activated by TGFβ in HepE14 cells.

A Luciferase assay. MEF2-luc or 8xGTIIC-luc reporters were transiently co-transfected in HepE14 cells treated or not with TGFβ, together with a Renilla expression vector. Luciferase activities were normalized for Renilla luciferase activity. The values, obtained in at least three independent experiments, are expressed as arbitrary units. Statistically significant differences are reported (**p<0,01; ***p<0,001).

B RT-qPCR analysis of Ctgf and Cyr61 gene expression in HepE14 cells treated or not with TGFβ. The values are calculated by the 2(−ΔCt) method and shown as means ± S.E.M. of at least three independent experiments. Statistically significant differences are reported (**p<0,01; ***p<0,001).

Starting from the several data described above, showing functional links between ERK5 and YAP and strongly supporting the hypothesis that ERK5 could be involved in the regulation of YAP activity, the aim of my work was to verify this hypothesis and eventually to identify the molecular mechanisms involved, also in the belief that the unveiling of a new regulator of YAP activity would be relevant for therapeutic approaches aimed at interfering with its function.

MATERIALS AND METHODS

Cell cultures and treatments

Resident liver stem cells (RLSCs) and hepatocytes E14 (HepE14) are immortalized and non-tumorigenic cell lines derived from murine liver explants at 14th days of development (*Amicone L et al. 1997; Conigliaro A et al. 2008; Conigliaro A et al. 2013*).

RLSCs are stem/precursor cells with typical stemness gene expression profile, self-renewing capability and multi-lineage differentiation potential. In fact, RLSC could spontaneously differentiate in terminally differentiated hepatocyte or cholangiocyte; moreover, they could also differentiate in various cell profiles (such as astrocyte or osteoblast) answering to precise differentiation stimuli (*Conigliaro A et al. 2008)*. RLSCs were maintained at 37 °C, in a humidified atmosphere with 5% CO2 on collagen I (Collagen I, Rat Tail; Gibco-Life Technologies) coated dishes in Dulbecco's modified Eagle's medium (DMEM; GibcoLife Technologies), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine (Gibco-Life Technologies) and antibiotics.

HepE14 are hepatocytes displaying a differentiated phenotype and a coherent gene expression profile. They have been used in a variety of studies of hepatocyte physiology being able to express a wide range of liver functions and products (*Gao C et al. 2021; Fitamant J et al. 2015; Ortega A et al. 2021*). HepE14 cells were grown at 37 °C, in a humidified atmosphere with 5% CO2 on collagen I (Collagen I, Rat Tail; Gibco-Life Technologies) coated dishes in RPMI-1640 medium (Gibco-Life Technologies), supplemented with 10% FBS, 2 mM glutamine (Gibco-Life Technologies), 50 ng/ml EGF, 30 ng/ml IGF II (PeproTech), 10 µg/ml insulin (Roche) and antibiotics.

Huh7 is a hepatocellular carcinoma cell line of well-differentiated hepatocytes, originally obtained from a tumorigenic liver of a 57-year-old Japanese male in 1982. HepG2 is a human hepatoma cell line, derived in 1975 from the liver tissue of a 15-year-old Caucasian male from Argentina with a well-differentiated HCC. The human liver carcinoma cell lines HuH7 and HepG2 were grown at 37 °C, in a humidified atmosphere with 5% CO2 on plastic (Corning) in Dulbecco's modified Eagle's medium (DMEM; Gibco-Life Technologies), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine (GibcoLife Technologies) and antibiotics (Gibco-Life Technologies). For the experiments in 3D cell culture, HuH7 cells

were grown in a low attachment substrate (0.6% agar) until the formation of suspended aggregates/spheroids (24 h).

Where indicated, cells were treated with 10 μM ERK5 inhibitor XMD8-92 (Selleckchem, Selleck Chemicals GmbH), 10 μM or 20 μM MEK5 inhibitor BIX02189 (Selleckchem, Selleck Chemicals GmbH), 10 μM of YAP-TEAD inhibitor Verteporfin or 4 ng/ml of TGFβ1 (PeproTech Inc., Rocky Hill, NJ, USA) for the indicated time. As previously reported, hepatocytes utilized in this study undergo EMT following TGFβ treatment (*Cozzolino AM et al. 2013; De Santis Puzzonia M et al. 2016).* The number of viable cells upon 16 h of treatment with MEK5/ERK5 inhibitors has been analyzed by CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega), following the manufacturer's protocol.

Cell transfections

ERK5-overexpressing cells were obtained by transient transfection with pCMV-ERK5 (carrying the human ERK5 cDNA, kindly provided by J.E. Dixon) and with pCMV-MEK5DD (carrying a phosphomimetic mutant sequence of human MEK5 cDNA, kindly provided by C.J. Marshall). Control cell lines were obtained by transfection with the

empty vector. Cells were transfected with Lipofectamine™ LTX Reagent with PLUS™ Reagent (Thermo Fisher Scientific), according to the manufacturer's protocol, and collected 48 h after transfection. YAP-overexpressing cells were obtained by transient transfection with pQCXIH-Myc-YAP or pQCXIH-Myc-YAP5SA (gift from Kunliang Guan, Addgene plasmids # 33091 and # 33093) (*Zhao B et al. 2007)*, respectively, using Lipofectamine™ LTX Reagent with PLUS™ Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Cells were collected 48 h after transfection or utilized for treatments. Notably, YAP5SA protein, carrying mutations of LATS1/2-dependent phosphorylation sites (S61A, S109A, S127A, S164A, S381A), is insensible to LATS inhibition and is therefore constitutively active (*Zhao B et al. 2007)*.

Luciferase assays

Luciferase Assay is a technique used to study the activity of a specific protein on a target promoter. In particular, utilize a reporter gene which encodes for the firefly Photinus Pyralis luciferase under the control of a specific promoter, object of the investigation. Luciferase is a monomeric protein capable of oxidizing the luciferin and emitting light in presence of ATP, Mg and O2. As control of the transfection

efficiency has been used a control reporter encoding for Renilla, a monomeric protein capable of emitting light when it oxidizes its substrate. Therefore, to analyze endogenous ERK5 and YAP activity, cells were plated in 60 mm plates and co-transfected by Lipofectamine™ LTX with PLUS™ Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol with the following constructs: MEF2-luc reporter (*Woronicz JD et al. 1995*) or 8XGTIIC-luc reporter (gift from Stefano Piccolo; Addgene plasmid # 34615) *(Dupont S et al. 2011)* (1 μg), Renilla expression vector (0.2 μg), pcDNA3 empty vector (4 μg). After 24 h, cells have been moved into 12-well plates and treated with MEK5 or ERK5 inhibitors or with their solvent DMSO, where indicated. All treatments were performed in triplicate. To analyze ERK5- and YAP-dependent transcriptional activity, cells were plated in 12-wells plates and co-transfected by Lipofectamine™ LTX with PLUS™ Reagent (Thermo Fisher Scientific) with the following constructs: MEF2-luc reporter or 8XGTIIC-luc reporter (0.5 μg), Renilla expression vector (0.1 μg), pCMV-ERK5/pCMV-MEK5DD (1.5 μg/0.5 μg) or the empty vector (2 μg). All transfections were performed in triplicate. Luciferase activity was measured by using the Dual-Luciferase Reporter Assay System kit (Promega Corporation, Madison, WI), according to the manufacturer's instructions and normalized for Renilla luciferase activity.

Immunofluorescence staining

For indirect immunofluorescence analysis, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X100, and incubated with mouse monoclonal α-YAP antibody (SC-101199, Santa Cruz Biotechnology, inc.; 1:50) or rabbit polyclonal α-ERK5 antibody (#3372, Cell Signaling; 1:50). Alexa CY3-conjugated secondary antibodies (1:400; Molecular Probes, Eugene, OR, USA) were utilized. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Calbiochem Merck, Darmstadt, Germany). Images were acquired with a Nikon Eclipse microscope (Nikon Corporation, Tokyo, Japan) equipped with a charge-coupled device camera. Digital images were acquired by Nikon NIS elements software (Nikon Corporation, Tokyo, Japan) and processed with Adobe Photoshop 7 software (Adobe Systems, Mountain View, CA). The same enhanced color levels were applied for all channels.

Western Blot Analysis

Cells were lysed in a RIPA buffer containing freshly added cocktail protease inhibitors (Sigma-Aldrich, St. Louis, MO). Equal amounts of proteins were loaded on 8% (for the analysis of ERK5 phosphorylation) or 12% acrylamide gels and then transferred to a

nitrocellulose membrane (Bio-Rad). Blots were probed with the following primary antibodies: mouse monoclonal αYAP (SC-101199, Santa Cruz Biotechnology, 1:1000), rabbit polyclonal αERK5 (#3372, Cell Signaling; 1:500) and mouse monoclonal α-GAPDH (MAB374, Millipore Corp., Bedford, MA, USA; 1:1000). Blots were then incubated with HRP-conjugated species-specific secondary antibodies (BioRad, Hercules, CA, USA), followed by Enhanced Chemiluminescence reaction (ECL, Bio-Rad Laboratories Inc., Hercules, CA, USA).

RNA isolation and quantitative RT-PCR

Total RNAs were extracted with ReliaPrep™ RNA Miniprep Systems (Promega) according to the manufacturer's protocol and reverse-transcribed using Biorad iSCRIPT cDNA Synthesis Kit (BioRad). cDNA was amplified by qPCR using GoTaq qPCR Master Mix (Promega Corporation, Madison, WI) in BioRad-iQ-iCycler. Relative amounts, calculated with the 2(−ΔCt) method, were normalized with respect to the housekeeping gene RPL34 (60 S ribosomal protein L34). The sequence of murine and human primers utilized are listed in Table 1 and Table 2, respectively.

Table 1. List of mouse primers used for RT-qPCR experiments.

Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')
Ctgf	ATCATGCTCGCCTCCGTCGC	TAGCAGGCCGGGTGCAGAGA
Cyr61	AGAGGCTTCCTGTCTTTGGC	CCAAGACGTGGTCTGAACGA
Ddit4	GCCGGAGGAAGACTCCTCATA	CATCAGGTTGGCACACAGGT
Erk5	TCTGACTCTGCAGCCTGCCCC	GGTGCACTGGGCCCATCTCTG
Rpl34	GGAGCCCCATCCAGACTC	CGCTGGATATGGCTTTCCTA

Table 2. List of human primers used for RT-qPCR experiments.

Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')
Ctgf	AGGAGTGGGTGTGTGACGA	CCAGGCAGTTGGCTCTAATC
Cyr61	AAGAAACCCGGATTTGTGAG	GCTGCATTTCTTGCCCTTT
Ankrd1	AGTAGAGGAACTGGTCACTGG	TGGGCTAGAAGTGTCTTCAGAT
Erk5	CTGTCTACGTGGTCCTGGAC	GCCTTGTCCAAGTCCAAGTC

Rpl34	GTCCCGAACCCCTGGTAATAG	GGCCCTGCTGACATGTTTCTT
Yap	GTCCCGAACCCCTGGTAATAG	GGCCCTGCTGACATGTTTCTT

RNA interference by short hairpin RNA (shRNA) and small interfering RNA (siRNA)

YAPand ERK5 genetic silencing has been performed by RNA interference (RNAi), by the use of two types of molecules: the chemically synthesized double-stranded small interfering RNA (siRNA) or vector-based short hairpin RNA (shRNA). In this work both types of molecules have been used.

pSUPER-shERK5 vector encoding shRNA specific for ERK5 was constructed according to Brummelkamp et al., 2002 (*Brummelkamp TR et al. 2002*). The target sequence in both mouse and human ERK5 mRNA was 5'-TGAGAACTGTGAGCTCAAG-3'. Cells were transfected with pSUPER-shERK5 or the empty vector (from OligoEngine, Seattle, WA, USA) and utilized after 48 h for the experiments. Knockdown efficiency was confirmed by Western Blotting and RT-qPCR.

For siRNA-based ERK5 and YAP silencing, cells were transfected with equal amounts (100 pmol) of ON-TARGET plus SMARTpool mouse

ERK5 siRNAs (L-040333-00-0005, GE Healthcare Dharmacon, Lafayette, CO, USA), ON-TARGET plus SMARTpool mouse YAP1 siRNA (22601; GE Healthcare Dharmacon, Lafayette, CO, USA), siRNA_YAP_hsa (5'-GACAUCUUCUGGUCAGAGAdTdT-3' + 5'-UCUCUGACCAGAAGAUGUCdTdT-3') using Lipofectamine RNAiMAX (Invitrogen) reagent in OptiMEM following the manufacturer's protocol. RNA and proteins were harvested and analyzed after 48 h. siRNA against GFP (Gene Pharma) or Silencer™ Negative Control No. 1 siRNA (#AM4611, Ambion) were utilized as negative controls.

Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) is used to study YAP protein interaction with DNA. For the immunoprecipitation 5 µg of the following antibodies have been used: rabbit polyclonal α-YAP (H-125X, Santa Cruz Biotechnology Inc.), or rabbit monoclonal α-YAP (D8H1X, 14074, Cell Signaling) or the negative control rabbit IgG (12370, Millipore Corp., Bedford, MA, USA). Equal amounts of immune-precipitated DNA and relative controls were used for qPCR analysis, performed in triplicate. The primers utilized are the followings: Ctgf promoter, forward 5'- CAATCCGGTGTGAGTTGATG-3' and reverse 5'-GGCGCTGGCTTTTATACG-3'; Neurogenin 1, forward 5'-

CCTCCCGCGAGCATAAATTA-3′ and reverse 5′-GCGATCAGATCAGCTCCTGT-3′. The promoter of Neurogenin1, a gene not expressed in liver cells, was used as negative control. qPCR analysis of the immunoprecipitated samples (IP) and of the negative controls (IgG) were both normalized to total chromatin input and expressed as (IP/IgG)/Input.

Co-Immunoprecipitation assay

To unveil protein-protein interactions a Co-Immunoprecipitation assay has been performed. In order to have a better immunoprecipitation efficiency, YAP protein level have been increased by a transfection of HuH7 cells by Lipofectamine 3000 (Invitrogen) with pQCXIH-Myc-YAP plasmid and treated with BIX02189 or DMSO at 24 h post-transfection. Cells were harvested 16 h after treatments and lysed in Triton lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 2 mM EDTA, 1% Triton-X 100, 10% glycerol) supplemented with protease and phosphatase inhibitors. For immunoprecipitation, protein extracts (1 mg) were precleared with protein protein G-Sepharose (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 1 h and then incubated with 5 µg of mouse antiTEAD antibody (ab58310, Abcam) or with normal mouse IgG

(12371, Millipore Corp., Bedford, MA, USA), at 4 °C overnight. Then, protein G was added and incubated for 2 h. The beads were then washed three times in NetGel buffer (150 mM NaCl; 50 mM Tris–HCl pH 7.5; 1 mM EDTA; 0.1% NP-40; 0.25% gelatin) and twice with Triton lysis buffer. The immune complexes were eluted and denatured with Laemmli buffer 1X. Total and immunoprecipitated proteins were resolved on SDS-PAGE and transferred to the nitrocellulose membrane. For immunoblotting, the following primary antibodies were used: mouse polyclonal α-YAP (SC101199, Santa Cruz Biotechnology, inc.; 1:1000), mouse polyclonal α-TEAD4 (ab58310, Abcam, 1:500), mouse monoclonal α-tubulin (B-7, sc-5286, Santa Cruz Biotechnology, 1:1000) Blots were then incubated with HRPconjugated species-specific secondary antibodies (Bio-Rad,Hercules, CA, USA) or Goat α-mouse IgG light-chain specific antibody (HRP conjugate, #91196, Cell Signaling Technology), followed by Enhanced Chemiluminescence reaction (ECL, Bio-Rad Laboratories Inc., Hercules, CA, USA).

Adhesion assay

For adhesion assays, HuH7 and HepG2 cells were seeded in non-coated plates for 24 h, then trypsinized, maintained in suspension for

10', and collected or plated in triplicate on collagen-coated plates (to promote cell adhesion) in the presence of DMSO or 10 μM BIX02189. After 3 and 4 h, respectively, cells were harvested and analyzed for gene expression.

Wound healing assay

For the migration assay, YAP5SA-expressing and parental HuH7 cells were plated at high density on 35 mm dishes. 24 h later, the confluent layer of cells was scratched with a sterile tip to create an artificial wound. After rinsing with PBS to remove unattached cells, a low serum medium (0.5% FBS) was added in the presence of 10 μM BIX02189 or DMSO for 16 h. Cell migration was then analyzed by an optical microscope. Images were captured by Optech Digital Camera (Optech Technology) and the distance between the edges of the wound was manually quantified and expressed as average gap width.

Mouse strains and treatments

For in vivo studies, transgenic mice that conditionally overexpress YAP have been used. YAP overexpression is under the control of the

doxycycline (Dox)–inducible TetON system. These mice showed hepatocyte-specific expression of YAP because the reverse tetracycline transactivator (rtTA) is under the control of the hepatocyte-specific ApoE promoter.

In the second experiment, a specific strain LATS fl/fl of C57BL/6J mice has been used. These mice have two LoxP sites flanking the LATS gene; therefore, after the Cre-expressing adeno-associated virus (AAV-Cre) injection, a conditional deletion of LATS1/2 encoding genes is obtained. AAV-Cre expresses Cre under the control of hepatocyte-specific thyroxine-binding globulin promoter (TBG promoter, UPenn, USA) and has been delivered in the liver by tail vein injection, diluted in 200 µl of phosphate buffered saline (PBS, VWR Chemicals, USA).

In the first experiment, mice (three/group) were treated daily with BIX 02189 (30 mg/Kg) or with Captisol 30%, by intraperitoneal injection for 12 days. In the second experiment five mice/group have been utilized.

Mice were housed, fed and treated in accordance with protocols approved by the committee for animal research at KU Leuven. All mouse experiments were approved by the institutional ethical

commission at KULeuven and performed in accordance with relevant institutional and national guidelines and regulations.

Processing of livers

At the end of the experiment, mice were sacrificed with an automated CO_2 program, and livers were explanted. The organs were kept shaking in formaldehyde solution or PFA (Sigma-Aldrich, USA) 4% in PBS for 48h at 4°C, then washed 3 times with PBS for 10'. One lobe was paraffin embedded and then sectioned for future H&E and Sirius Red staining. The remaining part of the liver is kept in ethanol (EtOH) 70%. Alternatively, RNAs were extracted by liver tissues RNAs with ReliaPrep™ RNA Miniprep Systems (Promega) according to the manufacturer's protocol, reverse-transcribed using Biorad iSCRIPT cDNA Synthesis Kit (BioRad) and then analyzed for RT-qPCR.

Haemaluin & Eosin (H&E) and Sirius Red staining

Samples were processed by the STP 120.3 Spin Tissue Processor by Microm (Walldorf) and then embedded in Surgipath Paraplast Plus

(Leica). Liver sections (4 µm) were obtained in the HM 355S Automatic Microtome (Thermo Fisher Scientific, USA).

Mayer's Haemaluin was prepared following the protocol of Langeron and Eosin 1% in aqueous solution was directly obtained from RAL Diagnosis (France). The Picrosirius Red stain (also called "Sirius red" stain) is one of the most used histochemical techniques to selectively visualize collagen networks. The staining was performed according to standard procedures. Mounting of the glasses was performed using DPX (Sigma-Aldrich). Pictures were automatically taken by the Axio Scan.Z1 Slide Scanner (ZEISS, Germany).

Statistical analysis

Statistical significance was determined by one-tailed paired Student's t test or one-sample t test using GraphPad Prism Version 5 (GraphPad Software).

RESULTS

1. ERK5 is required for gene expression driven by endogenous or overexpressed YAP in liver cells

To evaluate whether ERK5 function was only correlative or rather regulative of YAP activity, experiments of ERK5 inactivation/silencing were performed in RLSCs. To inactivate ERK5 kinase, we used two commercial and well-characterized chemical inhibitors, BIX02189, a MEK5 inhibitor (*Tatake RJ et al. 2008*), and XMD8-92, ERK5 inhibitor *(Yang Q et al. 2010*).

Firstly, to determine the correct doses of the inhibitors to obtain an effective inactivation of the enzyme without effects on cell viability, the specific IC50 (also EC50) of both inhibitors has been evaluated in a dose-response analysis of ERK5 activity in RLSC and HUH7 cells, by a luciferase assay with the MEF2-luc reporter. In the same experiments, the proliferation/vitality of cells has been also evaluated. As shown in Fig. 11, the effects on ERK5 activity and cell proliferation of increasing doses of XMD8-92 in RLSCs (left panel) and of BIX02189 in HuH7 (right panel) indicated 10 μM as the concentration of the inhibitors providing the most significant

inhibition of ERK5 activity without a significantly impact on cell proliferation/vitality.

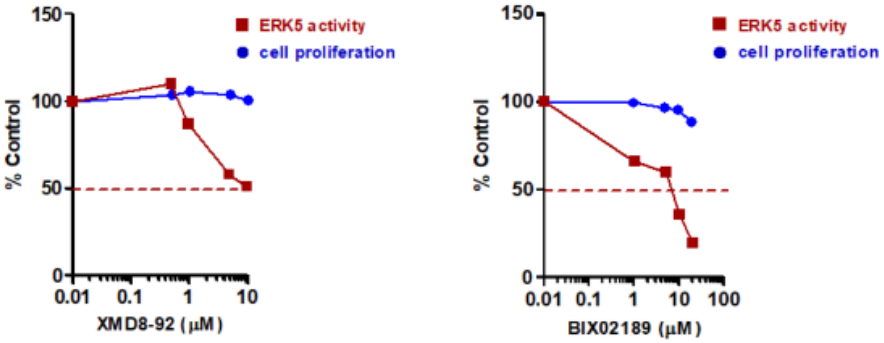

Fig. 11 Dose-response analysis of ERK5 activity and cell proliferation.
RLSCs (left panel) and HuH7 (right panel) cells treated with XMD8-92 and BIX02189, respectively. ERK5 activity has been assessed by luciferase assay with the MEF2-luc. Cell proliferation has been assessed by CellTiter 96® AQueous One Solution Cell Proliferation Assay. All analyses have been performed at 16 h of treatment.

The effect of ERK5 inactivation on YAP transcriptional activity has been therefore investigated in RLSCs treated with XMD8-92, transiently transfected with the ERK5- or YAP-responsive construct. As shown in Figure 12, when the ERK5 activity was inhibited, a significant decrease of YAP transcriptional activity both on its

exogenous reporter gene (Fig. 12A) and on its endogenous positive target genes Ctgf and Cyr61 (Fig. 12B) could be observed. A similar effect on the endogenous YAP activity was obtained after the treatment with the MEK5/ERK5 inhibitor BIX02189 (Fig. 12C). Importantly, the results obtained with the ERK5 chemical inhibition were confirmed in experiments of genetic inactivation, achieved with the use of specific small interfering RNAs (siRNA) (Fig. 12D) and of a vector expressing a silencing short hairpin RNA (shRNA) (Fig. 12E). To verify if the observed effects of ERK5 inhibition on YAP-dependent transcription could be dependent on a reduction of YAP expression, we performed a Western Blot analysis indicating that the YAP protein level was unaffected by both chemical inhibition and genetic silencing of ERK5 (Fig. 12F). Of note, also the expression of the TAZ protein has been found unchanged in the same experimental conditions (Fig. 12F).

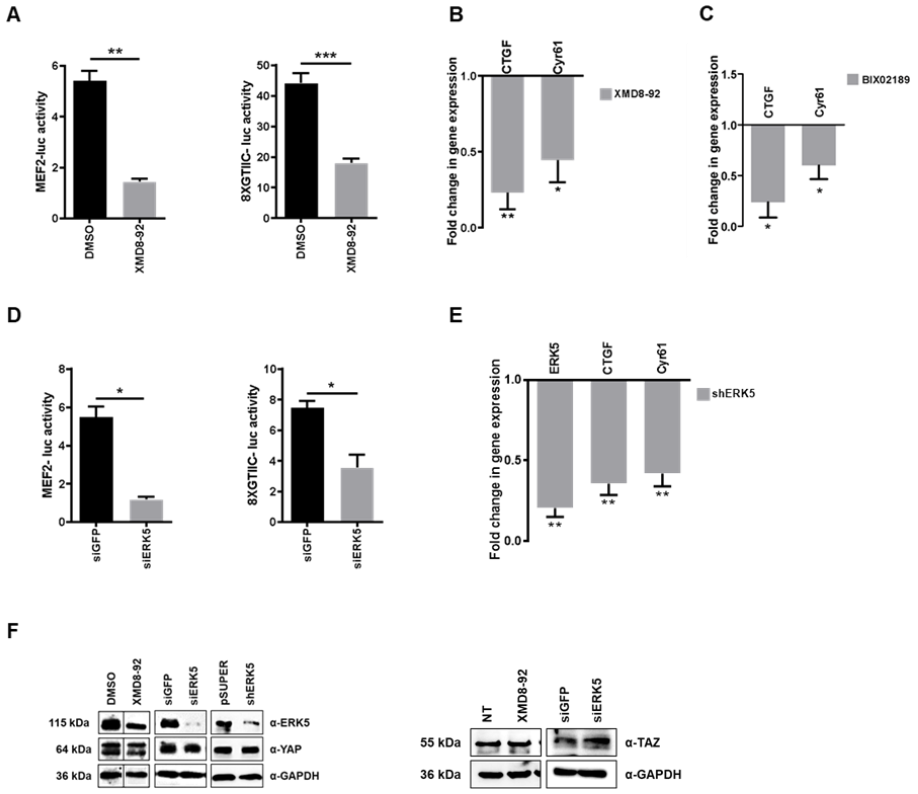

Fig. 12 ERK5 is required for the steady-state YAP transcriptional activity.
A Luciferase assay. MEF2-luc or 8xGTIIC-luc reporters were transiently co-transfected in RLSCs, together with a Renilla expression vector. Luciferase activities were normalized for Renilla luciferase activity and expressed as arbitrary units. Twenty-four hours post-transfection, cells were treated with 10 μM XMD8-92 or its solvent DMSO for 16 h. Statistically significant differences are reported (**p < 0.01; ***p < 0.001).
B RT-qPCR analysis of the indicated genes in RLSCs treated with XMD8-92 or DMSO. The values are calculated by the 2(−ΔCt) method, expressed as fold change in gene expression versus the control (DMSO, arbitrary value = 1) and shown as means ± S.E.M. of at least three independent experiments. Statistically significant differences are reported (*p < 0.05; **p < 0.01).
C RT-qPCR analysis of the indicated genes in RLSCs treated with 20 μM BIX02189 or DMSO. The values are calculated by the 2(−ΔCt) method, expressed as fold change in gene expression

versus the control (DMSO, arbitrary value=1) and shown as means ± S.E.M. of at least three independent experiments. Statistically significant differences are reported (*p<0.05).
D Luciferase assay. MEF2-luc or 8xGTIIC-luc reporters were transiently co-transfected in RLSCs, together with a Renilla expression vector and siERK5 or siGFP. Luciferase activities were normalized for Renilla luciferase activity and expressed as arbitrary units. Statistically significant differences are reported (*p < 0.05).
E RT-qPCR analysis of the indicated genes in RLSCs stably transfected with pSUPER or pSUPER-shERK5 vector. The values are calculated by the 2(−ΔCt) method, expressed as fold change in gene expression versus the control (pSUPER, arbitrary value = 1) and shown as means ± S.E.M. of at least three independent experiments. Statistically significant differences are reported (**p < 0.01).
F Western blot for the indicated proteins in RLSCs as in (A), (C), and (D). GAPDH has been utilized as loading control. WB images represent one indicative experiment of at least three independent ones.

Being YAP involved in the onset and progression of many types of human cancer, we verified the ERK5-dependent YAP regulation also in tumor cell lines. In the human hepatoma cell line, HUH7, the chemical inhibition of MEK5/ERK5 obtained with BIX02189 (Fig. 13A and 13B) and the genetic silencing of the kinase by shERK5 (Fig. 13C and 13D) significantly downregulated YAP-dependent transcription without affecting its expression (as well as that of its paralogous TAZ) (Fig. 13B and data not shown). Similar results have been obtained in a second human hepatoma cell line, HepG2 (Fig. 14A and 14B).

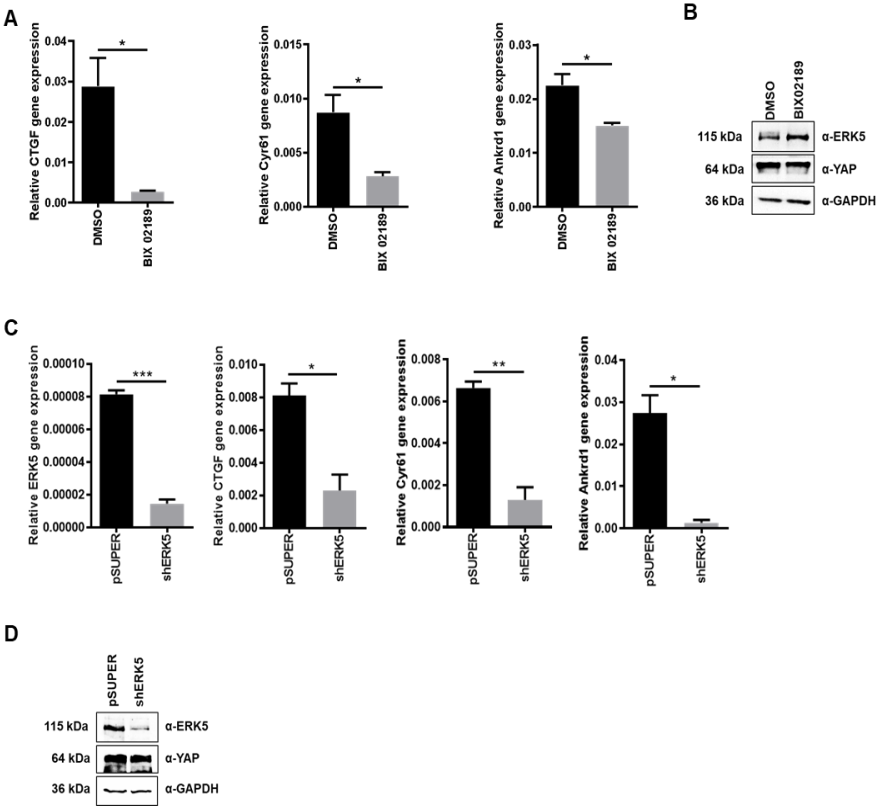

Fig. 13 ERK5 is required for endogenous YAP transcriptional activity in HuH7.
A RT-qPCR analysis of the indicated genes in HuH7 treated with 10 μM BIX02189 or DMSO. Data are expressed as relative gene expression and shown as mean ± S.E.M. of three independent experiments. Statistically significant differences are reported (*p < 0.05).
B Western blot for the indicated proteins in HuH7 as in (A). GAPDH has been utilized as loading control.
C RT-qPCR analysis of the indicated genes in HuH7 transfected with pSUPER or pSUPERshERK5 vector. Data are expressed as in (A). Statistically significant differences are reported (*p < 0.05; **p < 0.01; ***p < 0.001).
D Western blot for the indicated proteins in HuH7 as in (C). GAPDH has been utilized as loading control.

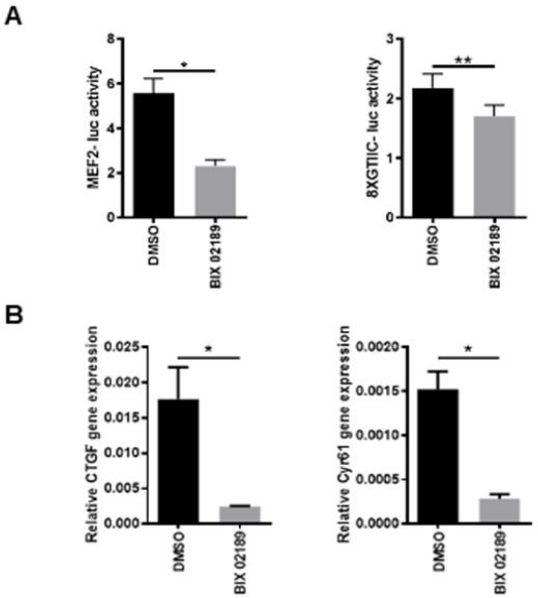

Fig. 14 ERK5 is required for endogenous YAP transcriptional activity in HepG2.
A Luciferase assay. MEF2-luc or 8xGTIIC-luc reporters were transiently co-transfected in HepG2 cells. Twenty-four hours post-transfection, cells were treated with 10μM BIX02189 or its solvent DMSO for 16h. Luciferase activities were normalized for Renilla activity and expressed as arbitrary units. Statistically significant differences are reported (*p<0.05; **p<0.01).
B RT-qPCR analysis of the indicated genes in HepG2 treated with BIX 02189 or DMSO. The values are calculated by the 2(−ΔCt) method, expressed as means ± S.E.M. of at least three independent experiments. Statistically significant differences are reported (*p<0.05).

Notably, the effect of the ERK5 inhibition has been assessed also in 3D cultured HuH7, an experimental condition that better simulates the drug uptake in vivo. Also in these experiments a downregulation of the YAP target genes CTGF and CYR61 can be observed, although,

as expected, at less significant level compared to that obtained in 2D cell culture (Figure 15).

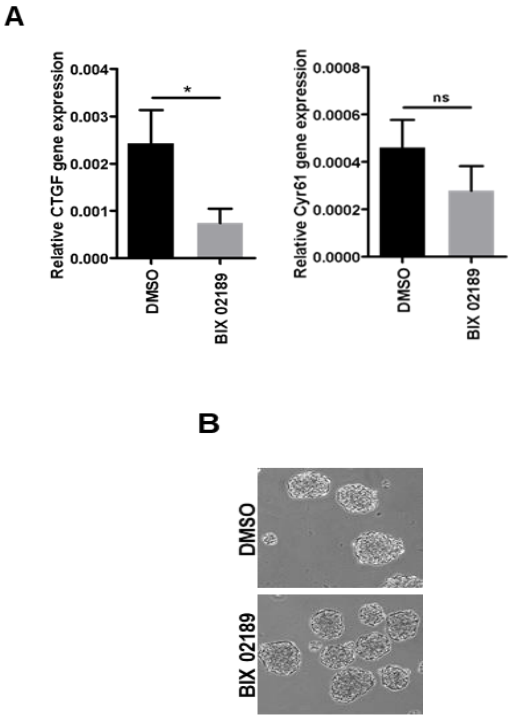

Fig. 15 ERK5 is required for endogenous YAP transcriptional activity in 3D cell culture condition.

A. RT-qPCR analysis of the indicated genes in HuH7 cells, grown as spheroids for 24 h and treated with 10 μM BIX02189 or DMSO. Data are expressed as relative gene expression and shown as mean ± S.E.M. of three independent 3D cell cultures. Statistically significant differences are reported (unpaired, one-tailed Student's t test; *$p < 0.05$; ns= not significant).

B. Representative images of spheroids in the two different cell conditions are shown.

Furthermore, to simulate a tumoral situation in which a very high level of YAP oncoprotein can be expressed, the effect of ERK5 chemical inhibition was tested also in HUH7 ectopically expressing the wild-type YAP protein. As shown in Figure 16, the YAP induced transcription of the reported target genes was impaired by treatment with BIX02189.

Overall, these data demonstrated that ERK5 activity is required for YAP target gene expression in liver stem/progenitor cells and in hepatoma cells.

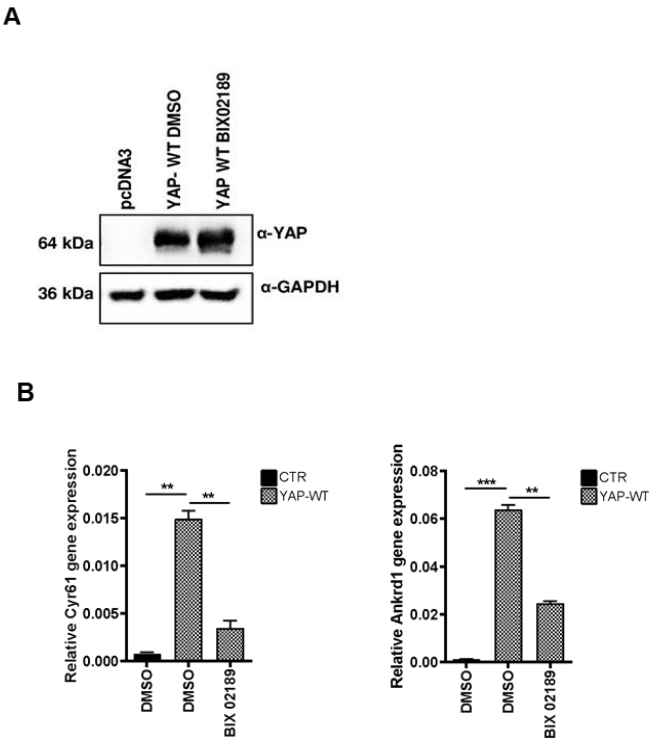

Fig. 16 ERK5 is required for exogenous YAP transcriptional activity in HuH7.
A Western blot for the indicated proteins in YAP WT mutant overexpressing HuH7, treated for 16h with DMSO or 10 μM BIX02189. GAPDH has been utilized as loading control.
B RT-qPCR analysis of the indicated YAP target genes in wild-type YAP-overexpressing HuH7 cells (YAP-WT) and in control cells (CTR), treated with 10 μM BIX02189 or DMSO. Data are expressed as relative gene expression and shown as mean ± S.E.M. of three independent experiments. Statistically significant differences are reported (**p < 0.01; ***p < 0.001).

2. ERK5 activation is sufficient to promote YAP transcriptional activity

To further investigate the role of ERK5 in the regulation of YAP activity, we performed experiments of ERK5 overexpression/activation. HepE14 cell line, characterized by low level of both YAP and ERK5 activity, have been transiently transfected with an ERK5 expressing vector together with a construct expressing a constitutively active mutant of the upstream kinase MEK5 (caMEK5) (*Barros JC & Marshall CJ 2005*) which activates specifically ERK5. As shown in Fig. 17, ERK5/caMEK5-overexpression induced in HepE14 a significant increase of the YAP-responsive reporter activity, indicating a functional activation of YAP by ERK5. Of note, ERK5 overexpression, as well as ERK5 knockdown, did not affect YAP expression at the protein level (Fig. 17B). However, the modulation of YAP endogenous target genes cannot be observed, suggesting that ERK5 activation is not sufficient to promote YAP -dependent target gene expression in HepE14. This result could be related to the terminally differentiated state of these cells where the chromatin state/configuration and the low endogenous level of YAP protein could not permit the expression of YAP target genes by ERK5. To verify this hypothesis, the experiment has been repeated in RLSCs where the experimental over-activation of MEK5/ERK5 signaling induced the upregulation of

Ctgf and Cyr61 and the downregulation of Ddit4, positive and negative YAP-target genes, respectively (Fig. 17C). Similar results have been obtained in HuH7 cells overexpressing caMEK5/ERK5, where a significant upregulation of the canonical YAP target genes CTGF, CYR61, and ANKRD1 has been observed (Fig. 17D).

These results further demonstrate an ERK5 dependent regulation of YAP target genes. Moreover, to exclude that this modulation could be independent from YAP and that ERK5 could be able to induce the transcription of these genes by an alternative way or also by itself, through its transactivation domain, the experiment of ERK5 overexpression has been carried out in condition of YAP inhibition. Specifically, ERK5/caMEK5 overexpressing cells were treated with Verteporfin, a chemical inhibitor of YAP activity, or genetically silenced with a siRNA specific for YAP. In both cases, the ERK5-dependent upregulation of YAP target genes has been impaired, thus excluding a YAP independent regulation of these genes by the kinase (Fig. 17D, E, F). Overall, these data demonstrate that the ERK5 activation is sufficient to promote YAP transcriptional activity in permissive liver cells.

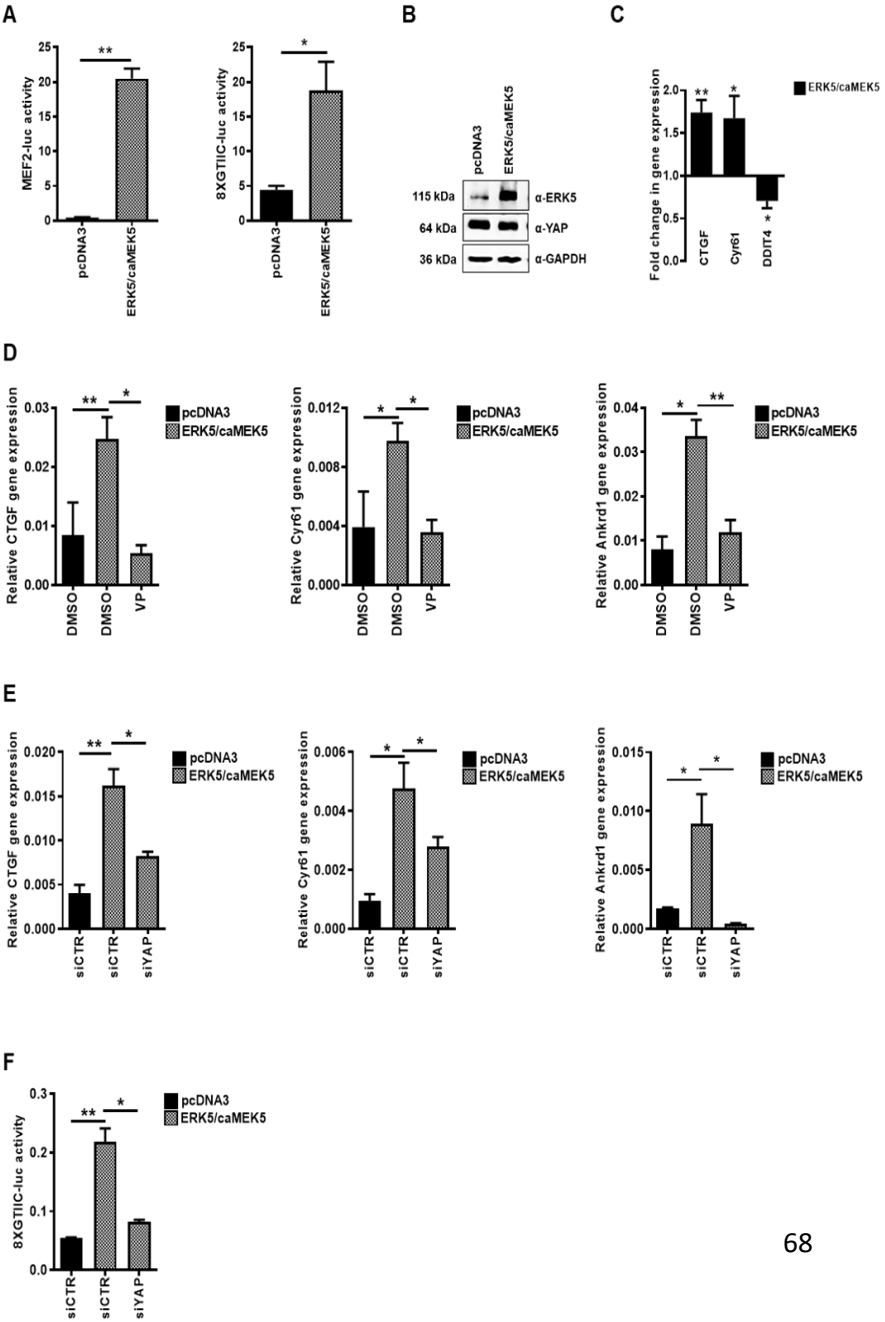
A
MEF2-luc activity
8XGTIIC-luc activity
pcDNA3
ERK5/caMEK5
B
pcDNA3
ERK5/caMEK5
115 kDa
64 kDa
36 kDa
α-ERK5
α-YAP
α-GAPDH
C
ERK5/caMEK5
Fold change in gene expression
CTGF
Cyr61
DDIT4
D
Relative CTGF gene expression
Relative Cyr61 gene expression
Relative Ankrd1 gene expression
DMSO
VP
E
siCTR
siYAP
F
8XGTIIC-luc activity

Fig. 17 ERK5 constitutive activation promotes YAP-dependent expression of target genes in HUH7.

A Luciferase assay. MEF2-luc or 8xGTIIC-luc reporters were transiently co-transfected in HepE14 cells, together with a Renilla expression vector, an ERK5 and a constitutive active MEK5 expressing vector (ERK5/caMEK5) or the empty vector (pcDNA3). Luciferase activities were normalized for Renilla luciferase activity and expressed as arbitrary units. Statistically significant differences are reported (*$p < 0.05$; **$p < 0.01$).

B Western blot for the indicated proteins in ERK5/caMEK5- overexpressing HepE14. GAPDH has been utilized as loading control. WB images represent one indicative experiment of three independent ones.

C RT-qPCR analysis of the indicated YAP target genes in ERK5/caMEK5-overexpressing RLSCs. The values are calculated by the $2(-\Delta Ct)$ method, expressed as fold change in gene expression versus the control (empty vector, arbitrary value = 1) and shown as means ± S.E.M. of at least three independent experiments. Statistically significant differences are reported (*$p < 0.05$; **$p < 0.01$).

D RT-qPCR analysis of the indicated YAP target genes in ERK5/caMEK5-overexpressing and in control HuH7 cells, treated with 10 μM of YAP-TEAD inhibitor Verteporfin (VP) or with DMSO. The values are calculated by the $2(-\Delta Ct)$ method and shown as means ± S.E.M. of three independent experiments. Statistically significant differences are reported (*$p < 0.05$; **$p < 0.01$).

E RT-qPCR analysis of the indicated YAP target genes in YAP-silenced ERK5/caMEK5-overexpressing HuH7 cells (siYAP), compared with cells transfected with control siRNAs (siCTR). The values are calculated by the $2(-\Delta Ct)$ method and shown as means ± S.E.M. of three independent experiments. Statistically significant differences are reported (*$p < 0.05$; **$p < 0.01$).

F Luciferase assay. 8xGTIIC-luc reporter was transiently co-transfected in HuH7 cells, together with a Renilla expression vector, an ERK5 and a constitutive active MEK5 expressing vector (ERK5/caMEK5) or the empty vector (pcDNA3), in the presence of siYAP or siCTR. Luciferase activities were normalized for Renilla luciferase activity and expressed as arbitrary units. Statistically significant differences are reported (*$p < 0.05$; **$p < 0.01$).

3. ERK5 activity is required for YAP/TEAD interaction and for YAP recruitment on DNA

The data reported so far showed that ERK5 is necessary for the transcriptional activity of YAP and its overexpression sufficient to trigger a YAP-dependent expression of the analyzed target genes. To unveil the mechanisms underlying this regulation we firstly verified if ERK5 could impact on YAP subcellular localization. By means of immunofluorescence assay we observed that, although the YAP immunostaining results improved both by transfection procedure and XMD8-92 treatment in RLSCs, nuclear localization of YAP appeared unaffected by ERK5 inhibition or silencing both in RLSC and HUH7 (Fig. 18A, B), thus indicating that YAP is still in the nucleus but less active on its target genes.

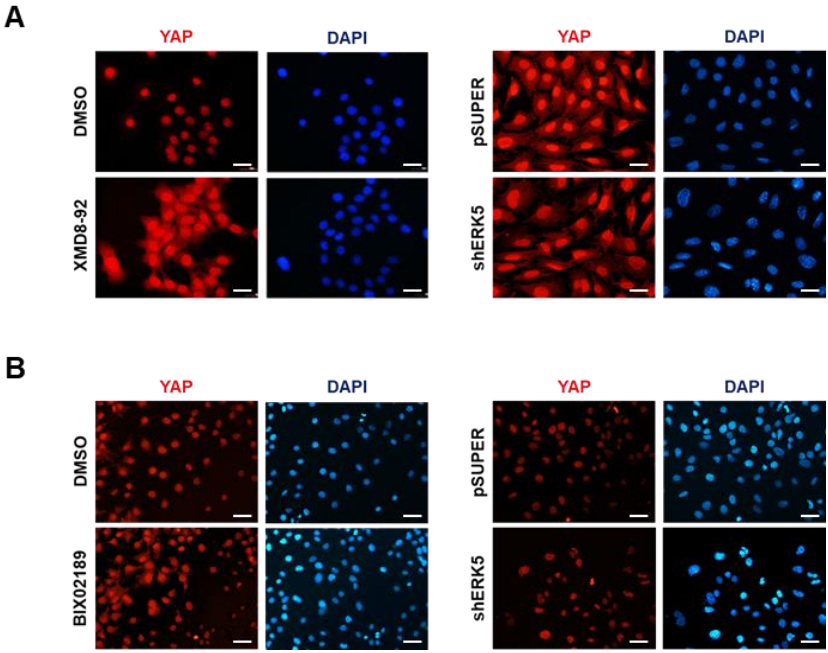

Fig. 18 ERK5 inhibition or silencing does not affect YAP subcellular localization.
A Immunofluorescence analysis of RLSC treated with XMD8-92 or with DMSO for 16 h (left panels) and of RLSC transfected with shERK5 or with the empty vector (right panels). Cells were stained with an anti-YAP antibody (red) and DAPI (nuclei, blue). Images are representative of three independent experiments. Scale bar: 50 µm.
B Immunofluorescence analysis of HuH7 treated with BIX02189 or with DMSO for 16 h (left panels) and of HuH7 transfected with shERK5 or with the empty vector (right panels). Cells were stained with an anti-YAP antibody (red) and DAPI (nuclei, blue). Images are representative of three independent experiments. Scale bar: 50 µm.

Thus, demonstrating that ERK5 inhibition or silencing does not affect YAP protein level or subcellular localization, we suppose an ERK5 involvement in YAP capability to bind chromatin. Therefore, we next

analyzed the DNA occupancy of YAP on specific chromatin sites in conditions of ERK5 activity inhibition. A Chromatin Immunoprecipitation (ChIP) assay was performed with an anti-YAP antibody on chromatin from RLSC cell line treated with the ERK5 inhibitor XMD8-92 or with its solvent DMSO. The TEAD binding site in the CTGF promoter, previously described as able to recruit YAP in these cells, has been explored by qPCRs. As negative control of YAP recruitment, the regulatory region of the Neurogenin 1 gene has been assessed. As shown in Fig. 19A, the lack of YAP binding on DNA was observed following ERK5 inactivation, thus indicating that ERK5 activity is required for the recruitment of YAP on target gene promoters.

To deeper analyze the mechanism underlying, we performed a co-immunoprecipitation assay in HuH7 cells treated with BIX02189. Interestingly, as shown in Figures 19B and 19C, ERK5 inactivation reduced the interaction between YAP and TEAD4 without affecting TEAD4 protein expression (Fig. 19B). Altogether these results indicated that ERK5 positively regulates YAP transcriptional activity by controlling its recruitment on target gene promoters and in particular modulating YAP interaction with TEAD4.

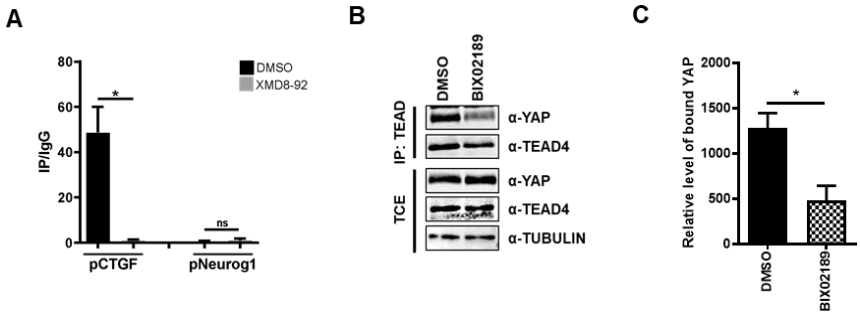

Fig. 19 ERK5 activity is required for the recruitment of YAP on target gene promoters and for YAP/TEAD interaction.

A qPCR analysis of ChIP assays with anti-YAP antibody (IP) and, as control, normal rabbit IgG (IgG) on chromatin from RLSC treated with XMD8-92 or with DMSO for 16 h. The TEAD consensus region embedded in the Ctgf gene promoter was analyzed. A YAP unbounded region of Neurogenin 1 promoter was utilized as negative control. Data are normalized to total chromatin input and background (control immunoprecipitation with IgG) and expressed as IP/IgG. Mean ± SEM of qPCR data obtained in triplicate from three independent experiments is reported. Statistical significance: *$p < 0.05$; ns = not significant.

B Co-immunoprecipitation of YAP and TEAD proteins. Total cell extracts (TCEs) and the anti-TEAD4 immunoprecipitates (IP) were analyzed by immunoblotting with anti-YAP and anti-TEAD4 antibodies. Tubulin has been utilized as loading control of TCEs. WB images represent one indicative experiment of three independent ones.

C anti-YAP IP from three independent experiments was quantified by densitometric analysis and normalized on the relative anti-TEAD4 IP. Statistical significance: *$p < 0.05$.

4. ECM-induced YAP activation requires ERK5 activity

So far, we demonstrated that ERK5 is required for YAP co-transcriptional activity, for its interaction with TEAD4 and, eventually, for its recruitment on the promoter of target genes. In the attempt to identify cellular processes in which this regulation can be involved,

we verified the effect of ERK5 inhibition in cell-extracellular matrix (ECM) adhesion dynamics, where the modulation of transcriptional activity of YAP has been well documented, both in the physiological and pathological cell response to mechanical stimuli as well as in cancer progression (*Cai X et al. 2021*). In particular, it has been previously reported that YAP activity is inhibited upon cell detachment and upregulated by cell adhesion (*Zhao B et al. 2012*). Starting from these observations, we investigated the effect of ERK5 inhibition on YAP target gene expression in cell detachment and adhesion dynamics. Therefore, trypsinized Huh7 cells were maintained in suspension for 10' and collected or re-plated on collagen-coated dishes and maintained in adhesion for 3 h, in the presence of MEK5/ERK5 chemical inhibitor BIX02189 or DMSO. As expected, in suspended cells a low level of YAP transcriptional activity was observed while cell adhesion induced YAP-dependent gene expression (Fig. 20A, B). The treatment of cells with BIX02189 significantly impaired the upregulation of YAP target genes CTGF and CYR61 induced by cell adhesion to ECM (Fig. 20A). Similar results were obtained in HepG2 cells (Fig. 20C). Altogether, these data indicate that cell/ECM interactions lead to an ERK5-dependent YAP activation.

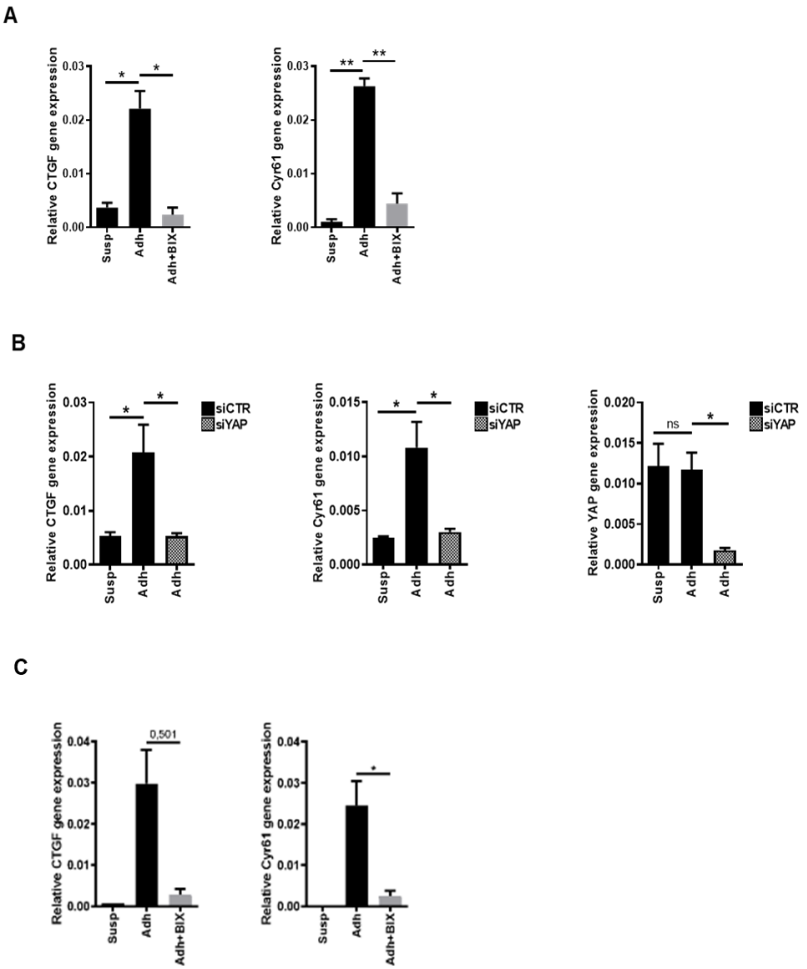

Fig. 20 Adhesion-mediated activation of YAP requires ERK5.

A RT-qPCR analysis of the indicated genes in HuH7. Cells were trypsinized, maintained in suspension for 10' and collected (Susp) or plated for 3 h on collagen-coated plates (Adh) in the presence or absence of ERK5 inhibitor BIX02189. Data are expressed as a relative expression and shown as means ± S.E.M. of at least three independent experiments. Statistically significant differences are reported (*$p < 0.05$; **$p < 0.01$).

B RT-qPCR analysis of the indicated genes in suspended and adherent YAP-silenced HuH7 (siYAP), compared with cells transfected with control siRNAs (siCTR). Data are expressed as a relative expression and shown as means ± S.E.M. of at least three independent experiments. Statistically significant differences are reported (*p < 0.05; ns =not significant).
C RT-qPCR analysis of the indicated genes in HepG2. Cells were trypsinized, maintained in suspension for 10' and collected (Susp) or attached for 4 hours on collagen-coated plates (Adh) in the presence of BIX02189 or DMSO. Statistically significant differences are reported (*p<0.05).

5. ERK5 mediates the TGFβ-induced YAP activation

Several lines of research unveiled a multilevel crosstalk between Hippo/YAP signaling and TGFβ pathway. However, while it has been shown that TGFβ-induced SMAD nuclear translocation is dependent on YAP (L*abibi B et al. 2020*) and that YAP knockdown strongly impacts on the cell response to the cytokine in terms of apoptosis and EMT *(Liu Y et al. 2017*), how the cytokine can control the YAP activity has not yet been elucidated. My research group has previously demonstrated that ERK5 is activated in the TGFβ-induced EMT of HepE14 hepatocytes and plays a crucial role in the Snail cytoplasmic stabilization (*Marchetti A et al. 2008*). We therefore asked whether ERK5 could also mediate YAP activation in hepatocytes in response to TGFβ treatment. To this aim, we analyzed the YAP transcriptional activity in TGFβ-treated cells in the presence of ERK5 chemical inhibition. As shown in Fig. 21, in HepE14 treated

with the cytokine, the expected ERK5 activation (Fig. 21A, left panel) correlated with the YAP functional activation, assessed both by the activity of YAP-responsive luciferase reporter 8XGTIIC-luc (Fig. 21A, right panel) and by the expression of its target genes, Ctgf and Cyr61 (Fig. 21B). The TGFβ-induced transcriptional activity of YAP is ERK5-dependent since the treatment of cells with XMD8-92 impaired both YAP dependent luciferase activity and target gene expression. Overall, these results demonstrate that the YAP transcriptional activation induced by TGFβ in hepatocytes undergoing EMT requires ERK5.

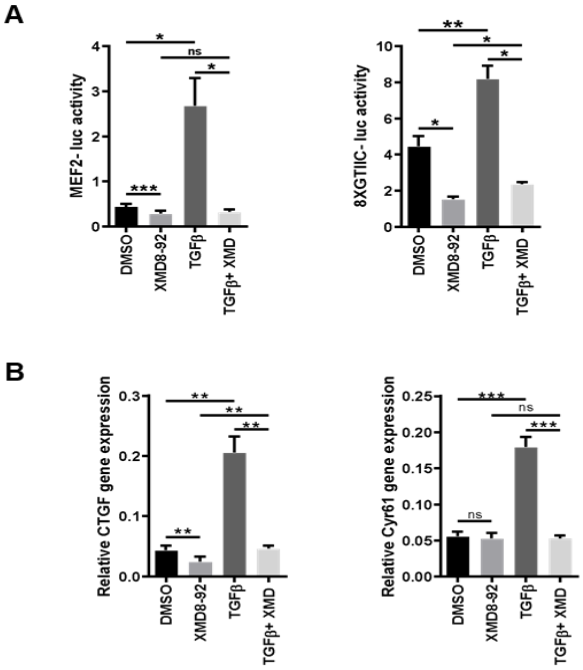

Fig. 21 TGFβ-mediated activation of YAP requires ERK5.

A Luciferase assay. MEF2-luc or 8xGTIIC-luc reporters were transiently co-transfected in HepE14, together with a Renilla luciferase expression vector. Twenty-four hours post-transfection, cells were treated with TGFβ1 or left untreated for 24 h, in the presence of 10 μM XMD8-92 or DMSO. Luciferase activities were normalized for Renilla luciferase activity and expressed as arbitrary units. Statistically significant differences are reported (*p < 0.05; **p < 0.01; ***p < 0.001; ns = not significant).

B RT-qPCR analysis of Ctgf and Cyr61 gene expression in untreated or TGFβ1-treated HepE14 cells. The values are calculated by the 2(−ΔCt) method and shown as means ± S.E.M. of at least three independent experiments. Statistically significant differences are reported (**p < 0.01; ***p < 0.001; ns = not significant).

6. YAP-induced motility of liver cancer cells requires the LATS1/2-independent ERK5 activity

The key role played by YAP in cell migration, both in development and in cancer metastasis, has been well documented (*Varelas X 2014; Chen Th et al. 2017; Takeda T et al. 2022*), but the molecular mechanisms involved in its regulation remain poorly described. We therefore tested the role of ERK5 in the YAP-induced migration of HuH7 cells that display, together with an epithelial phenotype, a low rate of motility. To this end, HuH7 cells were transfected with a constitutively active mutant form of YAP (Fig. 22A) and their migration has been assessed in a wound healing assay in the presence of the MEK5/ERK5 inhibitor BIX02189. As expected, YAP overexpression induced an increase in cell motility (Fig. 22C), confirming its role in cancer metastasis. The YAP-induced motility was significantly reduced by ERK5 inhibition, indicating the migration of tumor cells as a relevant functional readout of YAP regulation by ERK5.

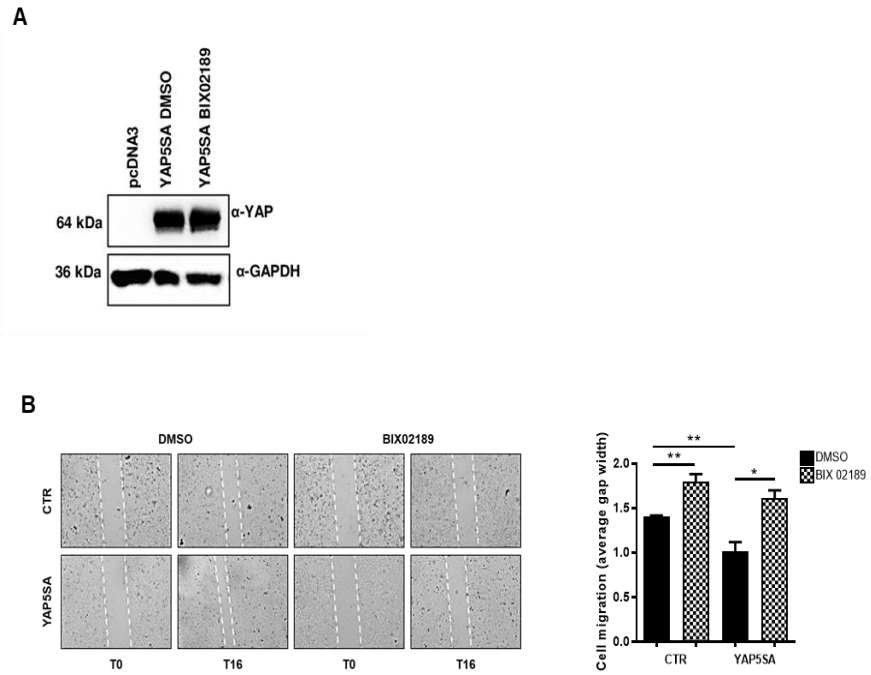

Fig. 22 ERK5 inhibition interferes with YAP-dependent migration of liver cancer cells in a LATS-independent manner.
A Western blot for the indicated proteins in YAP5SA mutant overexpressing HuH7, treated for 16h with DMSO or 10 μM BIX02189. GAPDH has been utilized as loading control.
B Wound healing assay of YAP5SA-expressing HuH7 cells and control cells treated with 10 μM BIX02189 or DMSO for 16 h (T16). The images are representative of two independent experiments performed in duplicate. Cell migration was manually quantified as average distance between the edges of the gap (gap width).

Interestingly, because the constitutively active mutant form of YAP utilized in the migration assay, named YAP5SA, carries five amino acid substitutions (five alanine instead of five serine) that make YAP non-

phosphorylatable by LATS1/2 (*Zhao B et al. 2007*), these results indicate that ERK5 can regulate YAP activity in a Hippo/LATS independent manner. To confirm what was observed at molecular level, HUH7 cells were transfected with YAP5SA and then treated with BIX02-189. As shown in Figure 23, YAP5SA overexpression leads to an upregulation of YAP target genes expression, which is unpaired by the chemical inhibition of the kinase. Overall, these results, while not entirely excluding that ERK5 could also interfere with the canonical pathway of Hippo, point to ERK5 as a new element of the Hippo/LATS-independent regulation of YAP-driven transcription and cellular outcomes.

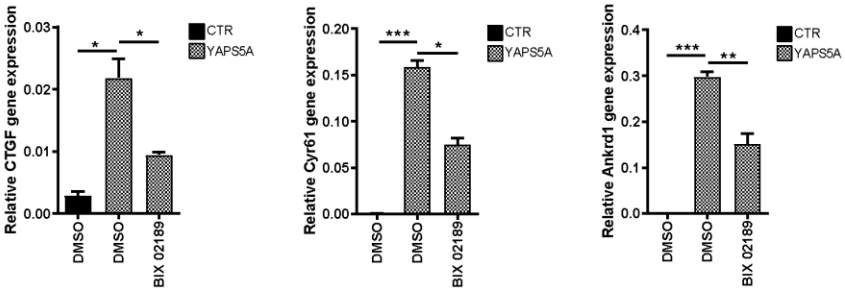

Fig. 23 ERK5 inhibition interferes with YAP-dependent target gene expression of liver cancer cells in a LATS-independent manner.
RT-qPCR analysis of the indicated YAP target genes in mutant YAP5SA-overexpressing HuH7 cells (YAP5SA) and in control cells (CTR), treated with 10 μM BIX02189 or DMSO. Data are expressed as relative gene expression and shown as mean ± S.E.M. of three independent experiments. Statistically significant differences are reported (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

7. In vivo study of YAP regulation by ERK5

We demonstrate a new role of ERK5 kinase in the regulation of YAP activity in liver cells, thus suggesting a new target against cancers characterized by a YAP overexpression or hyperactivity. The next step would be to verify the maintenance of ERK5-YAP signaling in animal models. Therefore, I spent five months in the laboratory of Dr. Georg Halder at the Campus Gasthuisberg (Leuven, Belgium), where mice models overexpressing YAP in hepatocytes were available and already characterized. They previously observed that YAP overexpression or hyper activation leads to liver overgrowth, fibrosis and mouse death. Starting from these observations, my aim was to verify if ERK5 inhibition could rescue or reduce these phenotypes.

The first experiment has been performed in three APOE-YAP transgenic mice, characterized by an overexpression of YAP protein in hepatocytes, treated with BIX02189 for 12 days (time point in which the effects of YAP overexpression appear evident). As control, three mice were treated with the solvent Captisol 30%. While no significant change in liver morphology and size have been observed (fig. 24A, B), the analysis of gene expression performed by RT-qPCR on the RNA collected from the livers of both groups of mice showed interesting differences that, although not statistically significant (presumably for the low number of

animals analyzed or for the too prolonged YAP-activation), confirmed the results obtained in cell culture. In fact, after ERK5 chemical inhibition, a decrease in the transcription level of YAP positive targets (Ctgf, Ankrd1, Snail) and an increase in one of YAP negative targets (Ddit4) have been observed (fig. 24C).

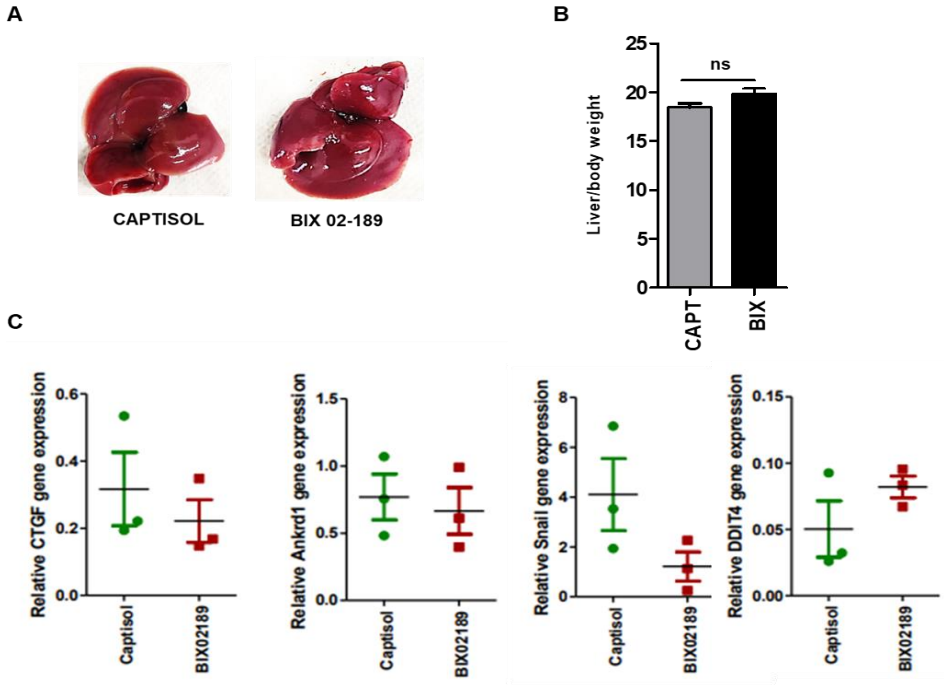

Fig. 24 ERK5 inhibition impacts on YAP-dependent expression of target genes of liver-specific YAP-overexpressing mice.
ApoE Rtt Tre YAP C57BL/6J mice were treated daily with BIX02189 or Captisol for 12 days.

A Representative pictures of livers from mice at the end of the experiment.
B Ratio between livers and mice weight.
C RT-qPCR analysis of the indicated YAP target genes in livers of animals treated with BIX02189 or Captisol. Data are expressed as relative gene expression and shown as mean ± S.E.M. of three independent experiments.

A second experiment has been performed in LATS flox/flox mice characterized by a hepatocyte specific knockout of LATS1/2, leading to YAP hyper activation. As well as in the previous experiment, no changes in liver size have been observed (Fig. 25A, B). Instead, and interestingly, as shown in Figure 25C, Haemaluin & Eosin and Sirius Red staining in livers from BIX02189-treated mice, showed a notable reduction of YAP-induced fibrosis compared to livers from control mice. Although further investigations are required, this data suggests a possible involvement of ERK5 in the liver fibrosis induced by YAP upregulation and reinforces the hypothesis of the independence of the ERK5-YAP axis from LATS kinases.

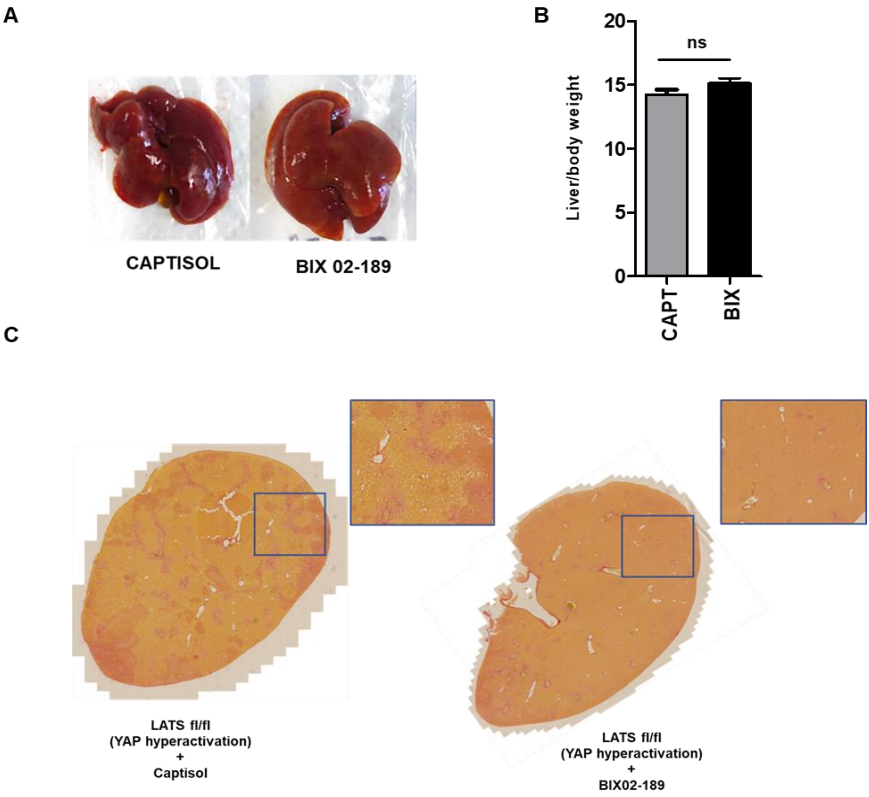

Fig. 25 ERK5 inhibition attenuates YAP-dependent fibrosis in LATS fl/fl mice.
LATS fl/fl C57BL/6J mice were treated daily with BIX02189 or Captisol for 12 days.
A Representative pictures of livers extracted from mice at the end of the experiment.
B Ratio between livers and mice weight.
C Sirius Red staining of liver sections. Figures report a representative image from 1 of 5 mice analyzed.

DISCUSSION AND CONCLUSION

In the last few years, there has been a growing interest in the identification and characterization of new regulators of YAP activity. In fact, despite the Hippo pathway being considered the main YAP regulator, an increasing amount of evidence highlights the presence of molecules and signaling that can regulate YAP activity by converging on the Hippo pathway at different levels or in a Hippo- and LATS-independent way. Therefore, the knowledge of the key components of these regulatory networks and of their functional role has particular relevance in the perspective of identifying new therapeutic targets and setting up new protocols for the treatment of YAP-dependent pathologies.

In this study, we unveiled ERK5/MAPK as a new regulator of YAP transcriptional activity. In particular, we demonstrated that ERK5 activity is required for (i) the maintenance of YAP transcriptional activity and the upregulation of specific target genes both in liver stem cells and in human hepatocellular carcinoma cell lines, (ii) the YAP activation in cell adhesion dynamics and TGFβ-induced EMT, and (iii) the YAP-dependent cell migration of cancer cells. Furthermore, we demonstrated that ERK5 signaling modulates the activity also of a YAP mutant non-phosphorylatable by LATS1/2, thus providing evidence of its, at least in

part, independence from the Hippo pathway. Mechanistically, data obtained by this work showed that ERK5 regulates YAP activity by allowing its physical interaction with the transcriptional partner, TEAD4, and, consequently, its recruitment on target gene promoters.

The here collected results are in line with and provide a mechanism for the correlation between ERK5 and YAP activity, previously described in several cellular processes, primarily in cancer. The dysregulation of both ERK5 and YAP, indeed, had been observed in several human cancers and related to an increased metastatic risk and less favorable outcome (*Zanconato F et al. 2016; Stecca B & Rovida E 2019*). In particular, in hepatocellular carcinoma, YAP is present in an active form in more than 85% of tumor samples (*Xu MZ et al. 2009*) and its inhibition restores hepatocyte differentiation and induces tumor regression in preclinical models (*Fitamant J et al. 2015*). On the other hand, ERK5 gene has been found amplified in primary HCC tumors (*Zen K et al.2009*) and the MEK5/ERK5 signaling pathway constitutively activated and associated with tumor growth *(Rovida E et al. 2015*).

Here, we showed that ERK5 is necessary and sufficient to induce a YAP-dependent expression of target genes in HCC cell lines, thus identifying YAP as a new target of ERK5 in cancer. Furthermore, we showed that ERK5 activity is required for hepatoma cell migration induced by the constitutive activation of YAP.

Interestingly, both proteins have been demonstrated to be directly involved in the EMT (*Bhatt AB et al. 2021; Ortega A et al. 2021*), a process crucial in carcinoma metastasization. Cancer cells overexpressing YAP undergo EMT, promoting tumor progression (*Cheng D et al. 2020; Yuan Y et al. 2016*). Moreover, YAP acts as a primary mediator of TFGβ-induced EMT (*Liu Y et al. 2017; Gao C et al. 2021*) and is required for the SMAD2/3 nuclear translocation (*Labibi B et al. 2020*). Furthermore, YAP induces EMT in epithelial cells thanks to its transcriptional activity on EMT/MET master genes. In my laboratory, in fact, it has been previously demonstrated that YAP directly controls positively the transcription of the EMT master gene Snail and negatively that of the MET master gene HNF4α (*Noce V et al. 2019*). At the same time, my research group has demonstrated that ERK5 is required for Snail protein stabilization in hepatocytes undergoing EMT following TGFβ treatment (*Marchetti A et al. 2008*). Here, we have extended these results suggesting that the new direct ERK5-YAP axis may contribute to the EMT process.

Regarding the molecular mechanism responsible for the effect of ERK5 activity on YAP function, we demonstrated that ERK5 is required for YAP recruitment on its target genes promoters and for its interaction with TEAD4. Further investigations will be also needed to deeper dissect the molecular basis of this control. ERK5, in fact, could directly phosphorylate YAP protein or its transcription partners, or act as

transcriptional factor. ERK5, in fact, shows a functional transactivation domain (*Kasler HG et al. 2000*) capable of driving gene expression through the interaction with transcription factors on DNA (*Madak-Erdogan Z et al. 2014*). To investigate this, a deeper analyses of YAP phosphorylation state after ERK5 inhibition or silencing is needed; moreover, the use of mutated ERK5 isoforms (for example with a deletion in the catalytic or in the TAD domains) will allow the identification of the mechanism by which ERK5 modulates YAP activity. Thanks to its TAD domain, the kinase could participate in the formation/activation/stabilization of a ternary transcriptional complex together with YAP and TEAD on target gene enhancers or promoters. Moreover, although the YAP regulated gene expression is mainly accomplished through the DNA binding mediated by TEAD family members, increasing evidence of additional transcriptional partners of YAP and of their role in triggering cell type- and context-specific cellular responses has been collected (*Lopez-Hernandez A et al. 2021)*. Therefore, the possible regulation by ERK5 of other YAP-containing transcriptional complexes cannot be excluded. As a matter of fact, the role played by ERK5 in complex YAP-dependent cell functions (i.e. motility), presumably involving the coordinate expression of several genes, enforces the hypothesis of a possible regulation by ERK5 of different transcriptional complexes containing YAP.

Interestingly, the described results have been confirmed also in a cell line overexpressing a mutated YAP, insensitive to LATS1/2 inhibition. Therefore, this suggests that ERK5 could, at least in part, modulate YAP activity in a LATS1/2 independent way. Further analysis in dKO-LATS1/2 cells will provide a formal demonstration of an ERK5 independence from Hippo pathway. Finally, preliminary data obtained in animal models of YAP overexpression enforced the biological relevance of the new axis ERK5/YAP described in cultured cells. Although the number of animals analyzed so far is small and the statistics still weak, in fact, we observed a modulation of the analyzed YAP target genes coherent with the ERK5 inhibition, obtained by treatment of mice with the chemical inhibitor BIX02189. Moreover, and interestingly, ERK5 inhibition reduced histological evidence of liver fibrosis induced by the overexpression of YAP in LATS fl/fl mice, so enforcing both the relevance of ERK5/YAP axis in liver pathology and the hypothesis of the independence of ERK5 activity from LATS proteins.

In conclusion, the results described in this thesis contribute to the knowledge of the molecular mechanisms underlying YAP activity and of their control in non-transformed hepatic stem/precursor cells, in human hepatoma cells and in animal models, indicating new perspectives in the cancer treatment.

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