UNIVERSITY OF VERONA

DEPARTEMENT OF MEDICINE

GRADUATE SCHOOL FOR HEALTH AND LIFE SCIENCES

Ph.D. PROGRAM IN

INFLAMMATION, IMMUNITY AND CANCER

Cycle: 29th

Dissecting the signaling of the MAP3 Kinases TAK1 and MEKK3 for the treatment of pancreatic cancer

S.S.D. MED/06

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Tesi di Dottorato
Verona, 20 Febbraio 2017

Sommario:

Il tumore pancreatico rappresenta una delle maggiori sfide per il XXI secolo a causa della sua prognosi infausta. Tra le vie di segnalazione che regolano l'aggressività del tumore pancreatico, la via delle MAPK gioca un ruolo chiave in questo processo. In particolare, è stato dimostrato che le MAP3Ks agiscono sia sulla chemioresistenza che sulle capacità metastatiche di diversi tipi di tumore regolando le vie di segnalazione di NFkB e di YAP/TAZ.
Abbiamo focalizzato la nostra attenzione su due specifici membri delle MAP3Ks, TAK1 (TGF-β-activated kinase 1) e MEKK3 (Mitogen-Activated protein Kinase Kinase Kinase 3), con lo scopo di capire se e come queste due chinasi fossero coinvolte nella regolazione dell'attività dei cofattori trascrizionali YAP/TAZ.
In primo luogo, abbiamo osservato che il silenziamento di TAK1 causa una deregolazione della via di segnalazione di HIPPO, andando a modificare i livelli proteici di YAP/TAZ. Nel nostro studio abbiamo dimostrato per la prima volta che TAK1 è in grado di regolare la stabilità di YAP/TAZ, indipendentemente dalla sua attività chinasica. Abbiamo osservato, infatti, che il silenziamento di TAK1 modificava i livelli di espressione di due importanti ubiquitin ligasi come TRAF6, che media l'ubiquitinazione K63, e ITCH/AIP4, che promuove l'ubiquitinazione K48.
A seguito di uno studio in cui viene dimostrato che l'inibizione farmacologica di GSK3 induce una riduzione dei livelli proteici di TAK1, abbiamo trattato le linee cellulari di tumore pancreatico con tre diversi inibitori di GSK3 ed abbiamo riscontrato un'importante riduzione dei livelli proteici sia di TAK1 che di YAP/TAZ. Inoltre, abbiamo dimostrato che il silenziamento farmacologico di TAK1 inibisce alcuni processi regolati da YAP/TAZ, come la proliferazione e la migrazione, riducendo così l'aggressività del tumore pancreatico.
Parallelamente, abbiamo studiato il ruolo di MEKK3 nel guidare l'aggressività del tumore pancreatico. A questo scopo, abbiamo deleto MEKK3 in diverse linee cellulari utilizzando la nuova tecnologia di CRISPR-Cas9. Successivamente, abbiamo valutato l'effetto della delezione sulle diverse caratteristiche del tumore pancreatico. In particolare, abbiamo osservato un'importante riduzione dell'invasività, della proliferazione e della capacità di formare colonie in cellule delete per MEKK3. Parallelamente, abbiamo valutato l'effetto della delezione di MEKK3 sull'attività delle vie di segnalazione di NFkB e YAP/TAZ. Sebbene non abbiamo riscontrato nessun'alterazione nella via di segnalazione di NFkB, abbiamo dimostrato che la delezione di MEKK3 riduceva l'attività trascrizionale di YAP/TAZ, senza alterare i loro livelli proteici.
Il ruolo emergente di YAP/TAZ nel guidare lo sviluppo e l'aggressività del tumore pancreatico sottolinea l'esigenza di identificare nuovi farmaci in grado di inibire la loro attività. Attualmente non esistono farmaci specifici per inibire YAP/TAZ e l'identificazione di farmaci in grado di bloccare l'azione di cofattori trascrizionali è molto complicata. I nostri dati dimostrano che, nel tumore pancreatico, l'attività di YAP/TAZ può essere ridotta attraverso l'inibizione farmacologica sia di GSK3/TAK1 che di MEKK3.

Abstract:

Pancreatic cancer (PC) remains one of the most lethal and poorly understood human malignancies and will continue to be a major unsolved health problem in the 21st century. The MAP3K pathway is one of most important pathways that regulate the aggressiveness of PC. In particular, MAP3Ks act by regulating NFkB and YAP/TAZ signaling, two of the most well characterized pathways sustaining the chemoresistance and EMT features of this cancer.

We focused our attention on two members of the MAP3K pathway, the TGF-β-activated kinase 1 (TAK1) and the Mitogen-Activated protein kinase kinase kinase 3 (MEKK3) with the aim to understand whether and how they could impact on YAP/TAZ.

We showed that TAK1 silencing affects the HIPPO pathway by modulating YAP/TAZ protein levels. We reported for the first time that TAK1 can regulate the stability of YAP/TAZ, independently on its kinase activity, by modulating the expression of E3-ubiquitin ligases, such as TRAF6 and ITCH/AIP4. Moreover, based on a recent report showing that the pharmacological inhibition of GSK3 caused a reduction of TAK1 levels, we treated our cells with GSK3 inhibitors and we observed a reduction of both TAK1 and YAP/TAZ proteins, as well as YAP/TAZ regulated genes. Pharmacological silencing of TAK1 impaired YAP/TAZ-regulated features, such as proliferation and migration.

As for MEKK3, we knocked out its expression in different cellular models by CRISPR-Cas9 technology. Then, we assessed the impact of MEKK3 knock-out (MEKK3 KO) on the aggressiveness of PC. We observed a decrease of proliferation and colony formation ability in MEKK3 KO cells. Simultaneously, we observed that MEKK3 KO affects the YAP/TAZ target genes expression, without altering YAP/TAZ protein levels or the NFkB pathway.

The emerging role of YAP/TAZ in orchestrating the development and the sustainment of PC opens the need for the discovery of drugs to inhibit their activities but, so far, no specific inhibitors of YAP/TAZ have been identified.

Our data open the path for targeting the YAP/TAZ pathway through pharmacological inhibition of GSK3/TAK1 and MEKK3.

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1. Background

1.1.Pancreatic Cancer

1.1.1 Epidemiology

Pancreatic cancer (PC) remains one of the most lethal and poorly understood human malignancies, and will continue to be a major unsolved health problem in the 21st century [1]. It has been estimated that in 2016 there were 53,070 new cases of pancreatic adenocarcinoma with approximately 41,780 deaths in the United States. Because of our inability to detect PC at an early stage and the lack of effective systemic therapies, only 1-4% of patients with adenocarcinoma of the pancreas will be alive 5 years after diagnosis. Thus, incidence rates are virtually identical to mortality rates. In the United States in 2016, PC will be the fourth leading cause of adult death from cancer and will be responsible for close to 7% of all cancer-related deaths [2].

1.1.2 Genetics and risk factors

Less then 10% of PC cases can be accounted for by hereditary genetic factors. Germline mutations in Serine Protease 1 (PRSS1), Serine/Threonine Kinase 11 (STK11), Cyclin Dependent Kinase Inhibitor 2A (CDKN2a), Breast Cancer Type 2 Susceptibility Protein (BRCA2), or mismatch repair genes may account for less than 20% of inherited PC [3]. Several genetic syndromes, such as hereditary pancreatitis, hereditary nonpolyposis colorectal cancer and familial breast cancer, have been associated with an increased risk of PC [4,5].

Pre-existing chronic pancreatitis has been associated with a 10-20 fold increased risk of PC [6]. Specifically, a multicentric cohort study of 2000 patients with chronic pancreatitis reported a 16-fold increased risk of PC [7].
Smoking has been consistently and convincingly linked to a marked increased risk of PC. In fact, studies have shown a correlation between the number of smoked cigarettes and the incidence of PC. In general, cigarette smoking has been estimated to account roughly 25-29% of the overall incidence of PC in the United States [8–10]. In particular, cigarettes smokers show up to 2,5 fold increased risk of developing PC as compared to nonsmokers [11].
Similarly, obesity is another important risk factor correlated with PC development and poor prognosis. In particular, it has been shown in three recent large pooled analyses that a BMI higher than 30 Kg/m^2 increases the risk to develop PC up to 20-50% as compared to normal BMI participants[12].

1.1.3 Histology

The most common malignancy of the pancreas is the infiltrating ductal adenocarcinoma. Infiltrating ductal adenocarcinoma, commonly known as "pancreatic cancer", is defined as an invasive malignant epithelial neoplasm with ductal differentiation [13]. The majority (60-70%) of PC arise in the head of the gland, most are poorly defined and they compromise the normal lobular architecture of the pancreas [14].
Pancreatic ductal adenocarcinomas (PDAC) are characterized by two remarkable features at the microscopic level. First, they elicit an intense desmoplastic reaction. As a result, most of the cells that comprise the mass produced by a PC are non-neoplastic fibroblasts, lymphocytes and macrophages. Second, despite the highly lethal nature of PC, most of these neoplasms are remarkably well-differentiated. Features supportive of a diagnosis of PC include perineural invasion, vascular invasion, a haphazard arrangement of the glands, nuclear pleomorphism, the presence of a gland immediately adjacent to a muscular artery, and luminal necrosis [13]. Immunohistochemical labeling can be used to characterize the direction of differentiation of the neoplastic cells. Most pancreatic cancers express several cytokeratins, such as CK7, 8, 13, 18, 19, carcinoembryonic antigen (CEA) and carcinoma antigen 19-9 (CA19-9) [15,16].

1.1.4 Molecular pathogenesis of pancreatic cancer

While our knowledge of the genetic events that underpin multistep carcinogenesis in PC has increased dramatically, and despite a steady identification of new targets and new drugs for clinical testing, researchers still continue to work with an incomplete understanding of how the complex molecular biology contributes to the aggressive behavior of this disease.
In contrast to many epithelial malignancies, PC is characterized by four genes that are altered in the majority of patients: the KRAS proto-oncogene is mutated and constitutively activated in >90% of cases, while the tumor suppressors cyclin-dependent kinase Inhibitor 2A (CDKN2A), p53, and DPC4/SMAD4 are mutated in >95%, 50-75%, and 55% of cases respectively [17]. In particular, has been demonstrated that constitutive activation of KRAS alone in the pancreatic epithelium drives the development of premalignant ductal neoplasias known as pancreatic interepithelial neoplasias (PanINs) in mice [18]. The consecutive loss or mutation of tumor suppressors, such as CDKN2A, p16 and p53 leads to the development of highly aggressive PDAC[19,20]. In addition, SMAD4 inactivation, that occurs in half of PC patients, is correlated to a highly aggressive metastatic phenotype of PDAC [21]. These alterations confer high genomic instability and aggressive features to PC.

1.1.5 Treatment

Despite efforts over the past century, conventional therapeutic approaches, such as surgery, radiation, chemotherapy, or combinations of these modalities, have not had much impact on the course of this aggressive disease [22]. The outlook of individuals with PC is dismal as described by the following statistics. Over 80% of patients with PC have advanced disease at time of diagnosis and are not candidates for a potentially curative resection. In this group of patients, approximately 20% will have locally advanced disease with a median survival of 8-12 months, and 50% will have metastatic disease with median survival of 3-6 months. Of the remaining patients who undergo a resection, the chance of long-term survival is low; 80-90% will go on to have recurrence. One-half of patients undergoing a potentially curative resection will be dead of disease in 18 months, and less then 20% will be alive at 5 years [23]. Certain pathologic features such as a tumor size less than 2 cm, absence of spread to regional lymphnodes, and a surgical margin free of carcinoma are good prognostic indicators [24]. Under ideal circumstances, in which all of these factors are favorable, 5-year survival is achieved in only 43% [23]. The major factors contributing to the lethality of this disease are the inability to detect early cancers and ineffective systemic therapy.
The only chance for long-term survival in patients with PC is with surgical resection. However, since the majority of patients have occult systemic disease at the time of resection, cure relies on systemic therapy. The first line treatment of

advanced PC consists in chemotherapies characterized by combination of gemcitabine with FOLFIRINOX, a cocktail of folinic acid, fluorouracil, irinotecan and oxaliplatin, or with nab-paclitaxel. This is the main front-line treatment option for patients with good performance status at the time of diagnosis. In spite of the aggressiveness of PC, nearly half of patients who have progressed on front-line therapy are able to receive second-line therapy. A fluorouracil-based regimen is used for patients who progress through gemcitabine, while patients who received FOLFIRINOX in the first-line, are treated with gemcitabine-based therapy in the second line [25].

1.2 Mitogen-activated kinase signaling

Mitogen-activated protein kinase (MAPK) signaling transduction pathways are ubiquitous and highly conserved mechanism of eukaryotic cell regulation [26]. Different MAPK pathways are able to integrate and coordinate the responses to different extracellular stimuli, including hormones, growth factors, cytokines, transforming growth factor (TGF)-β related agents and stresses, such as toxins, drugs exposure, change in cellular adherence, oxygen radicals and ultraviolet light [27].

The MAPKs phosphorylation cascade is orchestrated by three levels of activating phosphorylations mediated by Mitogen-Activated Protein Kinase Kinase Kinase (MKKKs or MAP3Ks) that phosphorylate Mitogen-Activated Protein Kinase Kinase (MKKs or MAP2Ks) phosphorylating in turn MAPKs [27]. In particular, MAPKs are activated via simultaneous Thr and Tyr phosphorylation within a distinct and conserved Thr-X-Tyr motif in the kinase activation loop. Conversely, MAP2Ks are activated by a pattern of different MAP3Ks through Ser-Thr phosphorylation in the conserved kinase domain [28]. MAP3Ks constitute the largest group of MAPKs, composed of at least twenty proteins, such as TAK1, MEKK3 and TPL2, as compared to seven MAP2Ks and eleven MAPKs, such as ERK1/2, c-Jun N terminal Kinases (JNKs) and p38 (Fig. 1) [29].

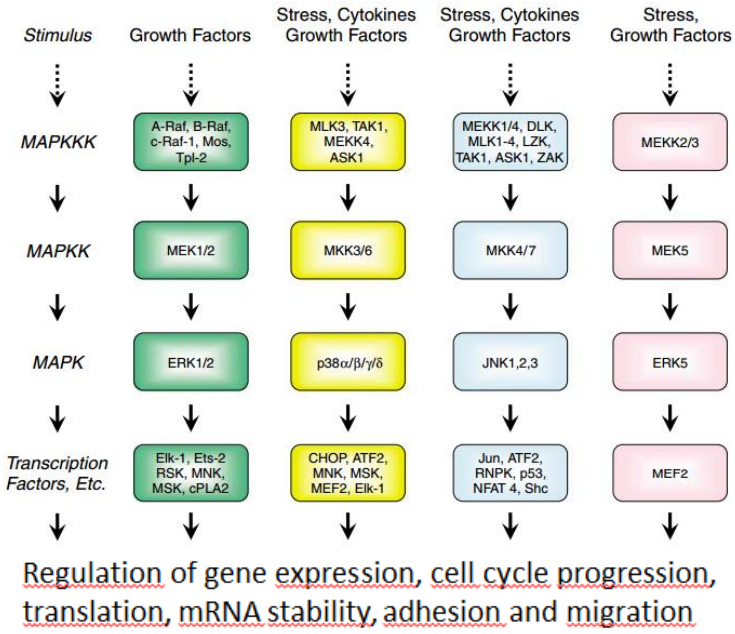


Fig 1. Mammalian MAPK Signaling Cascade. A broad range of extracellular stimuli including mitogens, cytokines, growth factors, and environmental stressors stimulate the activation of one or more MAPKK kinases (MAPKKKs) via receptor-dependent and -independent mechanisms. MAPKKKs then phosphorylate and activate a downstream MAPK kinase (MAPKK), which in turn phosphorylates and activates MAPKs. Activation of MAPKs leads to the phosphorylation and activation of specific MAPK-activated protein kinases (MAPKAPKs), such as members of the RSK, MSK, or MNK family, and MK2/3/5. These MAPKAPKs function to amplify the signal and mediate the broad range of biological processes regulated by the different MAPKs. Conventional MAPKs include the extracellular signal-regulated kinase 1 and 2 (Erk1/2 or p44/42), the c-Jun N-terminal kinases 1-3 (JNK1-3)/ stress activated protein kinases (SAPK1A, 1B, 1C), the p38 isoforms (p38α, β, γ, and δ), and Erk5. The lesser-studied, atypical MAPKs include Nemo-like kinase (NLK), Erk3/4, and Erk7/8 [30].

The protein kinases constituting MAPK signaling modules form a series of sequential binary interactions to create a protein kinase cascade. These protein kinases are organized into signaling complexes that determine the specificity of the activated pathways [31]. All MAPKs substrates are characterized by the presence of specific docking sites. Although the docking sites are not necessarily near the MAPK phosphor-acceptor sites, they are recognized by a complementary docking motif on the MAPKs and mediate a strong and selective interaction between specific MAPKs and their substrates. This specificity is increased by the binding of scaffold proteins, such as MEK-partner 1 (MP1) and β-arrestin 1 and 2 [32].

MAPK pathways are essential to regulate several physiological processes and carry out their functions by phosphorylating target proiteins and activating transcription factors, such as ETS transcription factor (Elk1), c-Jun, and activating transcription factor 2 (ATF2). Different studies have demonstrated the involvement of these pathways in orchestrating differentiation, proliferation and apoptosis. JNKs play a crucial role in the specification of CD4+ T-cells, regulating the balance between Th1 and Th2 phenotype. The lack of JNK1 and JNK2 impairs the production of IL-2 thus promoting the differentiation of CD4+ T-cells into Th2 phenotype. In addition, they promote cellular apoptosis following ultraviolet-C (UV-C) exposure and control cytochrome *c* release and mitochondrial apoptosis by regulating the activity of the anti-apoptotic protein Bcl2 [33]. On the contrary, p38 does not impact on the differentiation of CD4+ T-cells into Th1 or Th2 but it regulates Th1 responses and IFN-γ production [34]. Moreover, it has been demonstrated that p38α negatively regulates cell proliferation in multiple cell types and during liver cancer development. Indeed, the ablation of p38α in hepatocellular carcinoma cells is correlated with an up-regulation of the JNK-c-Jun pathway that promotes hepatocytes proliferation [35].

1.3. Transforming-growth factor-β (TGF-β) activated kinase 1 (TAK1)

The Transforming Growth Factor-β (TGF- β) activated kinase 1 (TAK1) is a serine/threonine kinase belonging to the MAP3K family. It has been demonstrated that TAK1 plays a critical role in orchestrating inflammatory response and cell survival control through the integration of different signaling pathways - including TGF-β, interleukin-1 (IL-1), Tumor Necrosis Factors α (TNFα) and Toll-Like Receptors (TLR). The main, and well characterized, effectors of TAK1 are two transcription factors - nuclear factor κ-B (NF-κB) and activated protein-1 (AP-1), and they are activated by specific extracellular stimuli [36]. In particular, TAK1 induces the activation of NF-κB by promoting the interaction between the NF-κB inhibitor kinase β (IKK β) complex and TNF Receptor Associated Factor 2 (TRAF2) or 6 (TRAF6) in TNFα and IL-1/TLR signaling pathways, respectively [37,38]. IKK β phosphorylates the IκB proteins leading to ubiquitination and, consequential degradation by the ubiquitinin-proteosome pathway. Upon degradation of IκB, NFκB is able to translocate into the nucleus and drive the expression of NFκB target genes [39].

1.3.1 Molecular mechanisms of TAK1 regulation

It has been demonstrated that TAK1 kinase activity is strictly regulated by multiple post translational modifications (PTMs), such as phosphorylation/dephosphorylation and ubiquitination, and these PTMs are orchestrated by TAK1 itself and by several TAK1 associated proteins [36]. TAK1 is constitutively bound to TAK1 Binding Protein 1 (TAB1), while it binds TAB2 or TAB3 only after IL-1 and TNFα stimulations [40]. All these binding proteins are required to regulate TAK1 activity. In particular, TAB1 contains a C-terminal domain and N-terminal pseudophospatase domain which are necessary to bind and activate TAK1, respectively [41]. On the other hand, TAB2 or TAB3 act by scaffold proteins and promote the interaction of TAK1/TAB1 complex to TRAF6 protein [40].

TRAF6 is an important ubiquitin ligase that promotes the K63-linked poly-ubiquitination of different proteins. Unlike K48-linked poly-ubiquitination, that is correlated with proteasomal degradation, K63-linked poly-ubiquitination has been demonstrated to regulate several signaling functions, such as protein kinase activation, DNA repair and vesicle trafficking [42]. Upon IL-1 stimulation, TRAF6 mediates a K63-linked poly-ubiquitination of TAK1 kinase complex which phosphorylates and activates IKK β [43]. In addition to TRAF6, the E3 ubiquitin ligase X-linked Inhibitor of Apoptosis (XIAP) has been shown to activate NF-κB pathway through the TAK1 ubiquitination [44].

TAK1 K63 poly-ubiquitination is tightly regulated by different deubiquitinatin (DUB) enzymes, such as Cylindromatosis (CYLD) and A20, which can negatively regulate TAK1, hence inhibiting NF-κB signaling [41]. The tumor suppressor protein CYLD contains an ubiquitin-carboxy-terminal-hydrolase (UCH) domain[45] through which it hydrolyzes the K63-linked polyubiquitin chains on TRAF6 and TRAF2, thereby inhibiting the activity of the TAK1 kinase complex [46]. Moreover, TAK1 stability is regulated by several E3 ubiquitin ligases mediating K48-linked poly-ubiquitination, such as ITCH/AIP4. The combined activity of ITCH and CYLD shifts TAK1 ubiquitination from K63-poly-ubiquitinationlinked to K48-linkedpoly-ubiquitination, thereby inducing TAK1 degradation and impairing the NF-κB-dependent inflammatory response [47].

1.3.2 Signaling pathways triggering TAK1 activation

TAK1 represents the cellular hub to which IL1, TGFβ and TLR signaling pathways converge (Fig.2).

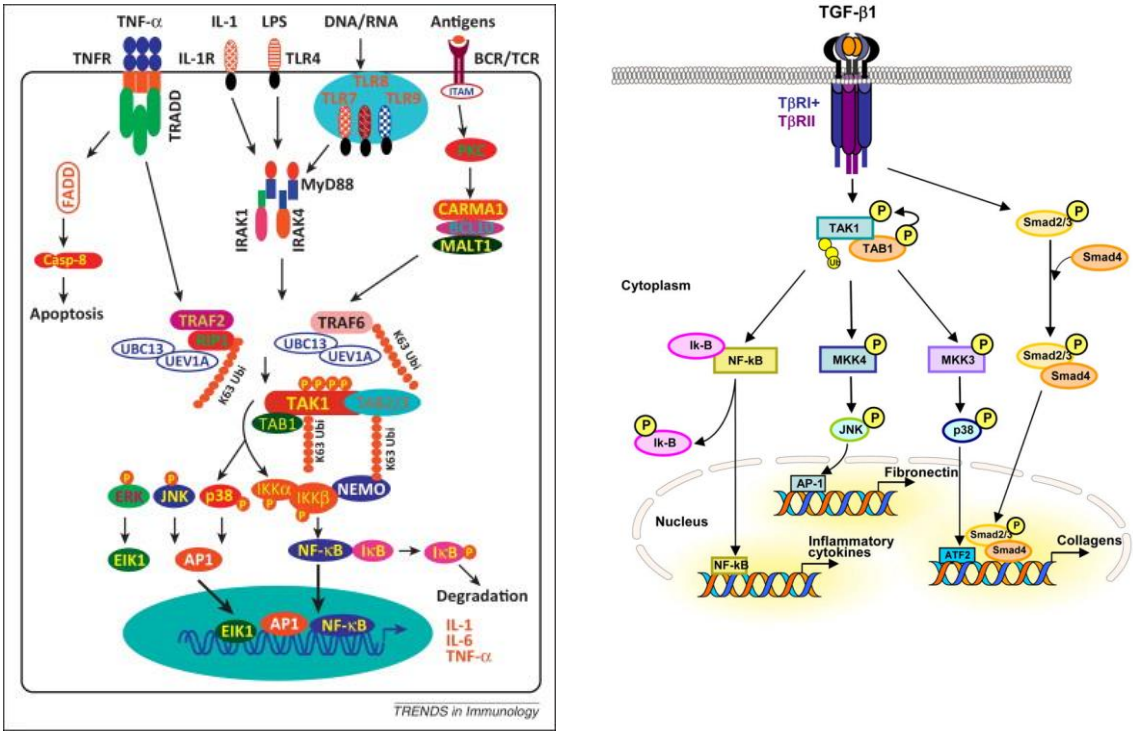


Fig 2. Transforming growth factor b-activated kinase 1 (TAK1) activation and downstream signaling pathways. TAK1 activation is triggered by several extracellular stimuli including IL-1, LPS and TGFβ. Stimulation of IL-1R and TLR4 promotes the activation of myeloid differentiation primary response gene 88 (MyD88) that, in turn, recruits IRAK4, IRAK1 and TRAF6. The MyD88/IRAK4/IRAK1 complex promotes the activation of TRAF6, which catalyzes K63-linked poly-ubiquitination of TAK1. Moreover for a full activation, TAK1 need to bind TAB1 and TAB2/3 proteins. This proteins promote the TAK1 activation by ubiquitination and auto-phosphorylation. Similarly, stimulation of TβRI and TβRII promotes by non-canonical pathway the activation of TRAF6 which, in turn, mediates the poly-ubiquitination and the activation of TAK1. Activated TAK1 drives the phopshorylation and the activation of both several MAPKs, including p38, JNK and ERK1/2, and NF-κB pathway [48,49].

IL-1 mediates inflammation and immunity responses through the activation of the NF-κB transcription factor [50]. The binding of IL-1 to its receptor, IL-1 receptor-1 (IL1R-1), leads to the activation of TAK1. Stimulation with IL-1 promotes the phosphorylation of TAK1 in Thr-178 and Thr-184 [51,52], both residing in its kinase activation loop, as well as the formation of a ternary TAB2-TAK1-TRAF6

complex resulting in TRAF6 mediated K63-linked poly-ubiquitination of TAK1[53,54].

The TGFβ growth factor is able to regulate several cellular functions by triggering a multitude of intracellular signaling pathways. TGF-β principally exerts its effects through the canonical Smad pathway; however, attention is also being focused on the non-canonical pathway, triggering MAPKs and NF-κB activation through the induction of TAK1 ubiquitination by TRAF6 [55]. In particular, TGFβ stimulation induces the dimerization of TGFβ receptor 1 and 2 (TβRI and TβRII) and the recruitment of TRAF6 to the receptor complex, which undergoes auto-ubiquitination and causes K63-linked poly-ubiquitination of TAK1 [56]. The outcome is p38 phosphorylation and the sustainment of several pro-survival pathways, such as NF-κB [57].

Another level of TAK1 regulation is exerted by Toll Like Receptors (TLRs). TLRs constitute a superfamily of pattern recognition receptors, well known for their role in host defense from infection [58]. Several studies demonstrated that TLRs are able to orchestrate key processes involved in tumorigenesis, such as inflammation, proliferation, migration and angiogenesis [59,60,61]. In particular, it has been demonstrated that, in response to lipopolysacchatide (LPS), TLR4, triggers the IL-1 receptor-associated kinase 1 (IRAK-1) dependent activation of TAK1 [62].

1.3.3 Role of TAK1 in normal tissues

The important role of TAK1 in orchestrating several cellular processes has been demonstrated through complete or conditional knock-out (KO) of TAK1, TAB1 or TAB2 in mice. Complete KO of any member of the TAK1 complex results in embryonic lethality, due to impaired development of neural tube [63], cardiovascular apparatus [64] and liver [65]. Conditional KO produces different outcomes. TAK1-/- Mouse Embryonic Fibroblasts (MEF) show reduced NF-κB and JNK signaling pathways [63]. Conditional KO of TAK1 in B-lymphocytes shows defect in both maturation and activation of B cells, due to the impairment of JNK activation [66,67]. Conditional KO in Natural Killer (NK) cells has shown a reduction of cytokines and chemokines secretion, caused by a decrease of NF-κB and JNK activity [68]. Conversely, TAK1 KO in myeloid cells induces splenomegaly and lymphomegaly, associated with neutrophilia and enhancement of both JNK and NF-κB activity [69]. NF-κB Conditional KO of TAK1 in Hematopoietic Stem/Progenitor Cells (HSPC) causes massive apoptosis in spleen, thymus, liver

and bone marrow, together with complete abrogation of both NF-κB and JNK signaling in bone marrow [70]. TAK1 KO in the liver parenchymal cells determines hepatocyte dysplasia and liver carcinogenesis [71], while epidermal-specific KO causes severe inflammatory skin condition and massive keratinocyte apoptosis, with impaired NF-κB and JNK signaling [72].

1.3.4 Role of TAK1 in cancer

Epithelial to Mesenchymal Transition (EMT) is the mean process by which cancer cells acquire the ability to invade and metastasize to other tissues. EMT is triggered by the interplay of soluble factors and cytokines, such as TGF-β and components of the extracellular matrix (ECM). The key event in EMT is probably the disruption of cadherin junctions, mostly due to the activity of several transcription factors repressing the expression of E-cadherin, such as the members of the ZEB, Snail and basic helix-loop-helix (bHLH) families. The signaling pathways triggering their regulation are constituted by SMADs, MAPK, Phosphatidylinositol 3-Kinase (PI3K), Glycogen Synthase Kinase 3 β (GSK3β) and NF-κB [73,74]. It has been reported that the TAK1/ NF-κB pathway induces downregulation of E-cadherin expression, thereby triggering EMT [75]. In addition, inhibition of TAK1 reverts IL-1β and TGF-β1 induced EMT by reducing the transcriptional activity of Smad1-5-8 in mesothelial cells [76], and by inhibition of Smad2/3 phosphorylation in retinal pigmental epithelial cells [77]. TAK1 promotes lymphatic invasion in breast cancer through increase in the expression of chemokine C-C motif receptor 7 (CCR7), whereas inhibition of its kinase activity by treatment with 5Z-7-Oxozeaenol (5Z-O) suppresses both lymphatic invasion and lung metastasis [78,79]. Similar results are observed in ovarian cancer, where TAK1 enhances tumor growth and metastatic capacity [80], and in colon cancer, where TAK1 has been shown to induce cancer cell migration and lung metastasis upon TNF-α stimulation by activating both JNK and p38 pathways [81].

It has been shown that TAK1 is one of the major regulators of chemioresistance in several types of tumors. Treatment of PC cells with the orally active TAK1 inhibitor, LYTAK1, reduced chemoresistance to oxaliplatin, gemcitabine and SN38. Moreover, it reduced tumor volume and prolonged survival in mice harboring PC xenografts and treated with the above mentioned drugs [57]. This effect is due to a NF-κB and AP1 dependent reduction of anti-apoptotic proteins, such as cellular inhibitor of apoptosis 2 (cIAP-2), which regulates programmed cell death by direct caspase inhibition and by promoting the degradation of pro-apoptotic proteins belonging to TNFα signaling [82] NF-κB[57].

1.4 Mitogen-activated kinase kinase kinase 3 (MAP3K3 or MEKK3)

Mitogen-activated kinase kinase kinase 3 (MAP3K3 or MEKK3) is a serine/threonine kinase belonging to the MEKK/STE11 subgroup of the MAP3K family that is costitutively expressed in several types of tissues [83]. It has been demonstrated an important role for MEKK3 in orchestrating cellular processes, such as proliferation, cell cycle progression[84], differentiation, migration, apoptosis [85,86] and inflammatory response [50], through the integration of different signaling pathways. MEKK3 is essential for both TNFα-induced IKK-NF-κB and JNK-p38 activation and IL1R-TLR4 induced IL6 productionNF-κB. The activation of the NF-kB and AP-1 transcription factors is exerted by different MEKK3-regulated kinases such as IKK, JNK1/2, p38, and ERK5 , upon specific extracellular stimuli [83,87,88].

1.4.1 Molecular mechanisms of MEKK3 regulation

The mechanisms regulating MEKK3 activation are still not fully understood. It has been demonstrated that several activating phosphorylations can occur within the kinase activation loop on Thr-516, Ser-520 and Ser-526, which might be due to either MEKK3 itself or other kinases following specific stimulation [89,90]. Several studies have demonstrated the involvement of accessory proteins, such as TRAF6 and 14-3-3, in promoting MEKK3 activation [50,91]. In particular, by interacting with p62, TRAF6 recruits MEKK3 on its zinc-finger domain, thereby promoting the oligomerization and the autophosporylation of MEKK3 in Ser-526 [50,92]. Phosphorylation in Ser-526 of MEKK3 promotes indirectly the recruitment

of 14-3-3ε to MEKK3. This interaction prevents dephosphorylation of Ser-526 by protein phosphatase 2 A (PP2A) thus sustaining MEKK3 activation [91 93]. Indeed, dephosphorylation of MEKK3 in Thr-516, Ser-520 and Ser-526 by PP2A is a main mechanism of inhibition of MEKK3-mediated signal transduction pathway [94]. However, it remains unclear how and which members of the protein serine/threonine phosphatase family inhibit MEKK3 activation.

1.4.2 Signaling pathways triggering MEKK3 activation

MEKK3 is a key signaling molecule downstream several pathways, such as TNFα [87], IL1 [50] and TLR4 [88], and its activity regulates, through NF-κB and AP1, the expression of many inflammatory response gene including pro-inflammatory cytokines, anti-apoptotic elements and growth factors [83,87,95].

Following IL-1 binding to its receptor, the formation of a receptor complex, constituted by IL1R-1 and IL1R accessory proteins, occurs. Subsequently, MyD88 is recruited to this complex and mediates the recruitment of IRAK1 and IRAK4 [96,97]. IRAK4 phosphorylates and activates IRAK1 which in turn recruits TRAF6 [98,99]. This multiprotein complex regulates two MEKK3 dependent pathways, the Ring finger and the Zinc finger pathways (Fig. 3).

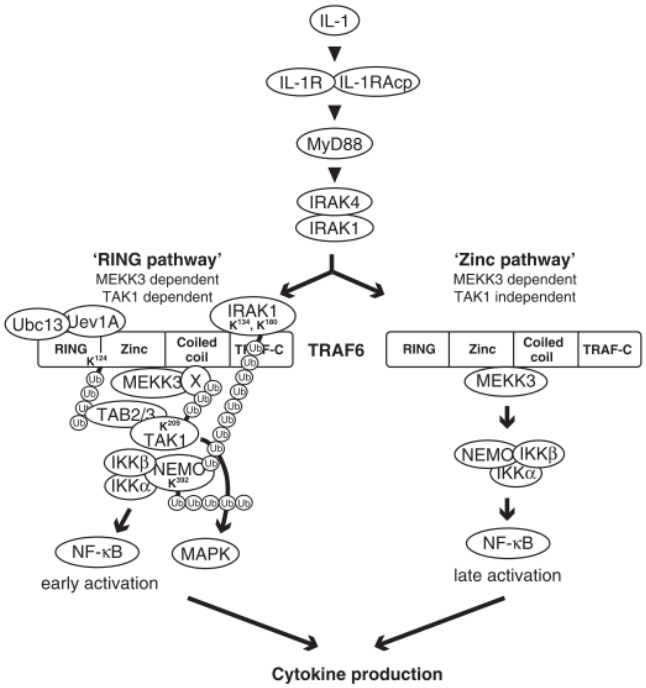


Fig. 3 IL-1 induces NF-κB activation by two mechanistically and temporally distinct pathways. The RING pathway transduces IL1 signaling by inducing the formation of a TRAF6/MEKK3/TAK1/IRAK1 signaling complex. K63-linked poly-ubiquitination of TRAF6 triggers the recruitment of TAB2/3 into the signaling complex, while K63-linked poly-ubiquitination of TAK1 promotes the binding of MEKK3 and its associated proteins. Within this complex, MEKK3 enhances TAK1 activity. In addition, following K63-linked poly-ubiquitination of IRAK1 IKK is recruited into the signaling complex thus activating the NF-κB pathway. On the other hand, the Zinc pathway is independent on TAK1 but MEKK3-dependent. Upon IL-1 stimulation, IRAK1 and 4 activate TRAF6 which, in turn, promotes the oligomerization and the autophosphorylation of MEKK3. Activated MEKK3 triggers the activation of the IKK complex and, thus, of the NF-κB pathway. While the RING pathway is triggered within one hour from IL-1 stimulation, the Zinc pathway drives NF-κB activation only afterwards [50].

In the Ring pathway, the IRAK1/TRAF6 complex translocates from the membrane to the cytosol where it binds both MEKK3 and TAK1 [41,88]. In this complex, MEKK3 may activate TAK1 by phosphorylation in Thr178, Thr184 and Thr187, thus promoting NF-κB activation [100]. In the Zinc pathway, instead, TRAF6 forms a complex with MEKK3 and promotes its oligomerization and, activation [50], which induces in turn the activation of the IKK complex by p38MAPK and promotes NF-κB signaling [88].

The proinflammatory cytokine TNFα is a soluble factor that strongly activates the NF-κB signaling pathway [101]. Upon TNFα treatment, both the IKK complex and MEKK3 are recruited to TNF receptor 1 (TNFR1) by TRAF2 and Receptor Interacting Protein (RIP) respectively [87,102]. It has been demonstrated that MEKK3 is necessary for the TNFα-induced activation of IKK complex and the NF-κB pathway. In accordance to this observation, MEKK3-/- Mouse Embryonic Fibroblasts (MEFs) show a downregulation of NF-κB-dependent antiapoptotic genes which makes them more sensitive to TNFα-induced apoptosis than MEF wild type [87].

LPS can activate NF-κB through IL-1R-TLR4 signaling in a MEKK3 dependent manner [103]. In particular, MEKK3 regulates the IL-1R-TLR4-induced IL-6 production through the activation of both JNK-p38 MAPK and IKK-NF-κB pathway. Knock-out of MEKK3 reduced the activation of MAPK kinase 6 (MKK6) and 7 (MEKK7), resulting in the loss of LPS-dependent p38 MAPK and JNK activation [104,88].

1.4.3 Role of MEKK3 in normal tissues

The important role of MEKK3 in orchestrating cellular processes has been also demonstrated through complete or conditional knock-out (KO) of MEKK3. Complete KO of MEKK3 causes embryonic lethality due to impaired cardiovascular development, endothelial cells proliferation and muscle cell formation [105,106]. Conditional KO mice harbored different phenotypes. Mice with T cell conditional ablation of MEKK3 have a significant reduction in peripheral T cells number, but do not present any alteration in the thymic T cells development and maturation [107]. Other studies have demonstrated that MEKK3 conditional KO in T cells leads to the accumulation of regulatory T (T reg) and Th17 cells in the periphery [108]. Conditional KO of MEKK3 in endothelial cells induces an increase of hemorrhages in multiple organs, especially in the brain [109].

1.4.4 Role of MEKK3 in cancer

Activation of the ERK pathway through hyperactivation of MAPKs signaling, is a hallmark of cancer development [110]. The epidermal growth factor (EGF) is involved in the pathogenesis of several types of tumors and its overexpression has been revealed in human carcinomas [111]. EGF receptor (EGFR) triggers an Erk5-dependent overexpression of the proto-oncogene c-Jun and consequent increase in cell proliferation [112]. It has been demonstrated that MEKK3 plays a major role in regulating EGF-induced cell proliferation by inducing endogenous Erk5 through the activation of MEK5 [84].

The role of MEKK3 in orchestrating many cellular processes important for the embryonic development is well known [113]. Only in the last years, the involvement of MEKK3 in driving the development and the malignancy of different tumors, such as lung, breast and esophageal cancers, has been studied [114–116]. More in details, MEKK3 is able to induce proliferation, migration and invasion of lung cancer cells, through the activation of AKT and GSK3β signaling pathways [114]. In particular, MEKK3 knock-out causes a reduction of cell proliferation and invasion, by downregulating genes such as CDC25A and CDK2, which promote cell proliferation, as well as DKK1 that promotes the invasiveness of cancer [114,117]. Overexpression of MEKK3 has been shown also in breast cancer and it is correlated with metastatization and survival of cancer cells. Silencing of MEKK3 reverts the malignant behavior of breast cancer cells; in particular MEKK3

knockdown in MCF7 cells impacts both on EMT through a downregulation of vimentin, and on cancer motility by a reduction of Intercellular Adhesion Molecule 1 (ICAM1) expression[118]. Moreover, the role of MEKK3 in driving the chemioresistance of ovarian and breast cancer has been also investigated; silencing of MEKK3 sensitizes cancer cell to apoptosis induced by both TNFα and chemotherapeutic agents, through the inhibition of the NF-κB pathway [115,118,119]. The clinical relevance of MEKK3 has been verified by Hasan *et al.*. They demonstrated that MEKK3 overexpression occurs in early stages of esophageal squamous cell carcinoma and, in combination with lymph-node positivity, it could be used as negative prognostic factor for this tumor [116].

1.5. The Hippo pathway

The Hippo pathway is crucial for the correct development of different organs and its dysregulation contributes to tumorigenesis [120]. Knock-out of the genes belonging to the Hippo pathway, such as Mammalian STE20-Like Protein Kinase 1 and 2 (MST1/2), Large Tumor Suppressor kinase 1 and 2 (LATS1/2) and Salvador Homolog 1 (SAV1), leads to increased organs size, due to both excessive cell proliferation and defective apoptotic program [121–123]. The Hippo pathway is constituted by the kinases MST1/2 and LATS1/2, the scaffold proteins SAV1 and MOB1A/B, and the transcriptional co-factors Yes-associated protein (YAP) and Transcriptional Coactivator With PDZ-Binding Motif (TAZ), which control the expression of Hippo target genes. Upon activation of the Hippo pathway, MST1/2 phosphorylate and activate LATS1 which, in turn, phosphorylates YAP and TAZ thereby inducing their cytoplasmic translocation and degradation [124,125] [126]. When the Hippo pathway is inactive, YAP and TAZ translocate into the nucleus and drive the expression of genes such as CTGF, AXL, CYR61, FosL1 and DKK1, involved in cell cycle progression, cell proliferation, differentiation, angiogenesis, stress response, apoptosis, and extracellular matrix formation [127–132] [120]. Being unable to bind DNA consensus sequences directly, YAP/TAZ form complexes with transcription factors, such as TEA domain family members (TEADs), AP1, SMADs and Nucleosome Remodeling Deacetylase (NuRD) complex, to exert their functions [127,133,134], and the binding to either transcription factor confers them characteristics of transcriptional co-activators or co-repressors.

1.5.1 Signaling pathways involved in YAP/TAZ regulation

The Hippo pathway activity is regulated by different stimuli including cell-cell and cell-matrix adhesion, matrix stiffness, mechanical stress and cell metabolism[120]. Moreover, YAP/TAZ is the hub for several signaling pathway such as EGF, TGFβ and canonical or non-canonical Wnt pathways [135,136]. In particular, the interplay between Wnt and YAP/TAZ pathway seem to be important in orchestrating several cancer processes including cell motility and tumorigenesis [137,138].

WNT is a family of growth factors that elicit diverse Frizzled receptor-mediated signaling pathways to control proliferation, stemness, EMT and tumorigenesis. Canonical WNT signaling is activated by Wnt3a ligand, and acts through β-catenin/TCF transcriptional activity. In the absence of Wnt activation, the so called 'destruction complex', containing the central scaffold protein Axin, adenomatous polyposis coli (APC), and Glycogen-synthase kinase-3 (GSK3) is assembled; GSK3 phosphorylates β-catenin and triggers its ubiquitination by βTrCP and consequent proteasomal degradation. Upon WNT activation, GSK3 is inactivated by phosphorylation, resulting in accumulation and nuclear translocation of β-catenin. Besides the canonical WNT signaling, a number of non-canonical, β-catenin-independent WNT pathways have been identified with the common activity to suppress canonical pathway, and Wnt5a/b are prototype of ligands activating these signaling [139]. Recent works highlighted a deep integration of YAP/TAZ in orchestrating canonical and non-canonical WNT responses. YAP/TAZ have been demonstrated as integral components of the β-catenin destruction complex, which serves as their functional sink. Activation of canonical WNT signaling causes rapid release of YAP/TAZ, leading to the activation of their transcriptional program. Moreover, YAP/TAZ incorporation in the destruction complex is essential for the recruitment of βTrCP and β-catenin degradation [140]. Conversely, in a non-canonical WNT pathway, Wnt5a induces a G-protein-mediated inhibition of LATS kinase activity towards YAP/TAZ. In turn, the stable YAP/TAZ/TEAD transcription complex drives the transcription of secreted factors such as CTGF, Wnt5a and DKK1, which inhibit canonical WNT pathway [137].

1.5.2 Role of YAP/TAZ in cancer

Growing evidence indicates that YAP and TAZ could be involved as key factors in different aspects of cancer, including tumorigenesis, metastasis, drug resistance, DNA synthesis and repair, control of cyclins for S-phase entry and completion of mitosis (Fig. 4) [141,142].

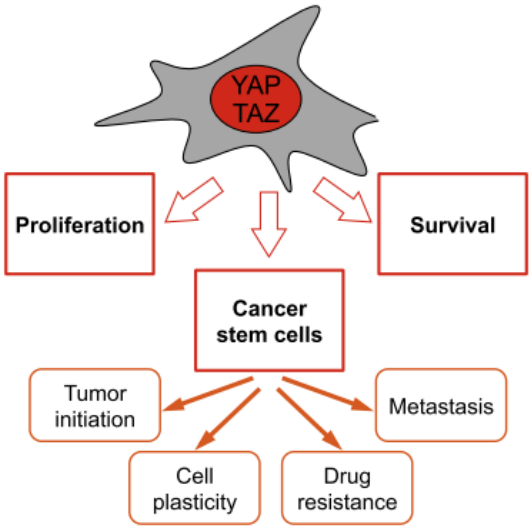


Fig. 4 YAP and TAZ confer aggressive features to cancer cells. Different works highlight the role of YAP/TAZ in regulating the development and the sustainment of most solid tumors. Their activation promotes cancer stem cell features, proliferation, chemoresistance and metastasis of cancer cells.

Overexpression of YAP/TAZ in normal cells promotes growth factor- and anchorage-independent proliferation, EMT and escape from apoptosis [143], by inducing c-Myc, or by promoting the expression of the Epidermal Growth Factor Receptor (EGFR) ligand amphiregulin (AREG) [127,144].

In breast cancer, YAP activation leads to a reduction of epithelial markers, such as E-cadherin, and an increase of mesenchymal markers, such as Vimentin and N-cadherin[143]. In addition, YAP/TAZ regulate several processes important for the metastatic spread of cancer, they promote matrix invasion and inhibit cancer cell death induced by loss of cell-substrate contact (anoikis)[120]. Knockdown of YAP in melanoma cells impairs their ability to invade the matrix, to escape from anoikis and produce lung metastasis in xenograft models [145]. Conversely, overexpression of YAP increases the metastatic potential of melanoma and breast cancer cells, and this process is highly correlated with YAP-TEAD interaction [146].

It is widely accepted that the growth of solid tumors requires the presence of cells with stem cell properties, known as Cancer Stem Cells (CSCs), which initiate and

promote tumor development. CSCs are characterized by self-renewal, metastatic ability, important chemoresistance and loss of differentiation markers [120,141]. Increasing evidences have shown YAP/TAZ are required and sufficient to endow cancer cells of these properties. It has been demonstrated that YAP interacts with the transcription factor SRF in breast cancer and promotes the transcription of genes typically expressed in mammary stem cells. This YAP-driven stemness requires IL-6 upregulation, and the YAP-SRF-IL6 axis is strongly activated in basal-like breast cancer, where YAP/TAZ protein levels are inversely correlated with patients survival [147]. Moreover, YAP has been recently demonstrated as a critical oncogenic effector of KRAS-induced PC development. In fact, YAP is essential for the development of the pre-neoplastic lesions PanIN into pancreatic ductal adenocarcinoma (PDAC) in Kras-mutant mice [148]. In addition, it has been shown that YAP is crucial for Kras-independent tumor recurrence in Kras-driven models of PDAC [149].

The role of TAZ in conferring stem cell properties has been investigated as well. Like YAP, TAZ is necessary for the self-renewal and the tumor-initiation capacities of breast cancer. Overexpression of TAZ in non-CSCs endows these cells of self-renewal capacity, while silencing of TAZ in patient-derived breast cancer stem cell (BCSC) lines reduces their tumorigenic and metastatic potential [150,151].

A main characteristic of CSCs is the intrinsic resistance to chemotherapy. YAP and TAZ have been shown to contribute to this feature. YAP/TAZ upregulation confers resistance to several types of anti-cancer drugs, such as anti-tubulin, anti-metabolite and DNA-damaging agents [142]. While TAZ drives the resistance to taxol and doxorubicin in breast cancer [151,152], YAP activity is correlated with the resistance of different cancer cell lines to 5-fluorouracil (5-FU) and its overexpression has been shown in therapy resistant colon and esophageal cancers [153–155]. The molecular mechanisms of YAP/TAZ chemoresistance in cancer cells are different and impact on several cellular processes, such as growth factor signaling, cell cycle progression, apoptosis, repair of DNA damage and EMT [142].

YAP/TAZ activation promotes the expression of genes involved in the growth factors signaling. In particular in esophageal cancer, YAP induces the expression of both EGFR and AREG, thus promoting EGF-independent survival and migration of cancer cells through the activation of EGFR signaling pathway [144]. Similarly, YAP is able to induce the insulin-like growth factor (IGF) pathway. It has been demonstrated that YAP-dependent IGF2/Akt activation promotes the cell survival of medulloblastoma upon irradiation, while the downregulation of IGF2 reverts this effects [156].

YAP/TAZ are able to reduce the effect of anti-cancer therapy by promoting cell cycle progression even in the presence of DNA damage [142]. It has been demonstrated in different models of PDAC and melanoma that the protein complex YAP/TAZ/TEAD cooperates with the transcription factor E2F1 to upregulate the expression of cell cycle/mitosis associated genes [149,157]. The consequence of this process is the accumulation of mutations in daughter cells and thus genomic instability of cancer cells[142].

Cancer cells are able to escape from apoptotic stimuli through YAP activity. YAP overexpression protects cancer cells from apoptosis upon chemotherapeutic treatment through the induction of anti-apoptotic genes, such as BCL2L1 and BIRC5[143,158,159]. Consistently, it has been reported that the development of resistance to RAF/MEK inhibitors is correlated with a YAP-dependent increase of BCL2L1 expression in several types of tumors [160].

2. Aims of the study

PC remains one of the most lethal and poorly understood human malignancy. Mitogen Activated Protein Kinase (MAPK) pathway is one of the most important cascades driving the aggressive features of PC. The activation of MAP3Ks leads to the regulation of cellular processes, such as cell cycle progression, adhesion, invasion and chemoresistance. Because of its pleiotropic role in PC biology, MAP3Ks pathway represents a critical area for the development of novel strategies for the treatment of PC.

Specific Aims:

Aim 1 – The role of TAK1 in regulating pancreatic cancer aggressiveness and treatment resistance through the activation of YAP/TAZ pathways.

TAK1 plays a central role in the NF-κB activation upon IL1 and TGFβ stimulation. Our recent paper has demonstrated that TAK1 orchestrates the chemoresistance of PC by sustaining NF-κB activation [57].
Several studies have demonstrated that the transcriptional cofactors YAP and TAZ, the main effectors of the HIPPO pathway, are able to regulate important cancer related processes, such as proliferation, tumorigenesis, stemness and drug resistance [161]. In particular, it has been revealed that YAP is a critical oncogenic effector of KRAS-induced PC development [148]. Although KRAS mutations are known to be a driver event for the development of different tumors, increasing evidences are accumulating about the development of a KRAS independent growth program in KRAS mutated fully competent tumors [162]. In details, two recent papers studied the mechanisms of tumor recurrence after KRAS inhibition in pancreatic and lung cancer models, and intriguingly linked the rescue of cell survival of initially KRAS addicted tumor cells to YAP1 activation. In genetically engineered $KRAS^{G12D}$: Trp53L/+ mouse models, spontaneous relapse of PC developed after $KRAS^{G12D}$ inactivation relied on YAP1 overexpression as a mechanism for KRAS independent growth [149,163].

The emerging role of YAP/TAZ in orchestrating the development and the sustainment of PC opens the need for the discovery of drugs able to inhibit their activities. However, so far, there are no drugs targeting specifically YAP/TAZ and the design of drugs, which could target transcriptional cofactors, is challenging.

My hypothesis was that TAK1 drives the aggressive features of pancreatic cancer, such as early metastatic behavior and chemoresistance, by sustaining YAP/TAZ activity. Inhibiting YAP and TAZ by targeting TAK-1 expression would revert the aggressiveness of pancreatic cancer.

Aim 2 – The role of MEKK3 in regulating the aggressive features of pancreatic cancer.

MEKK3, or MAP3K3, is a serine/threonine kinase downstream of three different receptors, IL1R, TNFαR and TLR8 [164], which regulates NF-κB, JNK and p38 MAPK pathways [88,103,104].

The role of MEKK3 in regulating embryonic development has been extensively studied [113]. Only recently, the involvement of MEKK3 in driving the development and the malignancy of lung, breast and esophageal cancers has been studied [114–116]. More in details, MEKK3 is able to regulate both pro-tumoral activities, such as proliferation, migration, invasion and chemoresistance, and anti-tumoral activity by sustaining the immune response. The clinical relevance of MEKK3 has been verified by Hasan *et al.*, who demonstrated that MEKK3 overexpression occurs in early stages of esophageal squamous cell carcinoma and, in combination with lymph-node positivity, it could be used as a negative prognostic factor for this tumor [116]. The role of MEKK3 in PC and, in particular, the features and the pathways regulated by MEKK3 in PC are still unknown.

My hypothesis was that MEKK3 plays an important role in orchestrating the aggressive behavior of pancreatic cancer, by sustaining the activation of both NF-κB and YAP/TAZ. Targeting of MEKK3 could reverse the intrinsic resistance of pancreatic cancer to chemotherapy, as well as its early metastatization.

3. Materials and Methods

3.1 Cell Lines and Reagents

Human pancreatic cancer (PC) cell lines AsPc-1, PANC- 1 were purchased from the American Type Culture Collection (Manassas, VA). MDAPanc-28 cell line was a kind gift by Dr. Paul J. Chiao. Panc1, AsPC1 and MDA-Panc28 PC cell lines silenced for the expression of TAK1 were established as described by Melisi *et al.* [57]. All cell lines used in this study were cultured as monolayers in high glucose Dulbecco's modified Eagle's medium (DMEM, 41966-029, Life Technologies, Gaithersburg, MD), supplemented with 10% heat-inactivated fetal bovine serum (FBS, 10270-106, Life Technologies), 2mM L-Glutamine (BE17-605E, Life Technologies), 100 IU/mL penicillin and 100 µg/mL streptomycin (DE17-602E, Life Technologies). Cell lines were grown at 37°C, 5% CO_2.

LYTAK-1 is a orally active TAK-1 kinase selective inhibitor (Ki = 13 nM; p38 Ki > 20 mM; IKKb Ki > 20 mM) generously provided by Eli Lilly Pharmaceuticals (Indianapolis, IN). For in vitro assays, LYTAK-1 was dissolved in 100% dimethyl sulfoxide (DMSO, A3672,0250, AppliChem, Darmstadt, Germany) at a stock concentration of 1 mM. The concentration of DMSO did not exceed 0.1% in any assay.

The TAK1 kinase activity was also targeted using (5Z)-7-oxozeaenol TAK1 kinase selective inhibitor (TOCRIS bioscience, Bristol, UK). For in vitro assays, (5Z)-7-oxozeaenol was dissolved in 100% DMSO at a stock concentration of 10 mM.

Lithium Chloride (LiCl, L4408-100G, Sigma), a non-specific oral GSK3 inhibitor (IC50=10mM) was purchased by AppliChem (Darmstadt, Germany). For *in vitro* assays, LiCl was dissolved in sterile water at a stock concentration of 5M. A working concentration of 20mM has been used in all the *in vitro* assays.

Gemcitabine (Accord), Oxalipatin (Accord), SN38 (Campto) and Abraxane (Celgene) were used at the indicated concentrations for the indicated time. The proteasome inhibitor MG132, (Z-Leu-Leu-Leu-al, C2211, Sigma-Aldrich, Saint Louis, MO) was dissolved in 100% DMSO and used at a 5µM concentration for 24h.

3.2 Generation of Knock-Out Cell Lines

In order to knock out MEKK3 in Panc1, AsPC1 and MDA-Panc28 PC cell lines, cells were transfected with plasmids expressing either control or MEKK3 targeting guide RNAs , as well as with Cas9-RFP vectors (Transomic, Huntsville, AL) using OMNIfect transfection reagent (# OTR1001, Transomic) following manufacturer's instruction. Briefly, one day prior transfection 3,0 x 10^4 cell were seed in 6-wells plate with DMEM 10% FBS without antibiotics. 200 µl of transfection solution, containing single guide RNA, Cas9-RFP and 4 µl of OMNIfect, were added to each well. 24 hours after transfection, transfected cells were selected with 2 µg/ml Blasticidin (A1113902, Life Technologies) for 72 hours. Cells were then trypsinized, washed with PBS, and re-suspended in DMEM with 2mM EDTA (A1104, AppliChem) and 1% penicillin/streptomycin. RFP-positive cells were single-sorted by FACS (FACS CANTO ARIA II, BD, Franklin Lakes, NJ, USA) into 96-well plates in 200 µL of DMEM containing 20% FBS and 1% penicillin/streptomycin. Single clones were expanded and screened for MEKK3 expression by protein immunoblotting.

Guide RNA sequences targeting MEKK3 are listed below:

Gene	Clone id	Sequence
MAP3K3	TEVH-1081756	GGACATTCGTGATTTCCGGA
MAP3K3	TEVH-1148898	CCTTGTGGTGCACAGACACG
MAP3K3	TEVH-1216040	ACAGACACGTGGTAGCGCCG
TELG1012	Non targeting control	GGAGCGCACCATCTTCTTCA

3.3 Gene Expression Microarrays and Pathway Analysis

Total RNA was extracted using Trizol reagent (Life Technologies, Carlsbad, California, US) following manufacturer's instruction. RNA quality was assessed by agarose gel electrophoresis. Total RNA was quantified by reading the absorbance at 260 nm using a NanoDrop (NanoDrop 2000, Thermo Fisher Scientific). Differences in gene expression between control and silenced TAK1

cells were examined using Illumina Human 48k gene chips (D-103-0204, Illumina, Milan, Italy). Briefly, synthesis of cDNA and biotinylated cRNA was performed using the IlluminaTotalPrep RNA Amplification Kit (AMIL1791, Ambion), according to the manufacturer's protocol using 500ng total RNA. cRNAs (750 ng) were hybridized using Illumina Human 48k gene chips (Human HT-12 V4 BeadChip). Array washing was performed using Illumina High Temp Wash Buffer for 10 minutes at 55°C, followed by staining using streptavidin-Cy3 dyes (Amersham Biosciences, Little Chalfont, United Kingdom). Probe intensity data were obtained using the Illumina Genome Studio software (Genome Studio V2011.1). Raw data were Loess normalized with the Lumi R package and further processed with Excel software. Each microarray experiment was repeated twice. Differentially expressed transcripts were tested for network and functional interrelatedness using the Ingenuity Pathway Analyses (IPA) software program (Ingenuity Systems, Redwood, CA).

3.5 Reverse Transcription PCR and quantitative Real Time PCR

Total RNA was extracted using Trizol reagent (Life Technologies) following manufacturer's instruction and quantified using Nanodrop (Thermo Fisher Scientific). 1 µg RNA was reverse-transcribed with High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems, Foster City, CA, USA) following manufacturer's instruction and 1/10 of the reverse transcription was subjected to Real-Time PCR using FAST PowerUp SYBR green mastermix (Applied Biosystems, Foster City, CA, USA). The following primers were purchased by Life Technologies and used at 0,2 µM final concentration:

Primer	Sequence
CTGF Forward	TACCAATGACAACGCCTCCT
CTGF Reverse	TGCCCTTCTTAATGTTCTCTTCC
DKK1 Forward	AAAAATGTATCACACCAAAGGACAAG
DKK1 Reverse	ATCCTGAGGCACAGTCTGATGA
BDNF forward	AGTTCGGCCCAATGAAGAAA
BDNF Reverse	GAGCATCACCCTGGACGTGTA
FOSL1 Forward	GCAGGCGGAGACTGACAAAC
FOSL1 Reverse	TTCCGGGATTTTGCAGATG
CYR61 Forward	GGATCTGCAGAGCTCAGTCAGA
CYR61 Reverse	CTTTCCCCGTTTTGGTAGATTCT
AXL Forward	TGCGCCAGGGAAATCG

AXL Reverse	AGGCATACAGTCCATCCAGACA
YAP Forward	CCACAGGCAATGCGGAATAT
YAP Reverse	CTGGCTACGCAGGGCTAACT
TAZ Forward	GGTGCTACAGTGTCCCCACAA
TAZ Reverse	TTTCTCCTGTATCCATCTCATCCA
Birc3 Forward	GACAGGAGTTCATCCGTCAAGTT
Birc3 Reverse	TCTGATGTGGATAGCAGCTGTTC
Bax Forward	TGGAGCTGCAGAGGATGATTG
Bax Reverse	GCTGCCACTCGGAAAAAGAC
Bcl2 Forward	GGCTGGGATGCCTTTGTG
Bcl2 Reverse	CAGCCAGGAGAAATCAAACAGA
B-actin Forward	GGCATGGGTCAGAAGGATT
B-actin Reverse	CACACGCAGCTCATTGTAGAAG

Primers for IL1α (QT00001127), IL1β (QT00021385), CXCL1 (QT00199752), CXCL2 (QT00013104), CXCL3 (QT00015442) and IL8 (QT00000322) were purchased from QIAGEN (Hilden, Germany).

To quantify the relative changes in gene expression, the 2-ΔΔCT method was used and reactions were normalized to endogenous control gene β-actin expression levels [165].

3.6 Protein extraction and western blotting

Total protein extracts were prepared by lysing cells in radioimmunoprecipitation assay buffer (50 mM Tris HCl [pH 8], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). All protein extracts were quantified by BCA Protein Assay Kit (23225, Thermo Fisher Scientific) and equal amounts (20-50 µg of protein extract) were loaded onto SDS-PAGE (8-10%), transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA) and subjected to immunoblot with the indicated antibodies. Antibodies to TAK1 (ab109526, 1:1000), CDH1 (ab40772, 1:10000), ITCH (ab109018, 1:1000), TRAF6 (ab94720, 1:1000) Ub-K63 (ab179434, 1:1000) were all purchased from Abcam (Cambridge, UK). YAP/TAZ (sc-101199, 1:1000), CTGF (sc-14939, 1:1000), GAPDH (sc-166545, 1:50000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MEKK3 (# 5727, 1:1000), AXL (# 3269, 1:1000), p65 (# 4764, 1:1000), p-p65 (# 3031, 1:1000), vinculin (# 13901, 1:1000) were purchased by Cell Signaling (Danvers, MA) and vimentin (M 0725, 1:4000) was purchased by DakoCytomation (Glostrup, Denmark). Secondary anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA, USA). All antibodies were diluted in 3% Non-fat dry milk dissolved in Tris Buffered Saline (TBS) or 5% bovine serum albumin/TBS/0.1% Tween-20. Immunoreactive proteins were visualized with Immobilion Western kit (EMD Millipore) according to the manufacturer's instructions. Images were acquired using ImageQuant LAS 4000 mini (GE Healthcare Life Sciences, Little Chalfont, UK).

3.7 Transwell migration and Wound healing assays

Cells were detached and counted. 5×10^4 cells, in a volume of 100 µl DMEM containing 0,1% FBS, were seeded in the upper chamber while the bottom chamber of the transwell was filled with 600 µl of DMEM with 10% FBS. Either LiCl or NaCl (control) was added to the upper chamber at a 20mM concentration . Cells were allowed to migrate for 20 hours. Then cells remaining in the upper chamber were scrubbed away with a cotton pad and cells remaining on the bottom layer of the upper chamber were subjected to DAPI staining as follows: the upper chamber was washed twice with PBS and then cells were fixed with 4% formaldehyde for 20 minutes, permeabilized with 0,2% Triton for 25 minutes and, after being washed with PBS, they were stained with 5 µM DAPI for 5 minutes. Each membrane was scanned by fluorescence microscopy (EVOS FL Auto, Thermo Fisher Scientific) and all the cells were automatically counted using ImageJ software. Each sample was assayed in triplicate. Graphs show percentage of cells relative to control. Student t-test has been used to calculate p-values.

Wound Healing assay was performed as described below. PC cell models were seed at a density of 6.0×10^5 in a 6-well plates. After 24 hours, a straight scratch was made using a pipette tip to simulate a wound. The cells were washed gently with cold phosphate-buffered saline (PBS) and rinsed with fresh complete medium. Photographs of at least three different points were taken immediately and every hour for the following 72 hours by automatic microscopy (EVOS FL Auto, Thermo Fisher Scientific). The Wound healing tool plugin has been used to measure wound opening.

3.8 Cell Proliferation assays

Control or MEKK3 KO PC cell lines were seeded at a density of 1.0×10^3 cells/well in 96-well plates. Cell proliferation was measured by using the Sulforhodamine B (SRB) assay. At specific time points (0, 24, 48 and 72 hours), cells were washed with PBS and fixed with 50% Trichloracetic acid solution in water. Following incubation for 1 hour at 4°C, cells were washed three times with distilled water and labeled with a solution of 0,4% SRB/1% acetic acid for 30 minutes. Labelled cells have been washed three times with 1% acetic acid solution and SRB was dissolved by adding 10mM TrisHCl pH 10,5. Assorbance at 540nm has been measured using the iMark microplate reader (Bio-rad, Hercules, CA, USA). Fold increase proliferation has been calculated by dividing the absorbance measured at 24, 48 and 72 hours by the absorbance measured at T_0.

3.9 Nude Mouse Orthotopic Xenograft Models

5-weeks old female athymic nude mice (Crl:CD1-Foxn1nu, CDNSSFE05S) were purchased from Charles River (Wilmington, MA, USA). All mice were housed and treated in accordance with the guidelines of The University of Verona Animal Ethic Committee, and maintained in specific pathogen-free conditions. To produce pancreatic tumors, PC cells were harvested from subconfluent cultures by brief exposure to 0.05% trypsin-EDTA (GIBCO, ref 25300-054, Life Technologies). Trypsin activity was quenched with medium containing 10% fetal bovine serum and tumor cells were resuspended in a solution of 1:1 Matrigel:PBS at 1.0×10^4 cells/µl concentration (Matrigel Matrix Growth Factor, 356230, BD, Franklin Lakes, NJ). Orthotopic injection of PC cells was performed as described below. Mice were anesthetized with a 3% isoflurane–air mixture. A small incision in the left abdominal flank was made, and the spleen was exteriorized. 5×10^5 tumor cells in 50 µl were injected subcapsularly in a region of the pancreas just beneath the spleen. A 30-gauge needle and 1 mL disposable syringe were used to inject the tumor cell suspension. A successful subcapsular intrapancreatic injection of tumor cells was identified by the appearance of a fluid bleb without intraperitoneal leakage. To prevent such leakage, a cotton swab was held over the injection site for 1 minute. One layer of the abdominal wound was closed with wound clips (Auto-clip; Clay Adams, Parsippany, NJ). Tumor growth was monitored by either bioluminescence with D-Luciferine Firefly (Part Number. #122799, PerkinElmer, Boston, MA) or fluorescence imaging performed using a

cryogenically cooled IVIS 100 imaging system coupled with a data-acquisition computer running the Living Image software program (Xenogen).

3.10 Colony-forming assay

5 x 10^2 cells were seeded in 6-wells plates and grown for 15 days. To maintain good growth conditions, the medium was changed every 48 hours. Cells were stained for 30 minutes with 0,1% crystal violet dissolved in 10% of formaldehyde. Cells were washed three times with water and colonies were counted by automatic microscopy (EVOS FL Auto, Thermo Fisher Scientific).

3.12 Chromatin Immunoprecipitation (ChIP) assay

Cells were crosslinked and chromatin was extracted and sonicated. Chromatin was incubated overnight with the following antibodies: IgG (Vector Laboratories, X0720, 1:50), anti-Phospho-NF-kB (#3033, 1:50), anti-Acetyl-H3 K9/K14 (Cell Signaling, #9677, 1:50), anti-YAP (Santa Cruz Biotechnology, sc-101199, 1µg). Dynabeads Protein G were blocked overnight with 1mg/ml Sonicated Salmon Sperm (Thermo Fisher Scientific, 15632011) and 1mg/ml Bovine Serum Albumin (Thermo Fisher Scientific, AM2616). 30µl Dynabeads Protein G (50% slurry) were used for each IP. Immunpecipitated chromatin was purified with DNeasy Blood & Tissue Kit (Qiagen, 69504) following manufacturer's instruction. Quantitative Real Time PCR was used to assess for DNA enrichment using FAST PowerUp SYBR green mastermix (Applied Biosystems). Immunoprecipitated chromatin was normalized to input chromatin (GAPDH gene). Values shown in the histograms have been normalized to IgG.

The following primer sequences were used:

Primer	Sequence
AXL Forward	GAGTGGAGTTCTGGAGGAATGTTT
AXL Reverse	GTGAGGCCGTGTCTCTCTATCC
DKK1 Forward	GCACCCAAGTTCCCAGAGTTC

DKK1 Reverse	CGAGCGTTATAGCAGACGACTTT
CTGF Forward	GCCATATTCAACATCTGCACACA
CTGF Reverse	TGAGGTCAGGACAAGGAAAGAATAG

4. Results and Discussions

4.1 Aim 1 - *To determine the role of TAK1 in PC aggressiveness and treatment resistance through the activation of YAP/TAZ.*

4.1.1 Silencing of TAK1 affects the HIPPO pathway.

In order to identify TAK1-regulated pathways, we used AsPC1, Panc1 and MDA-Panc28 PC cell lines previously transduced with lentivirus expressing TAK1-specific shRNA or scramble sequence as a control [57]. We compared gene expression profiles between TAK1-silenced and control cell lines by genome-wide differential gene expression analyses. Upon TAK1 silencing, we observed a deregulation of several pathways, including autophagy, WNT and HIPPO pathways. In particular, we identified a significant reduction in the expression of genes upregulated by the Hippo pathway, such as DKK1, CTGF, AXL (Fig. 5A). We validated our microarray data by quantitative Real Time (qRT) PCR and we measured a significant reduction of DKK1, CTGF and AXL expression in TAK1-silenced cells as compared to their controls (Fig.5B).

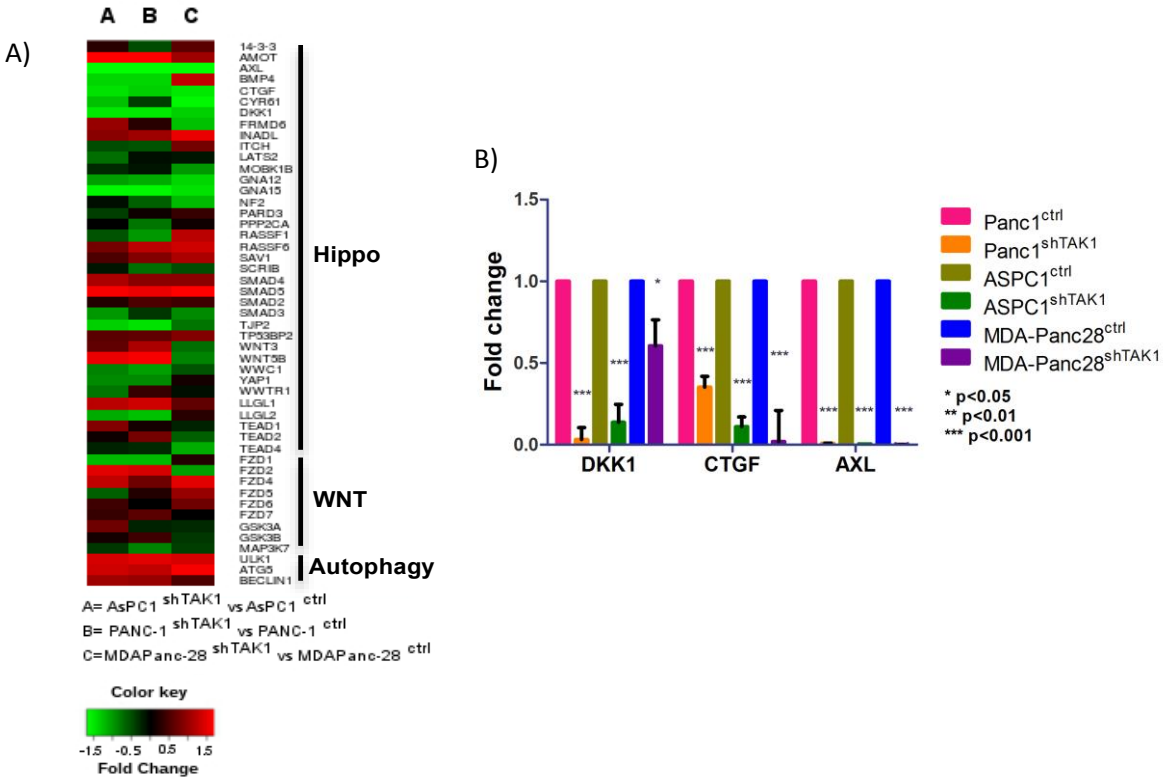


Fig. 5 TAK1 silencing affects different pathways in PC cell lines. A) Heatmap of TAK1-regulated pathways. The identification of relevant biological processes and genes was assessed by global transcript profiling. Signaling pathways enriched among genes differentially expressed in TAK1 silenced cell lines versus their respective controls were analysed. Gene expression levels and unsupervised hierarchical clustering of differentially expressed genes were performed. The log2of the gene expression levels are shown as colour-code heat map (green = decreased expression, red = increased expression). **B)** Expression of DKK1, CTGF and AXL in our TAK1 models. Histograms show the fold change in RNA expression between the gene of interest and β-actin as assessed by qRT-PCR. Mean values and SD from 2 independent experiments conducted in triplicate are shown. T-test has been used to perform statistical analysis.

4.1.2 TAK1 silencing decreases YAP/TAZ protein levels.

To further study the regulation of the HIPPO pathway by TAK1, we evaluated the expression of both YAP/TAZ and their regulated genes in PC cell lines stably interfered for TAK1 .We observed a strong decrease of YAP/TAZ protein levels in TAK1 silenced cells as compared to control cells (Fig.6A). Conversely, we could not detect any significant reduction in YAP and TAZ mRNA levels (Fig.6B). To corroborate our finding that the downregulation of YAP and TAZ proteins caused a reduction of their target genes DKK1, CTGF and AXL (Fig. 5B), we assessed CTGF protein levels by Western blot as well. We observed a strong decrease of CTGF in all shTAK1 cell lines as compared to their controls (Fig.6C).

To better evaluate whether YAP/TAZ reduction could be due to shRNA off-target effects or to a remodeling of cellular shape, previously reported as a consequence of TAK1 silencing [57], we transiently silenced the expression of TAK1 in AsPC1, Panc1 and MDA-Panc28 cells by using predesigned siRNA against TAK1. As early as 72 hours post transfection, when no change in cell shape could be detected, we observed a significant reduction of YAP/TAZ protein levels in PC cell lines (Fig. 6D), ruling out the possibility that reduction of YAP/TAZ protein levels could be due to a long-term adaptive effect of TAK1 silencing.

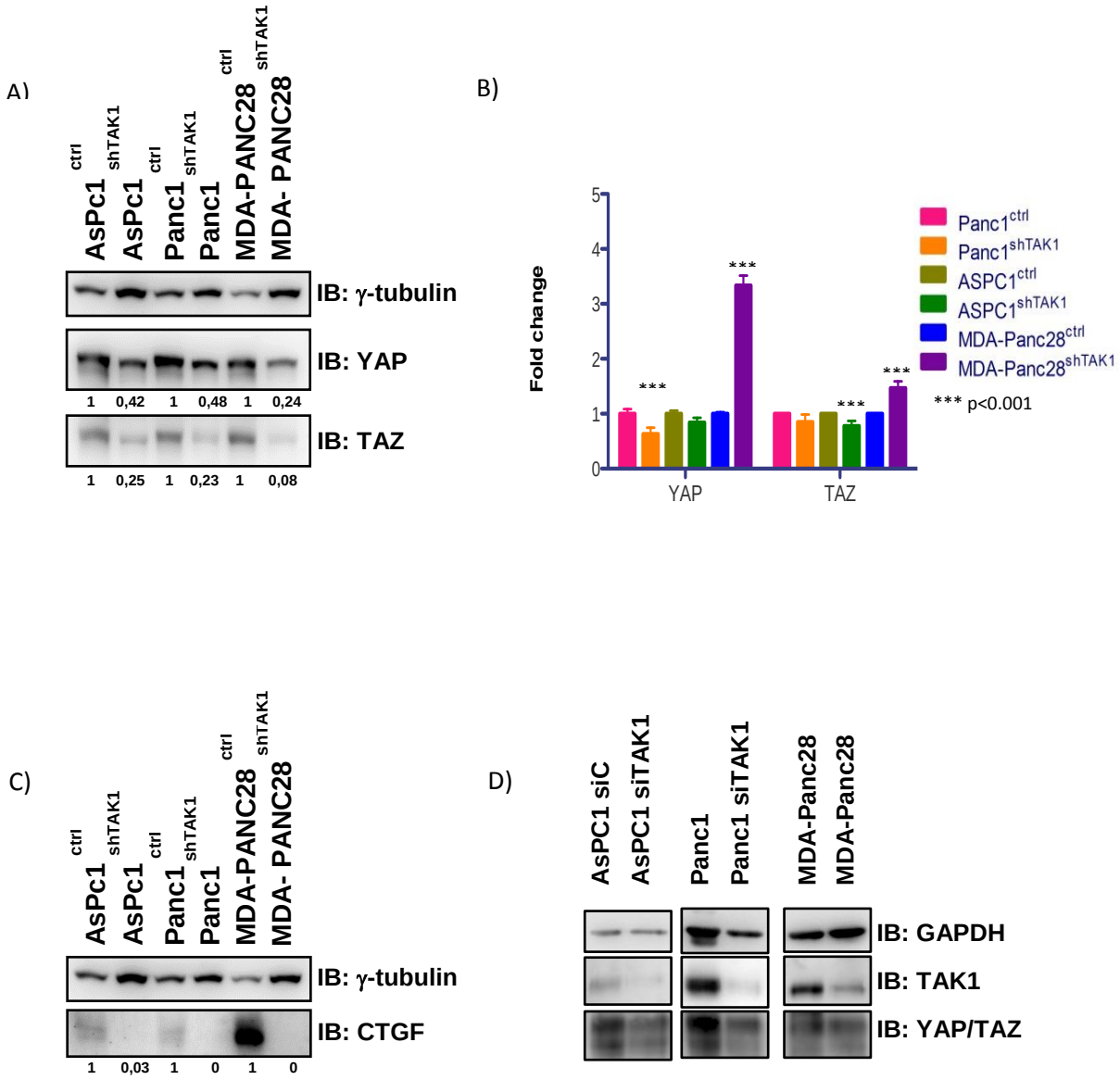


Fig. 6 TAK1 silencing reduces YAP/TAZ protein levels. A) Cell extracts from ASPC1, PANC1 e MDA-PANC28 control and shTAK1 cells were subjected to immunoblot with the indicated antibodies. All signals were normalized to γ-tubulin and densitometric analysis is shown below each immunoblot. **B)** qRT-PCR analysis of YAP/TAZ mRNA in ASPC1, PANC1 e MDA-PANC28 control and shTAK1 cells. Histograms show the fold change in RNA expression between the gene of interest and β-actin. Mean values and SEM from one independent experiment conducted in triplicate are shown. **C)** Cell extracts from ASPC1, PANC1 e MDA-PANC28 control and shTAK1 cells were subjected to immunoblot with the indicated antibodies. All signals were normalized to γ-tubulin and densitometric analysis is shown below each immunoblot. **D)** Cell extracts from ASPC1, PANC1 e MDA-PANC28 cells transiently transfected with either control or TAK1-targeting siRNA were subjected to immunoblot with the indicated antibodies.

4.1.3 TAK1 kinase activity is not involved in YAP/TAZ regulation.

In order to identify the mechanisms by which TAK1 regulates the YAP/TAZ protein levels, we first evaluated whether the reduction of YAP/TAZ could be related to TAK1 kinase activity. To this aim, we treated for 72 hours AsPc-1, PANC-1, and MDAPanc-28 cell lines with increasing doses of two TAK1 selective inhibitors, 5Z-7-oxozeaenol (2,5 and 5 µM) and LYTAK1 (5 and 10 nM). We observed that the pharmacological inhibition of TAK1 did not affect YAP/TAZ protein levels in any tested PC cell line (Fig. 7), suggesting that their levels depend on the presence of TAK1 rather than its kinase activity.

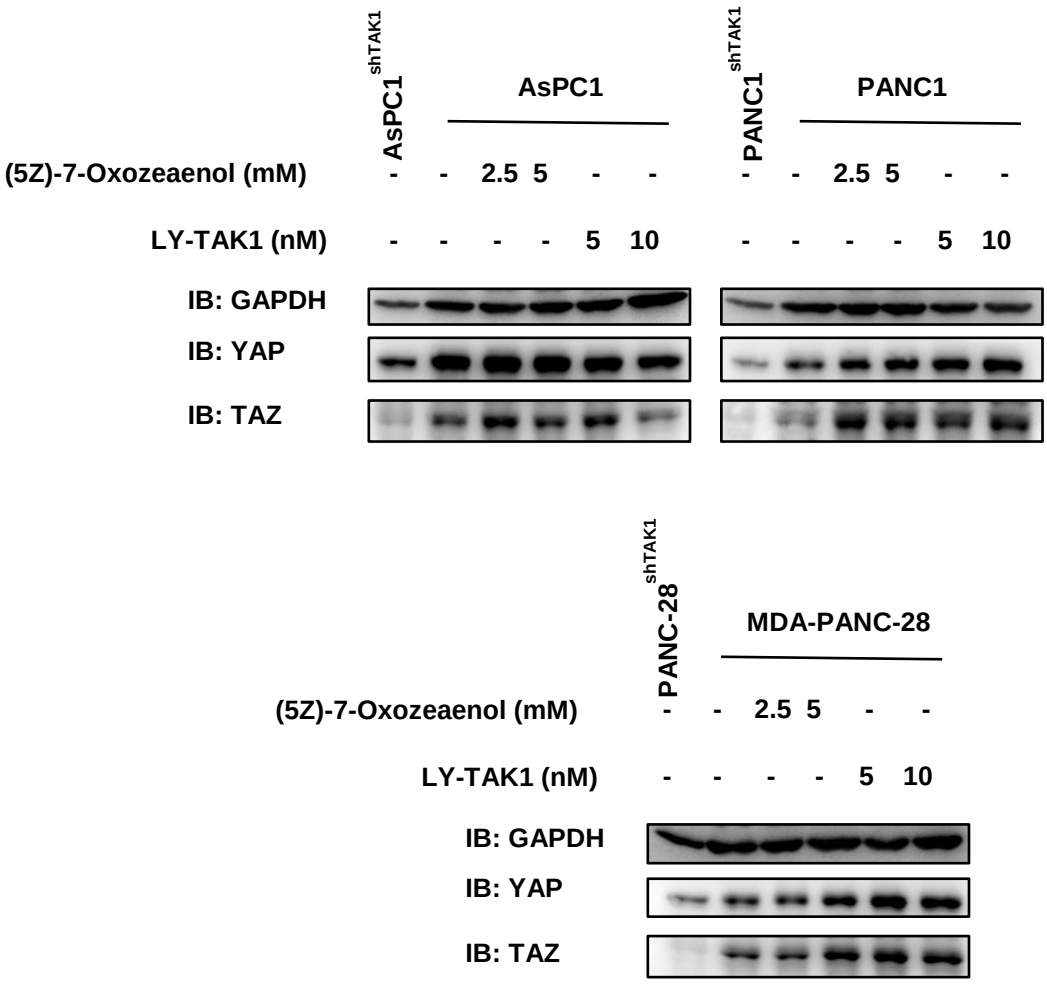
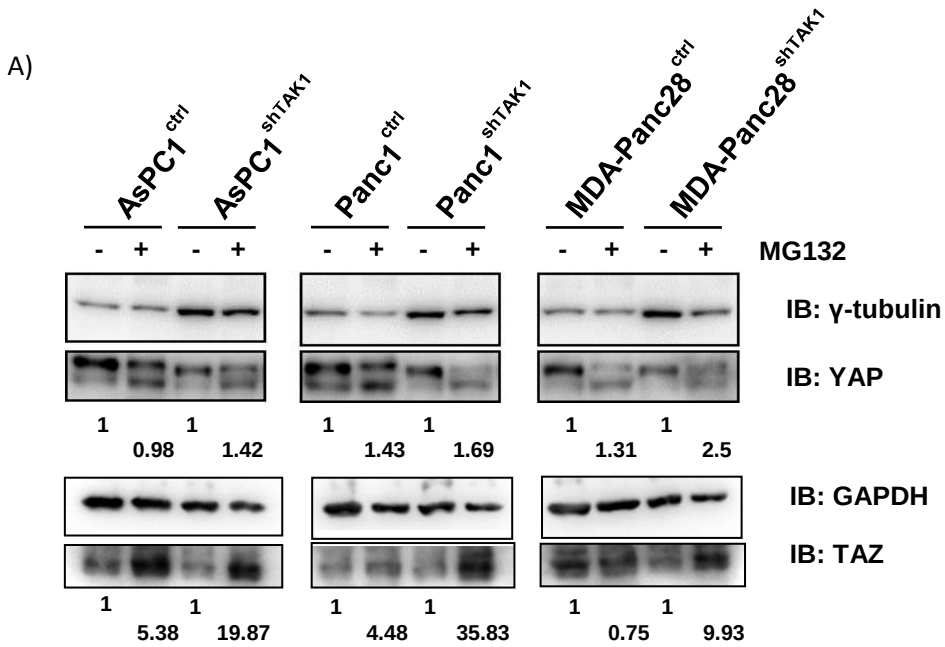


Fig. 7 TAK1 kinase activity did not regulate YAP/TAZ protein levels. Cell extracts from ASPC1, PANC1 e MDA-PANC28 cell lines treated with 5,7-Z-oxozeaenol (2,5uM and 5uM) and

LY26 (5nM and 10nM) for 72 hours were subjected to immunoblot with the indicated antibodies. All signals were normalized to vinculin.

In order to study whether TAK1 is able to protect YAP and TAZ from proteasomal degradation, we treated TAK1 silenced and control cells with the well-known proteasome inhibitor MG132. Interestingly, upon MG132 treatment we observed an increase of YAP and TAZ protein levels that was more significant in TAK1 silenced cells than in control cells. In particular, TAZ protein levels increased 19.87, 35.83 and 9.93 fold in shTAK1 AsPC1, Panc1 and MDA-Panc28 respectively (Fig. 8A). These data suggest that TAK1 could somehow protect YAP and TAZ from *proteasomal degradation. In order to investigate the role of TAK1 in modulating K63- or K48*-linked poly-ubiquitination, we analyzed the expression of TRAF6 and ITCH in TAK1 silenced cells. Interestingly, we observed an increase of ITCH protein, which mediates K48-linked polyubiquitination, and a reduction of TRAF6, that mediates K63-linked polyubiquitination, in shTAK1 AsPC1, Panc1 and MDA-Panc28 cell lines (Fig. 8B). Accordingly, we also revealed an important decrease of K63-linked poly-ubiquitinated proteins in TAK1 silenced cells as compared to their controls (Fig. 8C).



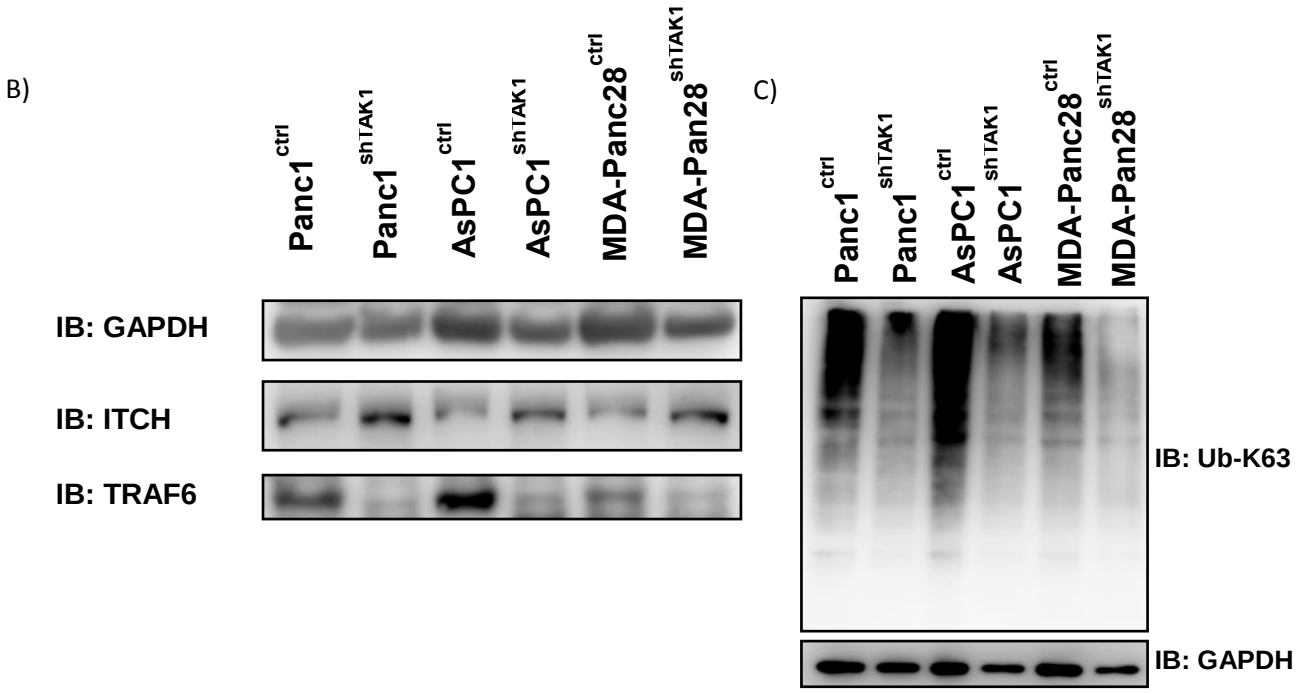


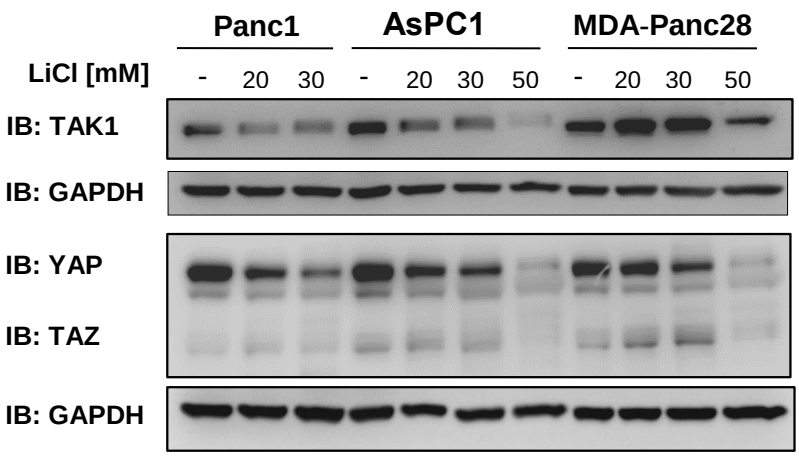
Fig. 8 TAK1 regulates YAP/TAZ proteasomal degradation. A) YAP/TAZ protein levels in ASPC1, Panc1 e MDA-Panc28 treated with MG132. AsPC1, Panc1 and MDA-Panc28 cell lines were treated with 5uM MG132 for 24h. Cellular extracts were subjected to immunoblot with the indicated antibodies. **B-C)** Cellular extracts from Panc1, AsPC1 and MDA-Panc28 control and shTAK1 cells were subjected to immunoblot with antibodies against ITCH and TRAF6 **(B)** and anti-K63-linked ubiquitin **(C).**

4.1.4 Targeting GSK3α activity downregulates both TAK1 and YAP/TAZ pathway.

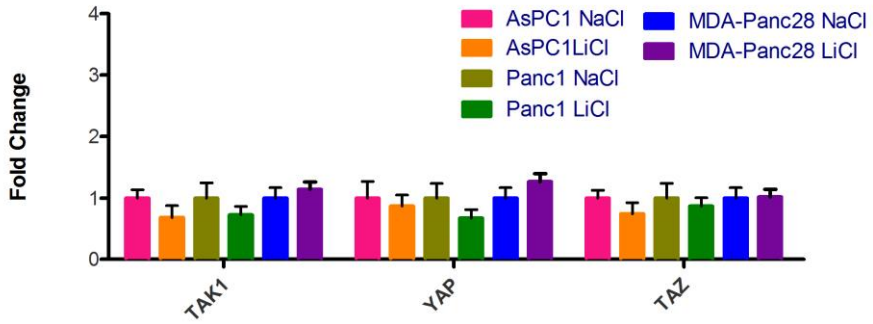
The emerging role of YAP/TAZ in orchestrating the development and the sustainment of PC opens the need for the discovery of drugs to inhibit their activities [148,149]. However, so far, there are no drugs targeting specifically YAP/TAZ and the design of drugs which could target transcriptional cofactors is challenging. As previously demonstrated, the well known TAK1 kinase inhibitors, 5,7-Z-oxozeaenol and LY26, are not able to reduce YAP/TAZ protein levels in PC cells, thus they can not be used in the clinics to modulate YAP/TAZ activities. Bang *et al*. have shown that inhibition of GSK3α affects the stability of TAK1 [166]. In light of this work, we asked whether GSK3α could regulate both the YAP/TAZ pathway and the aggressive features of PC by inhibiting TAK1.

We observed a decrease of both TAK1 and YAP/TAZ protein levels in AsPC1 and Panc1 PC cell lines, following inhibition of GSK3α activity with 20 mM LiCl. A higher LiCl concentration, such as 50mM, was needed to reduce TAK1 and YAP/TAZ protein levels in MDA-Panc28 cells (Fig. 9A). Like in TAK1 silenced cells, the decrease of TAK1 and YAP/TAZ proteins is not correlated with a reduction of their mRNA levels upon LiCl treatment (Fig. 9B). Because LiCl has several off target effects, we tested two more specific GSK3α inhibitors in PC cells, CHIR-99021 and LY2090314. Following treatment of AsPC1, Panc1 and MDA-Panc28 with 3 µM CHIR or LY2090314 for 72 hours, we observed a different reduction of YAP/TAZ and TAK1 protein levels (Fig. 9C), which was not correlated to a reduction of their mRNA (Fig. 9D, E). In particular, upon treatment with LY2090314, we observed a more prominent decrease of YAP in both AsPC1 and MDA-Panc28 cells, while only TAZ was downregulated in Panc1 cells (Fig. 9C).

A)



B)



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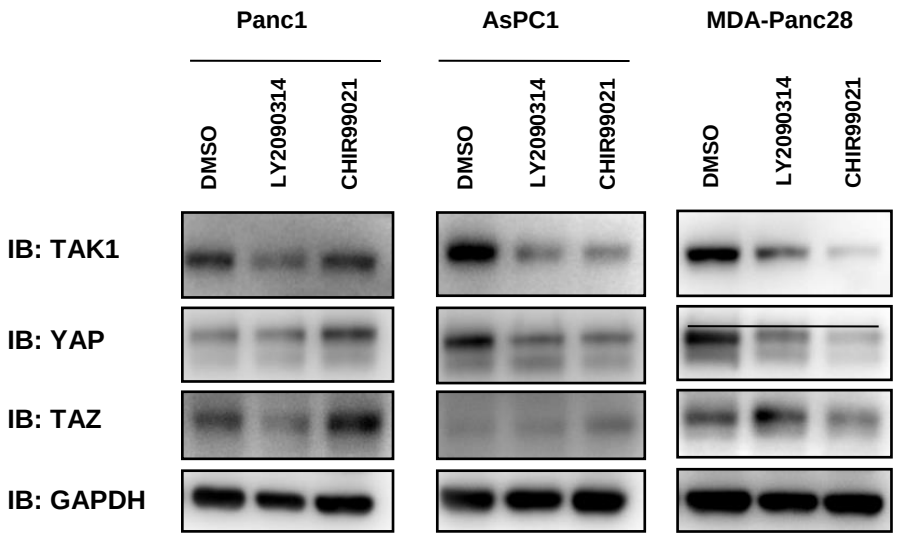
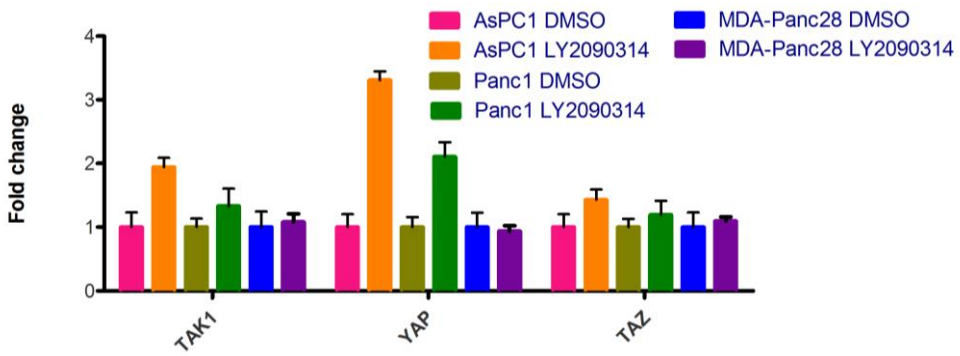


D)



E)

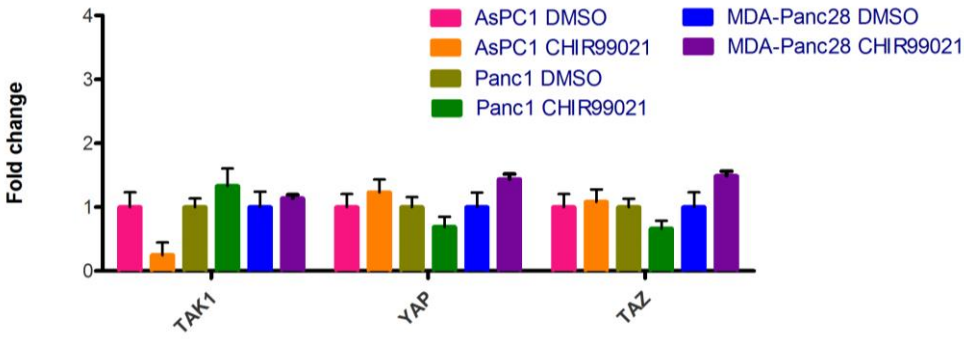


Fig 9. GSK3α inhibition reduces TAK1 and YAP/TAZ protein levels. A) Cellular extracts from Panc1, AsPC1 and MDA-Panc28 cells treated with 20, 30 and 50 mM LiCl for 72h were subjected to Western blot analysis for the expression of YAP/TAZ and TAK1. **B)** TAK1 and YAP/TAZ genes expression levels in Panc1, AsPC1 and Panc28 cells treated with 20mM LiCl for 72h were

evaluated by qRT-PCR. Histograms show mRNA levels of the indicated target genes over β-actin. Mean values and SD are shown. **C)** Cellular extracts from AsPC1, Panc1 and MDA-Panc28 cells treated with 3uM CHIR-99021 or LY2090314 for 72h were subjected to Western blot analysis for the expression of YAP/TAZ, TAK1. **D-E)** TAK1 and YAP/TAZ genes expression levels in Panc1, AsPC1 and Panc28 cells treated with 3 μM LY2090314 (D) or CHIR99021 (E) for 72h were evaluated by qRT-PCR. Histograms show mRNA levels of the indicated target genes over β-actin. Mean values and SD are shown.

To further investigate whether GSK3 inhibition could impair the YAP/TAZ pathway, we measured the expression of YAP/TAZ target genes such as CTGF, AXL, DKK1 and CYR61. In details, upon treatment with LiCl, we observed a 0,59 ($p<0,001$) and 0,47 ($p<0,001$) fold reduction in AsPC1 and MDA-Panc28 in CTGF expression, which is not significantly downregulated in Panc1 cell lines; AXL was downregulated 0,65 fold ($p<0.05$) in AsPC1, but not in Panc1 and MDA-Panc28 cell lines; CYR61 was reduced 0.63 fold ($p<0.05$) in AsPC1 cells, but not in Panc1 and MDA-Panc28 cell lines. Better results have been obtained by inhibiting GSK3 with LY2090314 and CHIR99021. In details, upon treatment with LY2090314 and CHIR99021, we measured a significant downregulation of CTGF, AXL, DKK1 and CYR61 expression in PC cell lines (Fig. 10) as reported in the table below. As shown, the treatments affect in different ways the expression of YAP/TAZ target genes in AsPC1, Panc1 and MDA-Panc28. This effect could be related to the different genetic background of PC cell lines, in which several mutations could drive the response to the treatment.

		AsPC1	Panc1	MDA-Panc28
LY2090314/DMSO	**CTGF**	0.08 ± 0.15	0.26 ± 0.23	0.23 ± 0.08
	AXL	0.18 ± 0.14	0.12 ± 0.22	0.45 ± 0.09
	DKK1	1.02 ± 0.14	3.47 ± 0.22	0.99 ± 0.08
	CYR61	0.44 ± 0.14	1.20 ± 0.22	0.23 ± 0.08
CHIR99021/DMSO	**CTGF**	0.18 ± 0.2	0.17 ± 0.14	0.33 ± 0.07
	AXL	0.25 ± 0.19	0.20 ± 0.13	0.46 ± 0.07
	DKK1	0.46 ± 0.19	0.53 ± 0.12	0.95 ± 0.06
	CYR61	0.33 ± 0.19	0.53 ± 0.14	0.29 ± 0.07

Table 1. Differential expression levels of YAP/TAZ target genes in AsPC1, Panc1 and MDA-Panc28 treated or not with LY2090314 and CHIR99021.

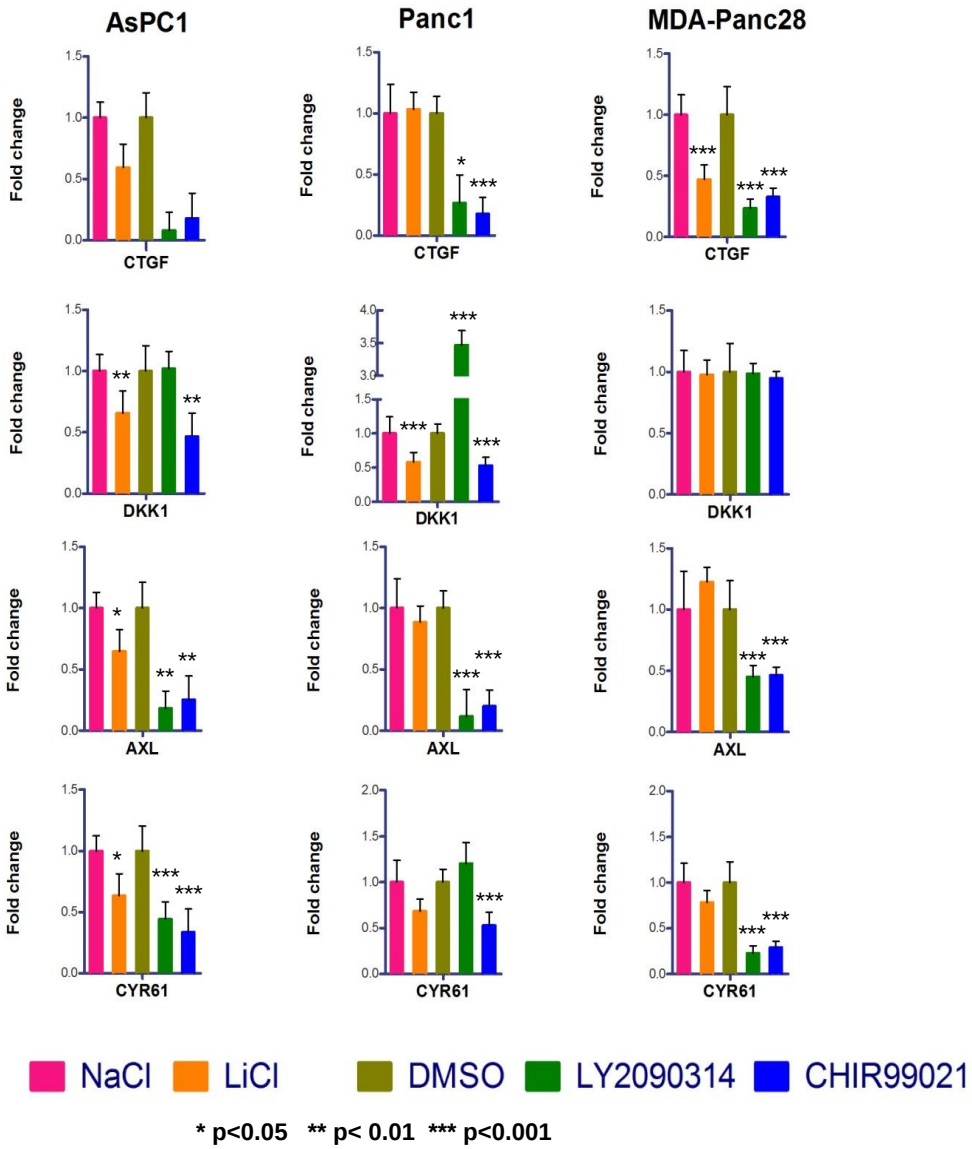
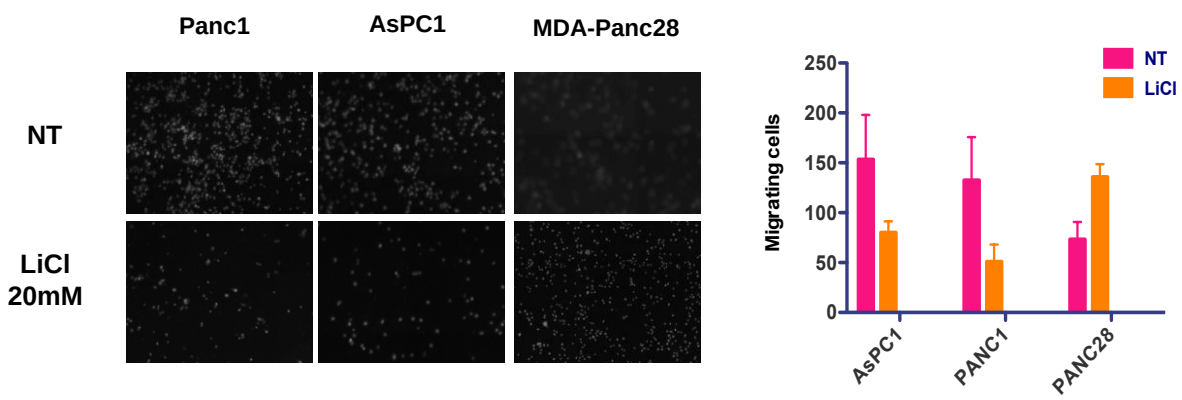


Fig 10. GSK3α inhibition affects YAP/TAZ-target genes expression. The expression of YAP/TAZ target genes was assessed by qRT-PCR in Panc1, AsPC1 and Panc28 cells treated with LiCl (20mM), LY2090314 (3µM) or CHIR99021 (3µM) for 72h. Histograms show mRNA levels of the indicated target genes over β-actin. Mean values and SD are shown from one experiment conducted in triplicate are shown. ***, P < 0.001; **,P<0.01; *, P <0,05, by t-test.

Because the YAP/TAZ pathway regulates several processes involved in tumor malignancy, we investigated whether GSK3 inhibition was correlated with a reduction of migration, proliferation and stemness of PC cells. Treatment of PC cells with 20 mM LiCl caused an strong reduction in their migration ability, amounting to 46% and 64% inhibition in AsPC1 and Panc1 respectively, as assessed by transwell migration assays (Fig. 11A). We also measured an important reduction of proliferation in PC cells treated with LiCl (20 mM). More in details, LiCl inhibited proliferation by 27% for AsPC1 ($p<0,001$), 12% for Panc1 ($p<0,001$) and 40% for MDA-Panc28 ($p<0,001$) respectively, as compared to untreated cells (Fig. 11B). Better results have been obtained by inhibiting GSK3 with LY2090314 and CHIR99021. In details, upon treatment with LY2090314, we measured a significant reduction of proliferation equal to 50.78% ($p<0,001$), 69.5% ($p<0,001$) and 30.47% ($p<0,001$) for AsPC1, Panc1 and MDA-Panc28, respectively. Likewise, upon treatment with CHIR99021, we observed a reduction in proliferation of 30.89% ($p<0,001$), 32.25% ($p<0,001$) and 18.98% ($p<0,01$) for AsPC1, Panc1 and MDA-Panc28, respectively. We could hypothesize that the decrease of TAZ in Panc1 cells following LY2090314 could account for a slightly more pronounced inhibition of proliferation (Fig. 11B).

A)



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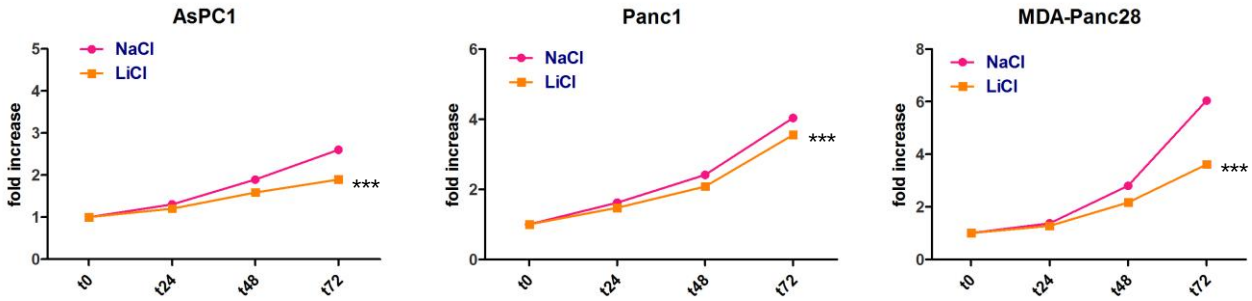

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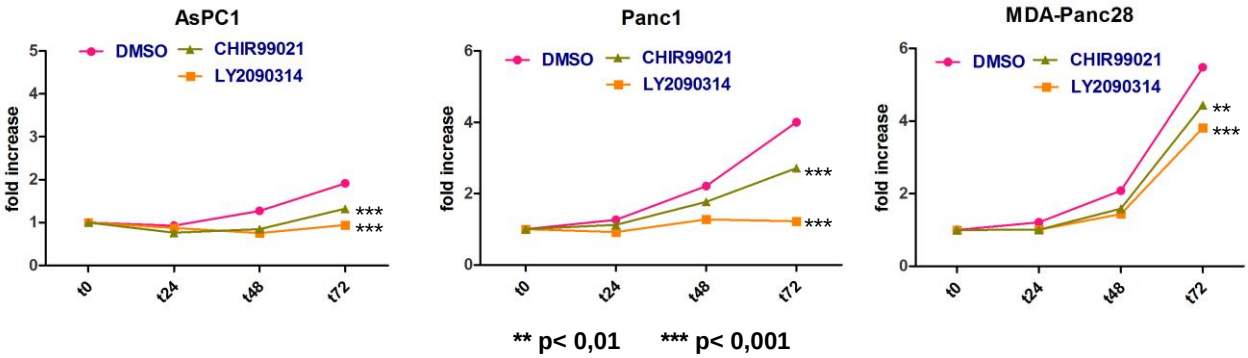


Fig 11. GSK3α inhibition impairs migration and proliferation of PC cells. A) A transwell assay was performed in AsPC1, Panc1 and MDA-Panc28 treated with 20 mM of LiCl for 24h. Images show the migrated cells. Histograms show the number of migrated cells. Mean and SD are indicated. **B-C)** Graphs show the results of cell proliferation assays in Panc1, AsPC1 and MDA-Panc28 treated or not with 20 mM LiCl **(B)**, 3 µM CHIR99021 and 3 µM LY2090314 (**C**). Measurements were conducted in octuplicate. Mean values and SD are shown from one experiment conducted in octuplicate are shown. ***, P < 0.001; **,P<0.01, by t-test.

4.1.5 Aim 1 – Discussion

PC is one of the most lethal human cancers and will continue to be a major unsolved health problem in the 21st century. One of the major challenges remains in developing effective therapeutic strategies that target the unique molecular biology of PC and to integrate these molecularly targeted agents into established combination chemotherapy regimens in order to improve patients survival. In this work we demonstrated a unique role for TAK-1 in the sustainment of YAP and TAZ.

TAK1 has recently emerged as a central regulator of diverse physiological processes including development, metabolism and immune and stress responses, leading to the activation of the transcription factors NF-κB and AP-1 [41]. The role of TAK1 in PC has been recently demonstrated [57]. Silencing or pharmacological inhibition of TAK1 reduces NF-κB activation, thereby strongly potentiating the activity of commonly used chemotherapeutic agents in PC cell lines [57].

Several studies have demonstrated that the transcriptional cofactors YAP and TAZ, the main effectors of the HIPPO pathway, are able to regulate important cancer related processes, such as proliferation, tumorigenesis, stemness and drug resistance [161]. In particular, it has been shown that YAP is essential for the development of PC in KRAS mutated mice, it drives EMT and promotes the tumor growth of PC even upon KRAS inactivation [148,149,163] . The emerging role of YAP/TAZ in orchestrating the development and the sustainment of PC opens the need for the discovery of drugs able to inhibit their activities. However, so far, there are no drugs targeting specifically YAP/TAZ and the identification of new molecules, which could target these transcriptional cofactors, is challenging.

To our knowledge, our present study is the first to identify TAK1 as a regulator of YAP/TAZ. In this regard, we demonstrated that silencing of TAK1 induced a significant downregulation of the HIPPO pathway (Fig.5A) and, in particular, a reduction in the expression of YAP/TAZ target genes DKK1, CTGF and AXL (Fig. 5B). Interestingly, we observed that silencing of TAK1 was correlated to a strong decrease in YAP/TAZ protein levels, but not to a downregulation of YAP/TAZ mRNA levels in PC cells (Fig. 6 A,B). In order to identify the mechanisms by which TAK1 could regulate the stability of YAP/TAZ, we evaluated whether TAK1 kinase activity was involved in this process. Interestingly, we observed that treatment with two TAK1 kinase inhibitors did not affect YAP/TAZ protein levels (Fig. 7), suggesting that TAK1 kinase activity was not involved in YAP/TAZ stabilization. Thus, we investigated whether proteasomal degradation was involved in YAP/TAZ degradation. Treatment with the proteasome inhibitor, MG132, led to an increase of TAZ protein levels that was stronger in shTAK1 cells than in their controls (Fig. 8A), suggesting that ubiquitination processes are involved in YAP/TAZ stabilization. Different kinds of ubiquitination exist and, in particular, K48-linked polyubiquitination mediates the proteasomal degradation of proteins, while K68-linked polyubiquitination prevents it [167,168]. Different studies have demonstrated that two important E3 ubiquitin ligases, ITCH and TRAF6, interact with and regulate TAK1 [169]. In particular, upon IL1 stimulation, TRAF6 binds to and promotes the activation of TAK1 by mediating its K63-linked poly-ubiquitination [43]. On the contrary, in

combination with CYLD, ITCH induces K48-linked poly-ubiquitination of TAK1 thereby inhibiting its activity [41]. Thus, we hypothesized that ITCH and TRAF6 could regulate YAP/TAZ degradation in PC cells. Interestingly, we observed a significant increase of ITCH and a reduction of TRAF6 in PC cells upon silencing of TAK1 (Fig. 8B), which was correlated to a strong decrease of total TRAF6-mediated K63-linked poly-ubiquitination in shTAK1 cells (Fig. 8C). Altogether, these data demonstrate for the first time that TAK1 regulates the proteasomal degradation of YAP/TAZ independently of its kinase activity, by modulating ITCH and TRAF6 expression.

In order to target YAP/TAZ activity by modulating TAK1 expression, we took advantage of a recent paper demonstrating that targeting GSK3α activity could affect TAK1 stability [166]. We observed for the first time that inhibition of GSK3 was correlated to a significant reduction of both TAK1 and YAP/TAZ protein levels in PC cells (Fig. 9 A, C). Like in shTAK1 cells, we did not measure any decrease in YAP/TAZ mRNA levels (Fig 9 B, D, E), but we observed an important downregulation of YAP/TAZ target genes upon GSK3 inhibition that was stronger upon LY2090314 treatment rather than CHIR99021 or LiCl treatments (Fig. 10). This diffenent effect on YAP/TAZ-regulated genes observed could be related to the diverse genetic background of PC cell lines, in which several mutations could drive the response to the treatment. These data further highlight that the presence of TAK1 is essential for YAP/TAZ stabilization rather than for their expression.

GSK3α/β are two of the main kinases regulated by canonical WNT pathway [139]. Recent works highlighted the role of canonical and non-canonical WNT pathways in regulating YAP/TAZ stability and activity. Azzolin *et al.* demonstrated that YAP/TAZ are integral components of the β-catenin destruction complex [140]. Activation of the canonical WNT pathway induces the recruitment of AXIN/β-catenin/YAP/TAZ complex to Low Density Lipoprotein Receptor-related Protein 6 (LRP6). This interaction induces a rapid release of YAP/TAZ from the complex, leading to their nuclear translocation and activation of YAP/TAZ/TEAD-dependent transcription. In the absence of WNT activation, YAP/TAZ drive the recruitment of the β-Transducin repeat Containing E3 ubiquitin Protein ligase (βTrCP) to AXIN/β-catenin/YAP/TAZ complex, thereby regulating the degradation of both YAP/TAZ and β-catenin. This work identifies the canonical WNT pathway and, in particular, the β-catenin destruction complex as the functional sink which mediates the degradation of both YAP/TAZ and β-catenin proteins [140]. Conversely, Park *et al.* demonstrated the role of non-canonical WNT pathway in regulating YAP/TAZ activity [137]. The stimulation of non-canonical WNT pathway mediated by Wnt5a/b induces the activation of $G\alpha_{12/13}$ proteins. The consequently activated phosphorylation cascade culminates in the inhibition of LATS and in the stabilization of YAP/TAZ. In turn, YAP/TAZ translocate into the nucleus where they regulate the expression of different secreted factors, such as DKK1, which can inhibit canonical WNT pathway. This work demonstrated that YAP/TAZ regulation by the non-canonical WNT pathway is independent on the destruction complex [137].

Different studies demonstrated a role for TAK1 in mediating non-canonical WNT signaling. Wnt1 stimulation resulted in autophosphorylation and activation of TAK1 in a TAB1-dependent fashion, resulting in the stimulation of a Nemo-like kinase (NLK)-MAPK cascade and in an inhibitory phosphorylation of TCF/LEF

[170,171]. The TAK1-NLK-MAPK cascade could be also activated by the non-canonical Wnt5a/Ca2+ pathway to counteract canonical β-catenin signaling. However, a kinase-inactive mutant of TAK1(K63W) only minimally reversed the blocking effect of Wnt5a on β-catenin activation [172], suggesting that other TAK1-regulated mechanisms could affect the canonical WNT pathway. *Our data identify the mechanism by which TAK1 promotes YAP/TAZ stabilization and impacts on the canonical WNT pathway.*

Interestingly, GSK3 inhibition, which simulates the activation of the canonical WNT pathway, induced a strong decrease in YAP/TAZ protein levels by modulating TAK1 expression (Fig. 9). We speculate that non-canonical [137] and canonical [140] WNT pathways can regulate the fate of YAP/TAZ by modulating TAK1.

Our study demonstrated for the first time that pharmacological silencing of TAK1, mediated by GSK3 inhibition, downregulates the YAP/TAZ pathway in PC cells. In order to investigate the biological relevance of this phenomenon, we evaluated the effect of GSK3-mediated YAP/TAZ dowregulation on different aspects of PC cells.

We observed that treatment with GSK3 inhibitors led to a significant reduction in proliferation and migration of cancer cells, that was more evident upon LY2090314 rather than LiCl or CHIR99021 treatment (Fig. 11). Similar results have been reported in two different papers. Marchand *et al.* demonstrated that silencing of GSK3 impaired the anchorage-independent tumor growth of PC cells [174], while Ying *et al.* identified in GSK3β one of the main players that drive the invasion of PC cells [173,174]. Additional studies will be necessary to evaluate the effects of TAK1 pharmacological silencing on YAP/TAZ-regulated oncogenic features, such as stemness, metastatization and drug resistance, in both *in vitro* and *in vivo* experiments.

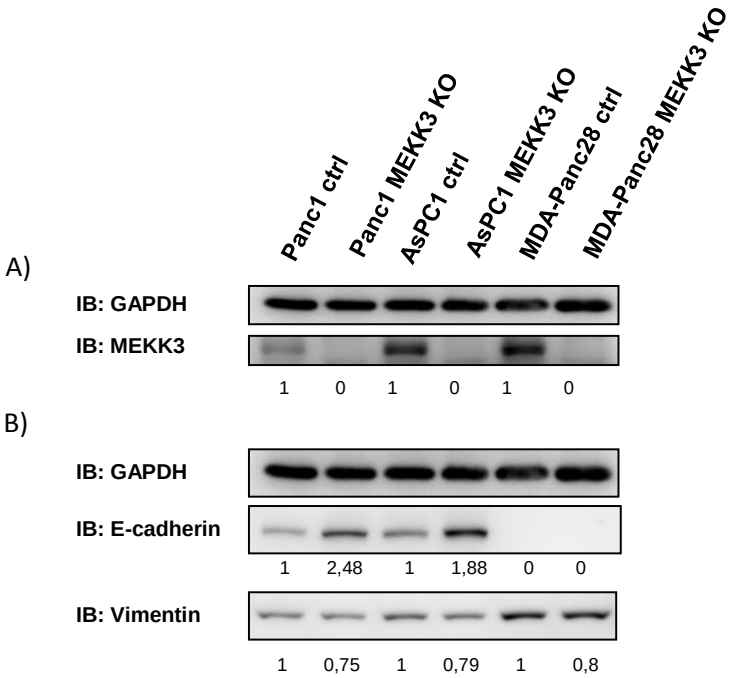
The past two decades have witnessed a major focusing of PC research on several molecules that are high in the signal transduction cascade, with a particular interest in membrane receptors such as the EGFR [175]. From the results of the clinical trials with inhibitors of this molecule in PC we learned that the single mutation of K-Ras - the most common genetic alteration in PC - is probably able to inactivate the antitumor activity of anti-EGFR approaches [176]. On the other hand, the design of drugs which could target transcriptional factors and cofactors, such as NF-kB and YAP/TAZ, is challenging. We believe that the pharmacological silencing of TAK1 could represent a better approach to reduce the aggressive behavior of PC. We concluded by observing that TAK1 is not a single enzyme, but it may be consider rather as the active component of a large protein signaling complex, characterized by different proteins, such as YAP and TAZ, and involved in the regulation of several signaling pathways.

4.2 Aim 2 - *To determine the role of Mitogen-Activated Protein Kinase Kinase Kinase 3 (MAP3K3 or MEKK3) in the metastatic behavior of PC.*

4.2.1 Knock-out of MEKK3 reverts EMT features in pancreatic cancer cells.

In order to study the role of MEKK3 in the metastatic behavior of PC, we established MEKK3 Knocked-Out (KO) cells by using CRISPR-Cas9 in Panc1, AsPC1 and MDA-Panc28. These cell lines were transfected with vectors expressing MEKK3-specific gRNA or control gRNA in combination with the DNA endonuclease, Cas9, which resulted in MEKK3 KO (Fig. 12B).

To determine the effect of MEKK3 KO on different EMT features, we evaluated both the expression of epithelial and mesenchymal markers and the migration of MEKK3 KO and control cell lines. We demonstrated that MEKK3 KO promotes the expression of the epithelial marker E-cadherin, and partially reverts the expression of the mesenchymal marker Vimentin, in all the three PC cell lines under study(Fig. 12B). Moreover, we observed that MEKK3 KO reduced the migratory abilities of Panc1 and AsPC1 by wound healing assay (Fig. 12C). Together, these data demonstrate that MEKK3 triggers EMT and migration in PC cells.



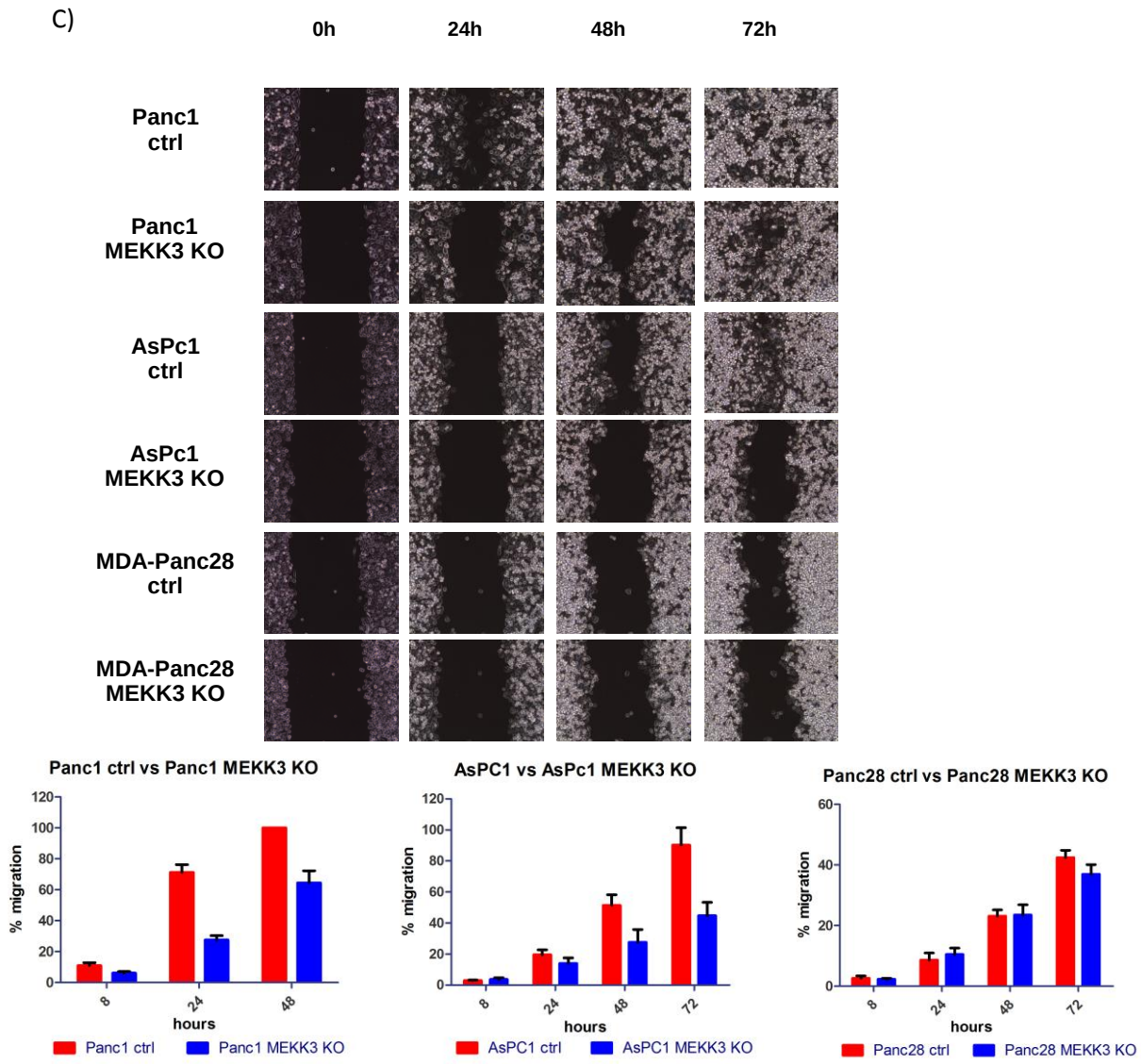


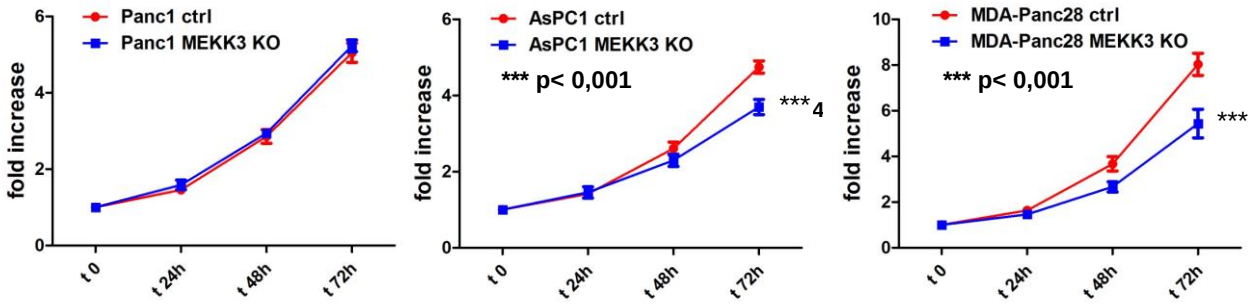
Fig.12 MEKK3 knock-out reverts EMT features in Panc1 and AsPC1 cell lines. A,B) Assessment of MEKK3 knock-out in Panc1, AsPC1, MDA-Panc28 cell lines. Cellular extracts from Panc1, AsPC1 and MDA-Panc28 cells wild-type or KO for MEKK3 were subjected to Western blot analysis with antibodies against MEKK3 (A), CDH1 and VIM (B). C**)** A scratch was performed in control and MEKK3 KO cells. Images show a time course of wound closure. Histograms show percentage wound closure. Mean and SD are indicated.

4.2.2 MEKK3 KO reduces proliferation and stemness of pancreatic cancer cells in *in vitro* experiments.

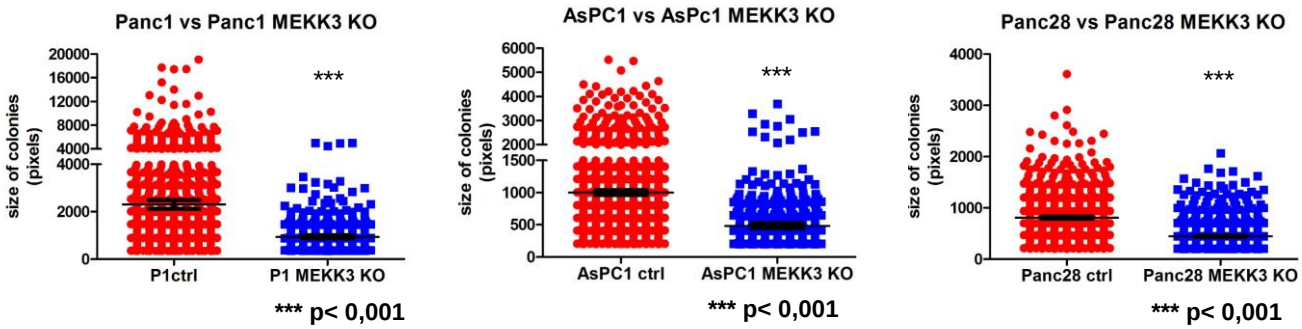
Aberrant proliferation and stemness are important features of cancer cells. In order to study the role of MEKK3 in orchestrating these processes, we first evaluated whether knocking-out MEKK3 impaired cell proliferation. We

observed that MEKK3 KO reduces cell proliferation at 72 hours by 22% in AsPC1 and 32% in MDA-Panc28 in comparison with their controls, while we did not observe any decrease in Panc1 cells (Fig. 13A). Then, we carried out colony formation assays in MEKK3 KO or control Panc1, AsPC1 and MDA-Panc28 cells and we observed a significant decrease in colonies size in MEKK3 KO cells. In details, MEKK3 KO induced *2,5*, *2,07* and *1,8* fold reduction in colony size (p<0,0001) in Panc1, AsPC1 and MDA-Panc28, respectively (Fig.13B). These data demonstrate that MEKK3 is a major regulator of proliferation and stemness of PC cells.

A)



B)



	Panc1 Ctrl	Panc1ctrl Panc1 MEKK3 KO Panc1 MEKK3 KO
Colonies size	2300 ± 98	900 ± 15
Fold decreased	1	2.5

	AsPC1 Ctrl	AsPC1 MEKK3 KO
Colonies size	999± 19.8	482 ± 20.6
Fold decreased	1	2.07

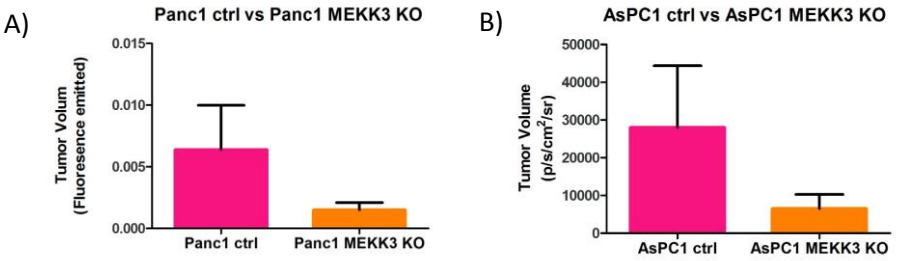
	MDA-Panc28 Ctrl	MDA-Panc28 MEKK3 KO
Colonies size	807± 12	447 ± 6.71
Fold decreased	1	1.8

Fig 13. MEKK3 knocked-out impairs on proliferation and stemness of PC cells. A) MEKK3 KO affects proliferation of PC cell lines. Cell proliferation assays in Panc1, AsPC1 and MDA-Panc28 control and MEKK3 KO cells. Mean and SD are shown in the graphs. Experiments have been conducted in octuplicate. ***, P < 0.001 as calculated by

t-Test. **B)** MEKK3 knocked-out reduces the colony size of PC cell lines. Colony Formation Assay of Panc1, AsPC1 and MDA-Panc28 control or MEKK3 KO cells. Mean and SD from 2 independent experiments conducted in triplicate are shown. ***, P < 0.001 as calculated by t-Test.

4.2.3 MEKK3 knock-out affects tumor growth and survival *in vivo* in Panc1 and AsPC1 pancreatic tumor orthotopic xenografts.

To demonstrate that MEKK3 is an important mediator for the malignancy of PC *in vivo*, we evaluated whether MEKK3 KO could affect orthotopic tumors growth. Six mice for each human PC cell line were orthotopically injected and the tumor growth was followed during all their lifespan. Only one out of six mice (1/6) injected with MEKK3 KO MDA-Panc28 cells while four out of six mice (4/6) injected with MDA-Panc28 control cells developed tumors. Hence, we could not use these animals for survival analyses. We observed that knocking out MEKK3 in Panc1 and AsPC1 reduced the tumor growth in *in vivo* experiments. In particular, we revealed that mice injected with MEKK3 KO Panc1 and AsPC1 presented a strong decrease in tumor volume if compared with mice injected with wild-type cells (Fig. 14 A,B). According to these data, we observed that mice injected with MEKK3 KO Panc1 demonstrated a significantly prolonged median survival duration as compared to mice injected with the control cell line, from 65 to 85 days for Panc1 (p=0.0306) (Fig 14 C). For MEKK3 KO AsPC1, we observed only a trend in prolonged median survival as compared with the control cell line, due to non-tumor related death of two mice. All these data demonstrated an involvement of MEKK3 in driving tumor malignancy *in vivo*.



C)

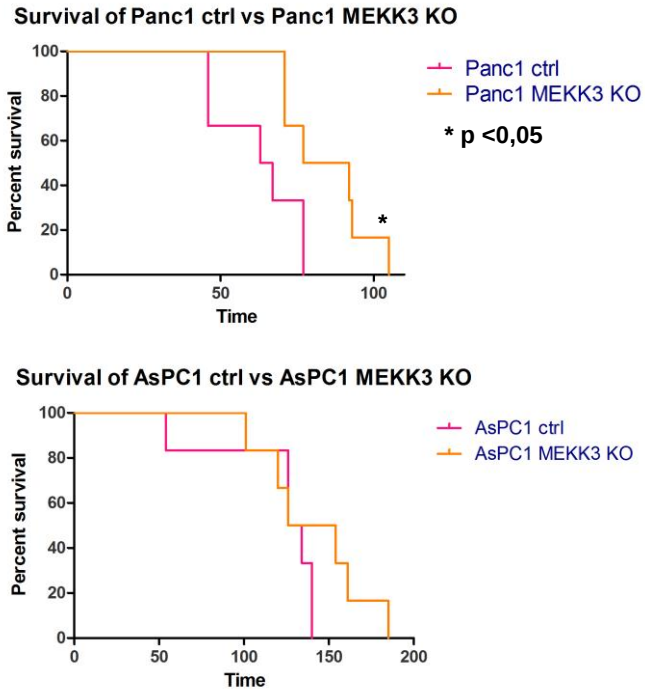
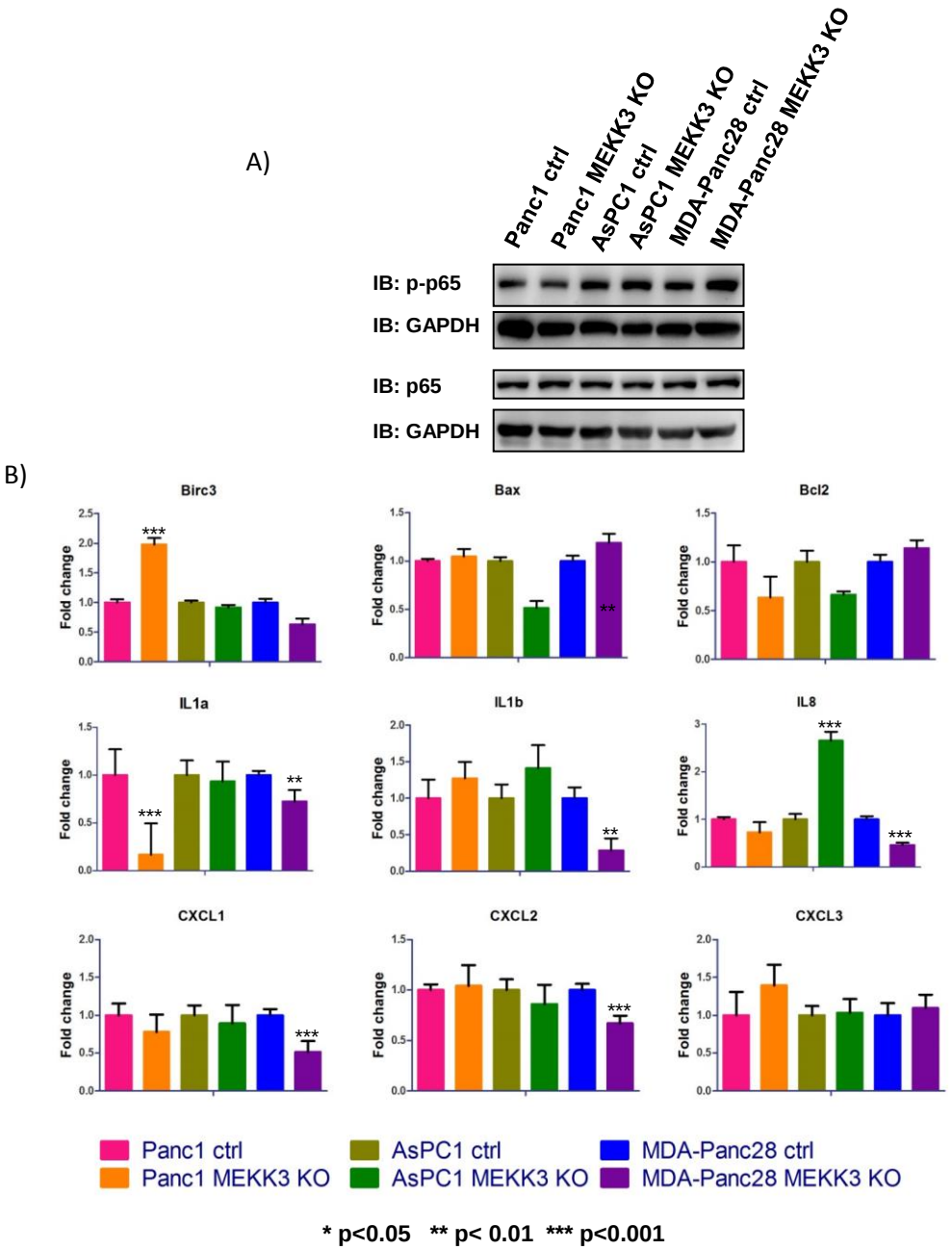


Fig. 14 MEKK3 KO affects tumors growth and survival of Panc1 and AsPC1 pancreatic tumors. A-B) Tumor volume was quantified as the average of fluorescence emitted in Panc1 (A) and as the average of all detected photons within the region of the tumor per second in AsPC1 (B). Error bars indicate SEM. **C)** Mice were killed by cervical dislocation when evidence of advanced bulky disease was present. Survival was estimated from the day of PC cells orthotopic injection until the day of death. Differences among survival duration of mice in each group were determined by log-rank test. *, $P < 0.05$.

4.2.4 MEKK3 does not affect the NF-κB pathway, while it inhibits YAP/TAZ activity in pancreatic cancer cells.

Previous studies have demonstrated that NF-κB is one of the major pathways regulated by MEKK3. As Carbone *et al*. have reported a main role for NF-κB in driving the aggressive features of PC [177], we investigated whether MEKK3 knock-out could impair the activation of the NF-κB pathway. First we analyzed the phosphorylation status of p65, one of the main subunits involved in NF-κB hetero-dimer formation, in MEKK3 KO cell lines. Unfortunately, we did not observed any alteration in p65 phosphorylation and

total protein levels upon knocking out MEKK3 in comparison to parental cell lines (Fig. 15A). To further investigate whether MEKK3 could regulate the transcriptional activity of NF-κB, we measured the expression of several NF-κB-target genes by qRT-PCR. As reported in Fig. 15B, we did not observed any even downregulation of NF-κB target genes in all three MEKK3 KO PC cell lines, suggesting that MEKK3 does not play a central role in the regulation of NF-κB pathway in PC.



*** p<0.05 ** p< 0.01 *** p<0.001**

Fig. 15 MEKK3 KO does not impair the phosphorylation of p65 and partially affects the NFkB target genes expression in PC cell lines . A) Cellular extracts from Panc1, AsPC1 and MDA-Panc28 control and MEKK3 KO cell lines were subjected to Western blot analysis of phosphorylated and total p65. **B)** Histograms show mRNA levels of the indicated target genes over β-actin as assessed by qRT-PCR. Mean values and SD are shown. T-test has been used to perform statistical analysis. ***, P*** 0.001; **,P<0.01; *, P <0,05.

The YAP/TAZ pathway is emerging as a master regulator of PC malignancy. In order to investigate whether MEKK3 could affect this pathway, we first investigated YAP/TAZ protein levels in all three PC cell lines upon knocking out MEKK3. By Western blot analyses, we did not observe any reduction of YAP/TAZ protein levels in MEKK3 KO cell lines in comparison with their controls (Fig. 16A). Likewise, we did not measure any reduction of YAP/TAZ mRNA upon MEKK3 KO (Fig. 16B), suggesting that MEKK3 in not involved in the regulation of YAP/TAZ stability

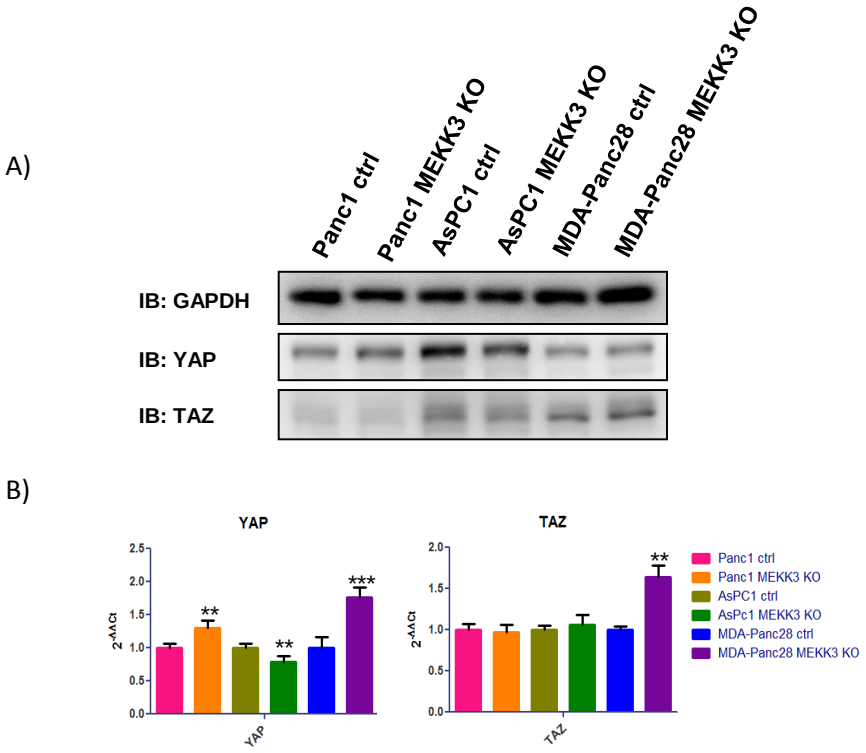


Fig. 16 MEKK3 KO does not affect YAP/TAZ expression. A) Cellular extracts from Panc1, AsPC1 e MDA-Panc28 control and MEKK3 KO cells were subjected to Western blot analysis for the expression of YAP/TAZ. **B)** Histograms show fold change in RNA expression of YAP/TAZ genes by qRT-PCR analysis. Mean values and SD are shown. T-test has been used to perform statistical analysis. ***, P < 0.001; **,P<0.01.

To further investigate whether MEKK3 could affect the activity of YAP/TAZ transcriptional cofactors, we measured the expression of well-known YAP/TAZ target genes. Interestingly, as reported in the table 2, we observed a downregulation of AXL, DKK1, CTGF, BDNF and FosL1 expression upon MEKK3 KO in all PC lines under investigation (Fig. 17A). Likewise, we revealed a decrease of AXL protein levels in MEKK3 KO cell lines (Fig. 17B).

	Panc1 MEKK3 KO/ctrl	AsPC1 MEKK3 KO/ctrl	MDA-Panc28 MEKK3 KO/ctrl
CTGF	0.70 ± 0.21	0.36 ± 0.19	1.25 ± 0.1
AXL	0.64 ± 0.21	0.34 ± 0.19	0.80 ± 0.065
DKK1	0.36 ± 0.2	0.62 ± 0.19	0.23 ± 0.05
FosL1	0.68 ± 0.12	0.42 ± 0.05	0.87 ± 0.09
BDNF	0.38 ± 0.24	0.46 ± 0.18	0.7 ± 0.1

Table 2 Different expression of YAP/TAZ target genes in Panc1, AsPC1 and MDA-Panc28 control and MEKK3 KO.

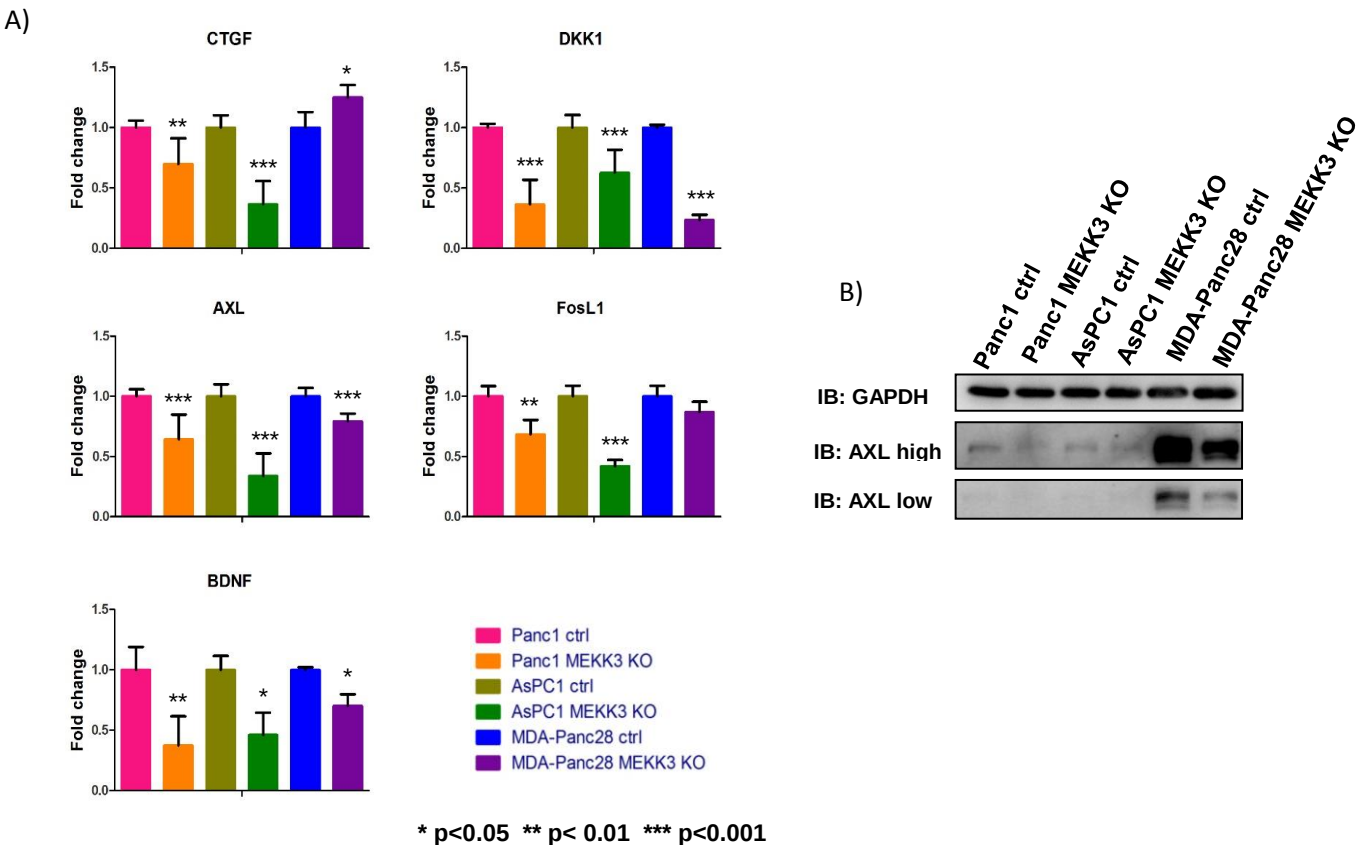


Fig. 17 MEKK3 KO reduces the expression of YAP/TAZ target genes. A) Histograms show mRNA levels of the indicated target genes over β-actin as assessed by qRT-PCR Mean values and

SD are shown. T-test has been used to perform statistical analysis. ***, P < 0.001; **,P<0.01; *, P <0,05. **B)** Western blot analysis for the expression of AXL in the indicated cells.

These data point out the possibility that MEKK3 might affect the recruitment of YAP/TAZ on different target promoters. To verify this hypothesis, we carried out a Chromatin Immunoprecipitation (ChIP) assay and we measured a reduction of YAP/TAZ binding to the promoters of AXL, DKK1 and CTGF in MEKK3 knocked-out Panc1 cells as compared to their controls (Fig. 18).

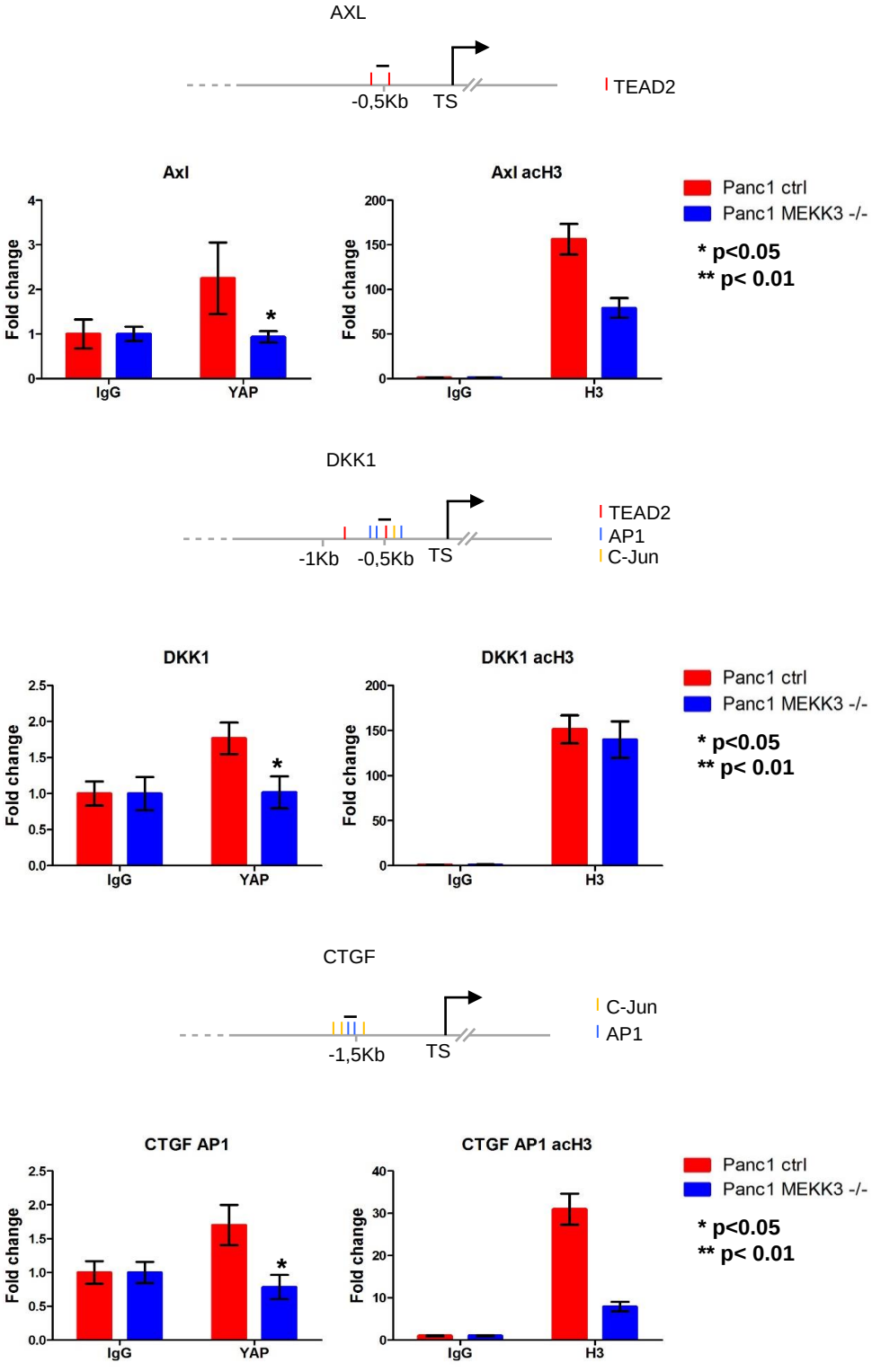


Fig. 18 MEKK3 KO reduces the binding of YAP/TAZ to the promoter regions of AXL, DKK1 and CTGF. Chromatin has been immunoprecipitated with the indicated antibodies and normalized to starting input chromatin as described in the materials and methods section. Histograms show fold increase over control (IgG). Mean values and SD are shown. T-test has been used to perform statistical analysis. *,P<0.05.

4.2.5 Discussion

MEKK3 is a serine/threonine kinase able to regulate embryonic development [113]. Only recently, it has been demonstrated that MEKK3 is important in orchestrating the aggressive behavior of different tumors [114,115]. To our knowledge, our study is the first to evaluate the role of MEKK3 in PC and, in particular, its involvement in regulating EMT features.

In breast cancer, overexpression of MEKK3 is correlated with metastatization of cancer cells. In particular, MEKK3 impacts on both EMT through an induction of mesenchymal marker vimentin, and migration by increasing ICAM1 protein levels [118]. In accordance to this study, we observed that MEKK3 drives EMT in PC cell lines. In particular, we demonstrated that knock-out of MEKK3 is correlated to both an increase of the epithelial marker E-cadherin (Fig.12C) and to a reduction in migration of PC cells (Fig. 12D).

Our data clearly demonstrated that the knock-out of MEKK3 impaired the proliferation of PC cells (Fig. 13A). Similar results have been reported in lung cancer cells as well. Silencing of MEKK3 impairs the proliferation of lung cancer cells by inhibiting the expression of some important cell proliferation inducing genes, such as CDK2, CDC25A, CCND1/2 and CCNE1 [114]. Their downregulation arrests cells in the G1/S phase and supports the evidence that MEKK3 could regulate the cell cycle. In addition, we observed a significant decrease in colony size (Fig. 13B) of PC cells knocked out for MEKK3. The reduction in stemness ability could be related to a downregulation of the YAP/TAZ pathway and, based on data by Tackhoon *et al.*, to a reduction in the expression of IL6 [147]. They have demonstrated that YAP cooperates with Serum Response Factor (SRF) in order to promote the expression of interleukin-6 (IL6), one of the main secreted factors that regulates the stemness-promoting activity of YAP [147]. IL6 plays a central role in stemness and metastasis of solid tumors, but the mechanisms underling IL6 regulation of these processes remain unclear. Wang *et al.* have demonstrated that IL6 triggers both the stemness and the metastatic potential of hepatocellular carcinoma through the induction of osteopontin (OPN) and other stemness-releated genes [178]. In addition to these studies, it has been shown that IL6-knockout mice display resistance to various tumorigenic insults to the liver [179], skin [180] and intestine [181], suggesting a role of IL6 in promoting carcinogenesis *in vivo* [147]. Interestingly, it has been demonstrated that MEKK3 is one of the main drivers in regulating the expression of IL6 in different cellular types [83,90]. Additional studies will be necessary to evaluate whether MEKK3 might regulate the expression of IL6 also in PC, thus promoting both the stemness and the metastatic behavior of this tumor.

Altogether, these data demonstrate for the first time that MEKK3 is able to regulate the aggressive features of PC cells in *in vitro* experiments.

In order to confirm these results and to validate MEKK3 as a druggable target for treatment of patients harboring PC, we evaluated the effect of MEKK3 on the growth of pancreatic tumors in *in vivo* experiments. We observed that knock-out of MEKK3 strongly reduced the tumor size of orthotopic tumors established from Panc1 (Fig. 14A) and AsPC1 (Fig.14B) cell lines. Moreover we measured a significant increase in the median survival of mice injected with Panc1 cells knocked out for MEKK3 (Fig. 14C). These data demonstrate that MEKK3 mediates the aggressive behavior of PC *in vivo*. Thus, MEKK3 could represent a good candidate for the design of drugs to treat PC.

Two of the most important pathways that orchestrate the aggressiveness of PC are NF-κB and YAP/TAZ pathways [148,149,163,177]. Our recent study has demonstrated that the activation of NF-κB mediated by TAK1 is the partially responsible for the chemoresistance of PC [57]. Different studies have demonstrated that MEKK3 could affect directly, or indirectly through TAK1, the activation of NF-κB transcription factors [50,87,88]. In this regard, we evaluated whether MEKK3 could regulate NF-κB activation also in PC. Our results demonstrated that the knock-out of MEKK3 did not affect NF-κB in PC, suggesting that TAK1 is the main regulator of NF-κB in this tumor. We also evaluated whether YAP/TAZ pathway could be affected by MEKK3 in PC. Although we did not measure any reduction in YAP/TAZ proteins (Fig. 16A) and mRNA levels (Fig. 16B), we observed a significant dowregulation of YAP/TAZ target genes upon MEKK3 knock-out (Fig. 17). Thus, we hypothesized that MEKK3 could regulate the recruitment of either YAP/TAZ themselves or additional cofactors on their target genes. Indeed, we found that knock-out of MEKK3 impaired the binding of YAP/TAZ to their target promoters, such as AXL, DKK1 and CTGF (Fig. 18). To our knowledge, our data demonstrate for the first time that MEKK3 regulates YAP/TAZ activity in cancer cells. Recently, Zhang *et al.* have demonstrated that ERK and another unknown kinase regulate YAP activity through specific phosphorylation and/or other post-translational modifications [148]. In light of our data, we can envision MEKK3 as the unknown kinase regulating YAP activities. However, additional experiments are necessary to identify the mechanisms by which MEKK3 affects YAP activation. In conclusion, *our work identifies MEKK3 as a regulator of the aggressiveness of PC, by sustaining the activation of YAP/TAZ* and opens the need to identify MEKK3 inhibitors in order to impair the invasiveness and metastatic behavior of PC.

5. Conclusions

In conclusion, our work shows the convergence of two MAP3Ks, TAK1 and MEKK3, in regulating the YAP/TAZ transcription cofactors. In particular, TAK1 promotes the stability of YAP/TAZ by modulating the expression of ITCH and TRAF6, while MEKK3 regulates the transcriptional activity of YAP and TAZ in PC cells by still unknown mechanisms. The emerging role of YAP/TAZ in orchestrating the development and the aggressiveness of PC opens the need for the discovery of drugs to inhibit their activities. However, so far, there are no drugs targeting specifically the YAP/TAZ pathway and the design of molecules which could target transcriptional cofactors is challenging. In light of this, our data open the path for targeting the YAP/TAZ pathway through pharmacological inhibition of MEKK3 and GSK3/TAK1. Most significantly, this study may candidate the suppression of TAK1 and MEKK3 as novel strategies to potentiate the clinical activity of chemotherapy for the treatment of PC patients.

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