Development of drug screening assays for identification of new molecules against pancreatic ductal adenocarcinoma

ILARIA CASTIGLIONI

PhD Thesis In Biomolecular Sciences

University of Trento

TUTOR/ADVISOR: Dr. Alessandro Provenzani

Academic Year 2012/2013

INDEX

ACRONYMS

PDAC: Pancreatic Ductal Adenocarcinoma

PKC: Protein Kinase C

Lats: Large tumor suppressor kinase

P-S127: phosphorylated Serine 127

TEAD: TEA Domain Family Member

CTGF: Connective Tissue Growth Factor

PK: Pharmacokinetic

BA: Bioavailability

PanIN: Pancreatic Intraepithelial Neoplasia

KRAS: Kirsten Rat Sarcoma viral oncogene homolog

p16INK4A/CDKN2A: Cyclin-Dependent Kinase Inhibitor 2A

TP53: Tumor Protein p53

SMAD4/DPC4: Mothers Against Decapentaplegic homolog 4

BRCA2: Breast Cancer type 2

CDK: Cyclin-Dependent Kinase

Rb-1: Retinoblastoma 1

TGF-β: Transforming Growth Factor-β

SH3 BM: Src homology domain 3 binding motif

RUNX: Runt-related transcription factor

MST1: Macrophage Stimulating 1

AJs: Adherens Junctions

TJs: Tight Junctions

AMOT: Angiomotin

AMOTL: Angiomotin Like

LPA: Lysophosphatidic Acid

S1P: Sphingosine 1-Phosphate

GPCR: G-Protein-Coupled Receptor

PTMs: Post-Translational Modifications

SN: Nucleophilic Substitution

CBP: CREB-Binding Protein

K: Lysine

S: Serine

Sirt: Sirtuin

PML: Promyelocytic Leukemia

MBP: Bone Morphogenetic Protein

AREG: Amphiregulin

FGF: Fibroblast Growth Factor

EMT: Epithelial-to-Mesenchymal Transition

ECM: Extracellular Matrix

PSCs: Pancreatic Stellate Cells

MEK/ERK: MAP kinse-ERK kinase

GSK: Glycogen Synthase Kinase

PPARγ: Peroxisome-Proliferator-Activated Receptor γ

CEBPα: CAATT Enhancer-Binding Protein α

MDR: Multidrug Resistance

MDRP1: Multidrug-Resistance like Protein 1

ChIP: Chromatin Immoprecipitation

DMSO: Dimethyl Sulfoxide

DMEM: Dulbecco's modification of Eagle's medium

FCS: Fetal Calf Serum

EGF: Epidermal Growth Factor

PFA: Paraformaldehyde

PBS: Phosphate Buffered Saline

DAPI: 4',6-diamidino-2-phenylindole

SDS-PAGE: Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis

PVDF: Polyvinylidene Difluoride

IEF: Isoelectric Focusing

DTE: 1,4-Dithioerythritol

IAA: Iodoacetamide

BBP: Bromophenol Blue

GAPDH: Glyceraldehyde-3-phosphate Dehydrogenase

IC50: Half maximal inhibitory concentration

ABSTRACT

Research activity within the frame of the FP7 European project PANACREAS:Integrating chemical approaches to treat pancreatic cancer: making new leads for a cure.

Identification of new potential drug candidates targeting the Yes Associated Protein, YAP, in pancreatic ductal adenocarcinoma

The yes-associated protein, YAP, is a transcriptional co-activator, able to regulate the expression level of a wide range of target genes. Despite deregulation of YAP has been associated with cancer etiology, no compounds are known to specifically modulate its functions. Here we identified an inhibitor of YAP functionality, called GF 10923X, and we proposed it as a potential lead molecule to be used against disease where YAP is found deregulated, pancreatic ductal adenocarcinoma (PDAC) included. GF 109203X is an already known kinase inhibitor, preferentially targeting PKCα. We developed a high throughput approach by which we identified this compound able to reduce YAP-induced proliferation and clonogenicity of PDAC cells*in vitro*, despite it leads to increased YAP nuclear levels. The Hippo pathway is the main inhibitor of YAP. One component of this signaling cascade,Lats1/2, phosphorylates YAP at Serine 127, thereby promoting its cytosolic retention and degradation.In line with YAP nuclear retention after the treatment, we observed that GF 109203X caused an increase of both acetylations and phosphorylations on YAP protein, with the exception of P-S127, suggesting a Hippo pathway-independent mechanism of action of the identified compound. TEAD is the major transcriptional factor partner in the functional activity of YAP and CTGF is considered one of the main representative target genes whose expression is regulated by this couple of transcriptional regulators.Chromatin immunoprecipitation experiments allowed us to demonstrate that GF 109203X interferes with YAP binding to CTGF promoter, without affecting the presence of TEAD at the same region. This inhibition is responsible of CTGF downregulation during GF 109203X administration and it can explain the phenotypic effects we observed. These effects, associated with the observed

toxicity in pancreatic cancer cells but not in immortalized HPNE cell lines, make GF 109203X a potential lead compound to be used in drug development for PDAC treatment.

INTRODUCTION

1. PANACREAS PROJECT

In the PANACREAS project, a team composed of clinicians, translational cancer researchers, chemists, and two pharmaceutical enterprises (one of them is a SME) has been built to synthesize, implement and test new drugs for PDAC.

The final aim consists in synthesizing new molecules, assessing their capacity to effectively block precise cancer-associated mechanisms and determining their anticancer activity *in vivo*. Compounds with strong Proof of Concept (PoC) activity will be developed up to the Investigational New Drug (IND) stage, and thus put on their way to clinical experimentation.

The phases of this project are:

- the synthesis and screening of new targeted inhibitors;
- the hit-validation and lead optimization phase;
- pre-formulation/formulation work and PK/BA studies of those compounds that appeared promising in terms of *in vitro* anticancer activity/potency/specificity, at the pharmaceutical companies;
- PoC study in PDAC models at the CIO Cologne-Bonn (where the project coordinator is located);
- IND-enabling study with one or more compounds that show PoC activity in these models, in particular those exhibiting an improved efficacy when compared to the current standard-of-care gemcitabine, in order to deliver by the end of the project a drug that is ready for clinical testing.

Our role and tasks

In the frame of this project, the laboratory of Genomic Screening is involved in two work packages (WPs). In one of these, the aim is to identify a potential new lead molecule that inhibits the Hippo pathway. In order to achieve this target, the proposal was to set up an immunofluorescent assay for detecting the subcellular localization of nuclear protein YAP. This assay should be used for the primary screening in order to evaluate those compounds able to relocate YAP protein in the cytoplasm since its cytoplasmic localization is related to the loss of its functionality. During my PhD course I have been primary involved in this work package, starting from scratch every activity and I have been responsible to set up and perform the proposed assay, characterize the molecular mechanism of action of the molecular entity identified and evaluate the importance of the YAP protein in PDAC cell lines.

2. PANCREATIC CANCER

Pancreatic cancer is a highly aggressive type of cancer and represents the 7th most frequent cause of cancer death worldwide with an approximate 265 000 deaths, out of 280 000 new cases in 2008 (Ferlay J et al. 2008: GLOBOCAN 2008). Incidence rates are higher in more high-income areas of the world, intermediate in South and Central America and Eastern Asia, and lowest in low-income areas (Figure 1). Mortality from pancreatic cancer has been increasing in high-income countries between the 1950s and the 1980s, and has been leveling off or declined (European Union) thereafter, particularly in men.

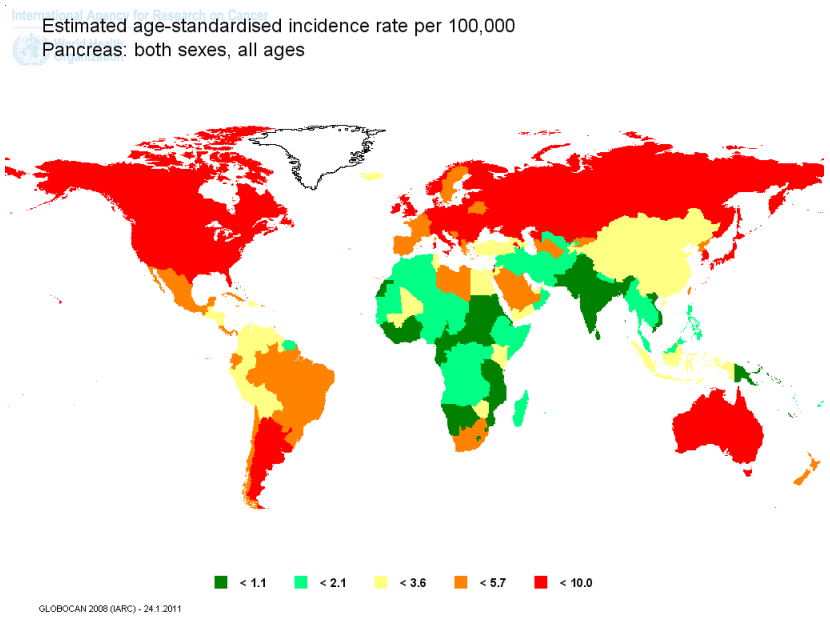


Figure 1. Estimated age-standardized incidence rate per 100,000 pancreas: both sexes, both ages _ *GLOBOCAN 2008 (ARC)*

2.1 Future prediction

Pancreatic cancer is unique among the top five cancer killers (currently lung, colorectal, breast, pancreas and prostate) in that both the incidence rate and death rate are increasing (Figure 2). The result of the combination of these factors is that both the projected numbers of new pancreatic cancer cases and pancreatic cancer deaths will be more than double by 2030. By as early as 2015, the number of deaths from pancreatic cancer will exceed those from breast and colorectal cancer, and be surpassed only by the loss of life from lung cancer.

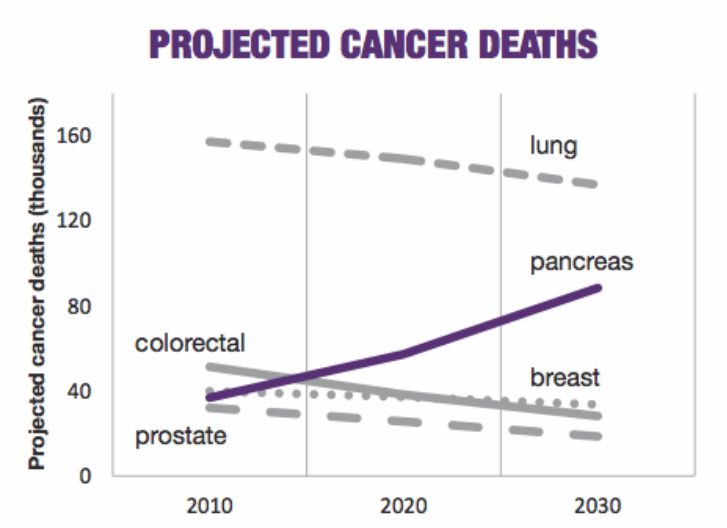


Figure 2. Projected cancer deaths for the major cancer killers. Projections were calculated by considering expected changes in the number of new cases for that cancer site and the average annual change expected in the death rate (number of deaths/100,000 population). Separate values were calculated for men and women and then combined to obtain the value for the total population. The projected number of new cases for 2020 and 2030 were calculated using age-, sex-, race-, and origin-specific incidence and applying the delay-adjusted average annual percent changes in incidence rate calculated by Eheman et al (Cancer 118:2338, 2012). These figures were further adjusted by the average annual percent changes in death rate calculated by Eheman et al (Cancer 118:2338, 2012, Table 2) _ *Pancreatic Cancer Action Network*

2.2 Epidemiology and risk factors

Pancreatic adenocarcinoma is generally thought to arise from pancreatic ductal cells; however, this remains an area of ongoing study (Hruban RH et al. 2001; Meszoely IM et al. 2001). The etiology of pancreatic adenocarcinoma remains poorly defined. Risk factors for developing pancreatic cancer include family history of the disease, age, chronic or hereditary pancreatitis, smoking, obesity and recent-onset diabetes. These and other risk factors are still being investigated. Pancreatic adenocarcinoma occurrence is associated with ageing (Anderson K et al. 2006), it is rare before the age of 40, it culminates in a 40-fold increased risk by the age of 80. Moreover, pancreatic cancer is about 30% more common in men than in women. The lifetime risk of developing pancreatic cancer is about 1.5% for both men and women.Figure 3 is related to the pancreatic cancer incidence in the United States, where the disease is a relevant problem, representing the fourth leading cause of cancer-related death.Examples of modifiable risk factors are: tobacco usage and obesity. Tobacco use is the most important known risk factor for pancreatic cancer; approximately 20% of pancreatic cancers are attributable to cigarette smoking (Iodice S et al. 2008). The risk of developing pancreatic cancer is about twice as high among smokers as among never smokers (Anderson K et al. 2006); risk increases with greater tobacco use and longer duration of smoking (Bosetti C et al. 2012). Obesity has also been fairly consistently linked to increased risk of pancreatic cancer. Obese individuals have a 20% higher risk of developing pancreatic cancer than those who are normal weight (Berrington de Gonzalez A et al. 2003; Arslan AA et al. 2010). At the genetic level, numerous studies have documented an increased risk in the relatives of pancreatic adenocarcinoma patients (approximately threefold), and it is estimated that 10% of pancreatic cancers are due to an inherited predisposition (Lynch, HT et al. 1996). However, unlike familial cancer syndromes for breast, colon and melanoma, pancreatic adenocarcinoma that is linked to a familial setting has a lower

penetrance (<10%) and maintains a comparable age of onset as sporadic cases in the general population.

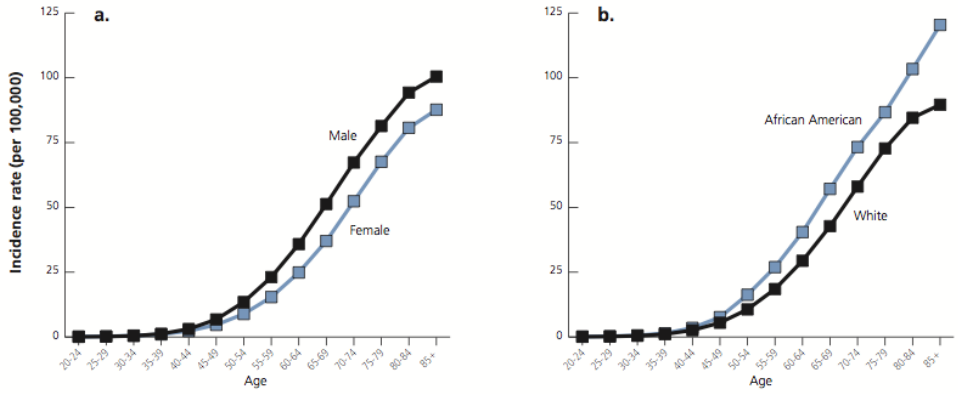


Figure 3. Pancreatic cancer incidence rates by age and sex (a) and age and race (b), US, 2005-2009. Source: North America Association of Central Cancer Registries (NAACCR). Data are collected by cancer registries participating in NCI's SEER program and CDC's National Program of Cancer Registries _ *American Cancer Society, Surveillance Research, 2013*

2.3 Molecular genetics of pancreatic adenocarcinoma

The pancreatic-duct cell is generally believed to be the progenitor of pancreatic adenocarcinoma. As defined in Cubilla and Fitzgerald's classic study (Cubilla AL & Fitzgerald PJ, 1976) the increased incidence of abnormal ductal structures (now designated pancreatic intraepithelial neoplasia, PanIN) (Klein WM et al. 2002) in patients with pancreatic adenocarcinoma, and the similar spatial distribution of such lesions to malignant tumors, are consistent with the hypothesis that such lesions might represent incipient pancreatic adenocarcinoma. Histologically, PanINs show a spectrum of divergent morphological alterations relative to normal ducts that seem to represent graded stages of increasingly dysplastic growth (Hruban RH et al. 2001) (Figure 4). Cell proliferation rates increase with advancing PanIN stages, which is consistent with the idea that these are progressive lesions (Klein WM et al. 2002). A growing number of studies have identified common mutational profiles in

simultaneous lesions, providing evidence of the relationship between PanINs and the pathogenesis of pancreatic adenocarcinoma. Specifically, common mutation patterns in PanIN and associated adenocarcinomas have been reported for K-RAS and for CDKN2A (Moskaluk CA et al. 1997). In addition, similar patterns of Loss Of Heterozygosity (LOH) at chromosomes 9q, 17p and 18q (harboring CDKN2A, TP53 and SMAD4, respectively) have been detected in coincident lesions (see below), and studies have consistently shown an increasing number of gene alterations in higher-grade PanINs (Yamano M et al. 2000). Intriguingly, there seems to be an ordered series of mutational events in association with specific neoplastic stages. A characteristic pattern of genetic lesions in evolving pancreatic adenocarcinoma has been revealed. The field is now facing with the challenge of understanding how this signature of genetic lesions — mutations of K-RAS, CDKN2A, TP53, BRCA2 and SMAD4/DPC4 — contribute to the biological characteristics and evolution of this disease.K-RAS is one member of a family of three *ras* oncogenes that also includes the Harvey- and N-*ras* oncogenes. The *ras* oncogenes encode for closely related GTP-binding proteins that can acquire transforming potential when altered in one of the critical positions at codons 12, 13, or 61. Under normal circumstances, ras proteins are involved in growth signal transduction within the cell, similarly to “second messenger” G-proteins (Mc Cormick FR et al. 1995). The mutated K-RAS encodes a protein locked in a constitutively active state, leading to persistent downstream signals such as activation of the RAF-MEK-ERK (extracellular signal-regulated kinase) cascade (Roberts PJ and Der CJ. 2007). The commonly occurring mutations of K-RAS alter the ability of the K-RAS protein to hydrolyse GTP. Thus the K-RAS protein becomes constitutively active, persistently stimulating its downstream targets and acting as though under constant mitogenic stimulus. In PDAC these mutations occur almost exclusively in codon 12 and to a lesser extent in codons 13 and 61. K-RAS mutations have been observed in 70–100% of pancreatic cancers. This is the highest incidence of K-RAS mutation found in any cancer. Interestingly, mutated K-RAS is present in both invasive cells and the earlier non-invasive cells. Evidence for this comes from a number of studies (Luttges J et al. 1999). The tumor suppressor gene p53 is the most commonly mutated gene in human cancer and has been found to be mutated in 50–70% of human pancreatic

adenocarcinomas(Pellegata NS et al. 1994). Common types of p53 mutations detected in pancreatic tumors include single nucleotide changes, intragenic deletions, and microdeletions(Pellegata NS et al. 1994). The protein encoded by p53 has a transcriptional regulator role in which it modulates the expression of genes involved in cell cycle control, DNA repair, and apoptosis (Levine AJ et al. 1997). Cells without active p53 continue to divide and will either die or accumulate additional errors. This latter phenomenon may explain why tumor cells show a progressive increase in DNA damage, such as deletions, translocations, and amplified genes.The p16INK4A/CDKN2A gene, located on the short arm of chromosome 9 (9p), is one of the most frequently inactivated tumor suppressor genes in PDAC. Virtually all pancreatic carcinomas have loss of p16INK4A/CDKN2A function, in 40% of pancreatic cancer through homozygous deletion, in 40% by an intragenic mutation coupled with loss of the second allele, and in 15% by hypermethylation of the p16INK4A/CDKN2A gene promoter (Schutte M et al. 1997). p16 belongs to the cyclin-dependent kinase (CDK) inhibitors family and prevents the phosphorylation of cyclin D-CDK4 and cyclin D-CDK6 complexes, which in turn inhibit Rb-1 phosphorylation, modulating cell-cycle progression. In PDAC, p16INK4A inactivation leads to inappropriate phosphorylation of Rb-1, allowing progression of cell cycle through the G1/S transition (Sellers WR et al. 1995). DPC4/SMAD4 is a tumor suppressor gene on chromosome 18q inactivated in about 55% of the cases of PDAC. Inactivation of DPC4/SMAD4 occurs either through homozygous deletion, in approximately 30% of the cases, or loss of one allele coupled with an intragenic mutation in the second allele in approximately 25% of the events (Koorstra JB et al. 2008).SMAD4 is a downstream effector of the transforming growth factor-β (TGF-β) pathway that acts in the nucleus as a transcription factor promoting growth inhibition and stopping inappropriate G1/S transition (Heldin CH et al. 1997). TGF-βs are multifunctional polypeptides that inhibit the growth of epithelial cells and exert multiple effects on the extracellular matrix. TGF-β1 acts through a family of transmembrane receptors that have intrinsic serine/threonine kinase activity. The inhibitory Smads, Smad6 and Smad7, associate with the activated TGF-β receptor type I, thereby blocking access and phosphorylation of Smad2 and possibly Smad3. This prevents their interaction with Smad4, and blocks their subsequent nuclear translocation (Imamura T et

al. 1997).Crane et al. studied disease progression and SMAD4 expression in locally advanced PDAC in a phase III clinical trial after treatment with cetuximab, gemcitabine, and oxaliplatin followed by chemoradiation and cetuximab (Crane CH, et al. 2011). The Breast Cancer Type 2 (BRCA2) has been implicated in regulation of gene transcription, chromatin remodeling, cell growth, DNA damage repair and chromosomal instability (Van der Heijden MS et al. 2005). Inherited mutations in the BRCA2 gene caused familial breast/ovarian cancer syndrome which is associated with an increased risk of breast cancer both in men and women and a subset of these families also have an increased risk of PDAC (Berman DB et al. 1996). 7 e 10% of PDAC harbor an inactivating intragenic mutation of one copy of the BRCA2 gene, coupled with loss of heterozygosity germline mutations of the BRCA2 gene (13q12-13) identified in 4 e 17% of familial pancreatic cancer (Naderi A et al. 2002).

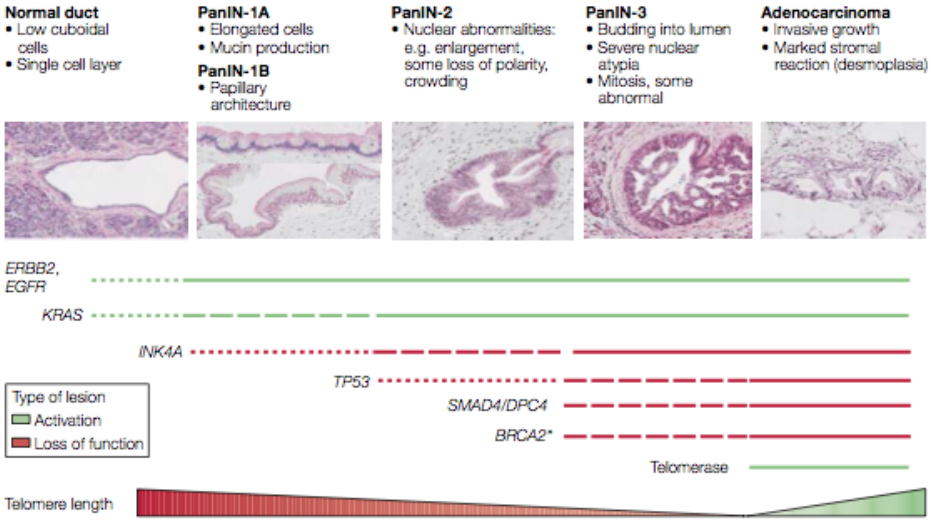


Figure 4. Genetic progression model of pancreatic adenocarcinoma. Pancreatic intraepithelial neoplasias (PanINs) seem to represent progressive stages of neoplastic growth that are precursors to pancreatic adenocarcinomas. The genetic alterations documented in adenocarcinomas also occur in PanIN in what seems to be a temporal sequence, although these alterations have not been correlated with the acquisition of specific histopathological features. The stage of onset of these lesions is depicted. The thickness of the line corresponds to the frequency of a lesion _ *Pancreatic cancer biology and genetics. Bardeesy N, DePinho RA. Nat Rev Cancer 2002; 2(12):897-909*

2.4 Treatments

Pancreatic cancer is a leading cause of cancer death largely because there are no efficient diagnostic tools for early detection or effective treatment options, because of the complex biological features of the disease. Early stage pancreatic cancer usually has no symptoms, or only vague disorders that could indicate many different conditions within the abdomen or gastrointestinal tract. Due to a lack of specific symptoms and limitations in diagnostic methods, the disease often eludes detection during its formative stages. When symptoms do occur, the tumor has usually spread to surrounding tissues or distant organs. To date, there is no single, reliable test for the early detection of pancreatic cancer; therefore, screening the general population is not recommended by any health agency(Greenhalf W et al. 2009). Existing screening programs have been limited to research settings with a focus on detecting precancerous lesions among high-risk individuals (Shin EJ et al. 2012). Treatment options for pancreatic cancer are limited. In adenocarcinoma, the most common type of pancreatic cancer, surgical removal of the tumor is possible in only approximately 15% of patients. Postoperative (adjuvant) chemotherapy either alone or in combination with radiation has been proven to improve progression-free and overall survival in both randomized controlled trials and observational studies (Neoptolemos JP 2011). The role of radiation therapy by itself in the adjuvant setting remains unclear (Abrams RA et al. 2012). Treatment with chemotherapy or chemoradiotherapy prior to surgery (neo-adjuvant) is an emerging strategy. There are three FDA-approved drugs for the treatment of pancreatic adenocarcinoma, and two FDA-approved drugs to treat advanced pancreatic neuroendocrine tumors. The treatment for patients with advanced disease focuses on managing symptoms and relieving pain and suffering (palliative care). Treatment options include chemotherapy alone or in combination with radiation. The chemotherapy drug gemcitabine has been a standard initial treatment for patients with metastatic pancreatic cancer for more than 15 years. However, the National Comprehensive Cancer Network's

Guidelines for the treatment of pancreatic cancer stated that clinical trials are the preferred option for treatment. Numerous clinical trials have tested new drugs, either alone or in combination with gemcitabine, in these patients. The chemotherapy regimen known as FOLFIRINOX is the only treatment that has been shown in a clinical trial to improve the survival of patients with metastatic pancreatic cancer. In an international randomized phase III trial, patients with metastaticpancreatic cancer who were treated with a combination of albumin-bound paclitaxel (nab-paclitaxel [Abraxane®]) and gemcitabine (Gemzar®) lived longer than patients who were treated with gemcitabine alone. Patients who received both drugs also lived longer without their disease getting worse (progression-free survival). Nab-paclitaxel is a form of the chemotherapy drug paclitaxel that is bound to the human protein albumin and contained in nanoparticles. Binding paclitaxel to albumin eliminates the need for solvent that keep paclitaxel soluble once injected into the body but that can also cause allergic reactions and side effects. Albumin also plays a key role in delivering nutrients to dividing cells. Tumor cells require an abundance of nutrients to survive, so binding paclitaxel to albumin helps deliver paclitaxel to tumor cells. Studies in mice with tumors derived from human pancreatic cancer cells suggested that the combination of gemcitabine and nab-paclitaxel was more effective than either drug alone. Results from a phase I/II trialstrongly suggested that the combination could be effective in previously untreated patients with metastatic pancreatic cancer (Von Hoff DD et al. 2013).

3. YAP

Marius Sudol and colleagues in 1995 characterized a novel protein: the Yes-associated protein, YAP (Sudol M et al. 1995). YAP protein consists of 454 amino acids, with a molecular weight of 65 kDa (Sudol M et al. 1994).YAP has a proline-rich (P-rich) region at the N-terminal, two tandem WW domains in the middle followed by an Src homology domain 3 binding motif (SH3 BM)

PVKQPPPLAP, a coiled-coil domain (CC), and a C-terminal capped by TWL sequence, a PDZ domain ligand.The proline-rich region is responsible of the interaction with SH3 domains of c-Yes and many other proteins. The WW domain contains two tryptophan residues, which appear to be conserved along evolution and that play an important role in the domain structure and function (Sudol M et al. 1995; Sudol M and Hunter T 2000). This domain binds to short stretches of prolines (PY motif), and therefore mediating the interaction between proteins. YAP has been found to interact with many proteins, whose function often is quite substantially different, and the majority of these interactions is mostly mediated by the WW domain (Bertini et al. 2009).The N-terminal of YAP was mapped to be the TEAD family transcription factors interaction domain, and the C-terminal of YAP rich in serine, threonine, and acidic residues was shown to be a strong transcription activator.The human YAP1 coding sequence consists of 1364 bp from the start codon to the stop codon. A differentially spliced isoform of YAP1 (9 exons), with two WW domains known as YAP2 also exists (Sudol M et al. 1995). Regulation of the switch between the two YAP isoforms is not clear. In general, YAP mRNA is ubiquitously expressed in a wide range of tissues, except peripheral blood leukocytes (Komuro A et al. 2003). YAP is also expressed in the full developmental stages from blastocyst to perinatal (Morin-Kensicki EM et al. 2006). However, the function of YAP remained enigmatic until it was shown to be a transcription co-activator (Yagi R et al. 1999). YAP does not have any obvious DNA binding domain. However, when fused to Gal4 DNA binding domain, YAP could strongly activate luciferase, therefore, it is categorized as a transcription co-activator. The transcription activation domain of YAP was further mapped to the C-terminal region. Interestingly, this region was found to be truncated in possibly dominant-negative YAP isoforms specifically expressed in neurons (Hoshino M et al. 2006). YAP interacts with the PPXY (Proline-Proline-x–Tyrosine) motif of transcription factor PEBP2α (RUNX1 and RUNX2). Peptide ligand containing PPXY consensus motif is specific for binding WW domain. Besides that, YAP has also been reported to co-activate other PPXY-motif-containing transcription factors, including ErbB4 cytoplasmic domain (Komuro A et al. 2003) and p73 (Strano S et al. 2001). YAP also binds to TEAD family transcription factors (Vassilev A et al. 2001), which have four highly homologous proteins sharing a

conserved DNA-binding TEA domain, found in N-terminal of particular gene regulatory proteins, in human and mouse (see below). TAZ (transcriptional co-activator with PDZ-binding motif) is also referred to as WWTR1 (WW domain containing transcription regulator 1). TAZ is homologous to YAP with 46% amino acid sequence identify (with YAP isoform 3) and displaying similar domain organization but having only one WW domain. Both YAP and TAZ are homologous to fly Yki, which was identified as a downstream target of the Hippo pathway in 2005 (Huang J et al. 2005) (Figure 5).

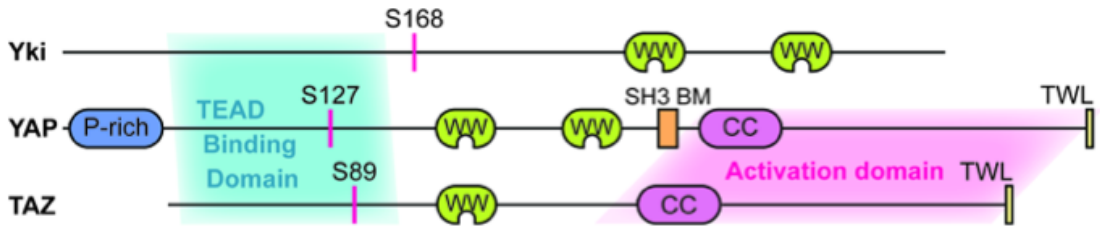


Figure 5. A schematic view of YAP, TAZ, and Yki.YAP has a proline-rich (P- rich), two tandem WW domains in the middle, followed by a Src homology domain 3 binding motif (SH3 BM) PVKQPPPLAP, a coiled-coil domain (CC), and a C-terminal capped by TWL sequence, a PDZ domain ligand. The N-terminal (aa 47-154 in human YAP2, shaded in blue) of YAP was mapped to be the TEAD family transcription factors interaction domain, and the C-terminal of YAP (aa 292-488, shaded in pink) rich in serine, threonine, and acidic residues was shown to be a strong transcription activator. The Lats phosphorylation and 14-3-3 binding critical S127 in human YAP2 and its equivalent in Yki and TAZ are also shown. The topology of Yki and TAZ are shown in similar fashion and the proteins are drawn in scale _ *The Hippo-YAP pathway: new connections between regulation of organ size and cancer. Zhao B et al. Curr Opin Cell Biol 2008; 20(6):638-46.*

3.1 REGULATION

3.1.1. Hippo pathway

The Hippo signaling pathway was initially defined by genetic studies in *Drosophila* to regulate tissue growth and organ size and is highly conserved in mammals (Zhao B et al. 2010) (Figure 6). The Hippo core kinase cassette

contains four proteins, two of which are kinases: Hpo and Wts in the fly and Mst1/2 and Lats1/2 in mammals. The other two proteins, Sav and Mats in the fly, and WW45 and Mob in mammals, act as adaptors/activators. In mammals, Mst1/2 in association with WW45 is activated by phosphorylation in response to upstream regulators. The activated Mst1/2-WW45 can phosphorylate and activate LATS1/2-Mob complex. The major target of the Hippo core kinase cascade is Yki transcription co-activator in the fly and YAP and TAZ in mammals. Mechanistically, Mst2 and LATS2 were shown to act coordinately to phosphorylate YAP at HXRXXS motifs, which is defined as a Lats recognition motif, with the S127-containing motif being the major site whose phosphorylation created a binding site for 14–3–3 proteins. TAZ and Yki have a corresponding motif with S89 and S168 as the major site, respectively. Phosphorylation of YAP and TAZ by the Hippo pathway leads to their sequestration in the cytoplasm by interaction with 14–3–3 proteins and ubiquitination-dependent proteasomal degradation. Therefore, the Hippo pathway acts to restrict the availability/functionality of YAP and TAZ in the nucleus by governing its distribution and protein levels. In the fly, Yki binds Scalloped (Sd) and activates transcription of downstream target genes like Diap, bantam and cycE. In mammals, YAP and TAZ interact primarily with transcriptional factors TEAD1–4 (TEADs) and activate expression of target genes such as CTGF, IGFBP3, ITGB2, Birc5/Survivin, Gli2, and Axl.

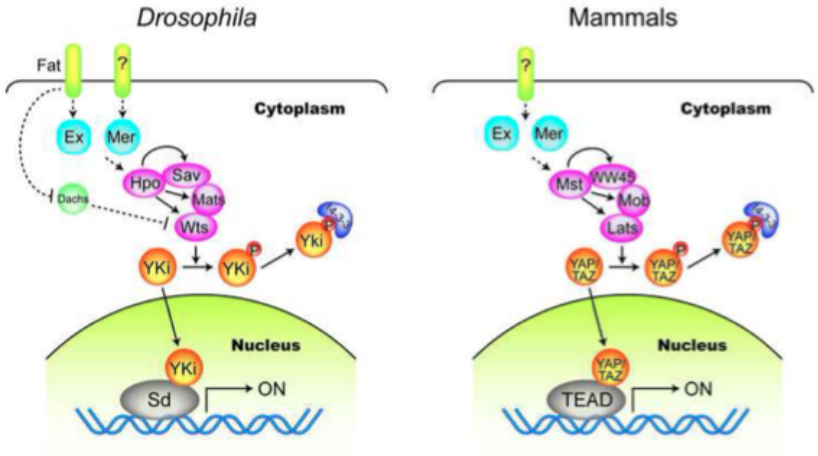


Figure 6. The Hippo pathway in Drosophila and mammals. Corresponding components in *Drosophila* and mammals are shown in the same color. The abbreviations used are as follows: Ex (Expanded), Mer (Merlin, also called NF2), Hpo (Hippo), Sav (Salvador), Mats (Mob as tumor suppressor), Wts (Warts), Yki (Yorkie), Sd (Scalloped), Mst (Mst1/2, also called STK4 and STK3, Hpo homolog), WW45 (Sav homolog), Mob (Mps One Binder kinase activator-like 1A/B, MOBKL1A/B, Mats homolog), Lats (Lats1/2, Wts homolog), YAP (Yes-associated protein, Yki homolog), TAZ (transcriptional co-activator with PDZ-binding motif, also called WWTR1, Yki homolog), and TEAD (TEA domain family member 1/2/3/4). Dashed arrows indicate unknown

biochemical mechanism and question marks denote unknown components _ *The Hippo-YAP pathway: new connections between regulation of organ size and cancer. Zhao B et al. Curr Opin Cell Biol 2008; 20(6):638-46.*

3.1.2. Cell density / Cell contact

A fundamental property of a normal cell is to cease proliferation upon reaching confluence, a phenomenon referred to as cell contact inhibition (Eagle H and Levine EM 1967). In contrast, cancer cells are able to escape cell contact inhibition, which enhances their ability to invade host tissues and metastasize (Hanahan D and Weinberg RA 2000). Several lines of evidence support the function of YAP in mediating cell contact inhibition. First, cell density regulates Lats kinase activity and YAP nuclear/cytoplasmic shuttling. The Hippo pathway-induced phosphorylation of YAP is utilized in YAP regulation by cell density and is likely conserved in *Drosophila*. At low density, YAP is predominantly localized in the nucleus. In contrast, YAP translocates to the cytoplasm at high density. This phenomenon was observed in different cell lines, such as in the MCF10A human breast epithelial cells and in NIH-3T3 murine embryonic fibroblast (Zhao B et al. 2007). Second, scratching of confluent cultured cells induces YAP nuclear localization in cells at the wound edge. Those cells with nuclear YAP also enter the cell cycle. Third, YAP-overexpressing cells fail to exit the cell cycle when confluent and grow to a much higher density. Fourth, expression of dominant-negative YAP restores contact inhibition in a human cancer cell line bearing deletion of Sav. Fifth, YAP regulates many genes in a manner opposite to high cell density. Furthermore, YAP is inhibited by Merlin, which has been implicated in mediating cell contact inhibition (Okada T et al. 2005).

Epithelial cells usually adhere to one another through cell–cell junctions such as adherens junctions (AJs), desmosomes, and tight junctions (TJs) (Figure 7). TJs and AJs, with help from different polarity complexes, divide the plasma membrane into an apical domain and a basolateral domain and thereby establish an apical–basal polarity in epithelial cells (Martin-Belmonte F and Perez-Moreno M 2012). Interestingly, many upstream regulators identified for the Hippo pathway are known components of TJs, AJs, or apical–basal polarity protein complexes. In *Drosophila*, Mer, Ex, and Kibra colocalize at the apical domain of polarized epithelial cells (Yu et al. 2010) and this complex may recruit

the Hippo pathway kinases to the apical plasma membrane for activation (Genevet et al. 2010). The regulation of apical–basal polarity on the Hippo pathway is largely conserved in mammals. Disruption of TJs or AJs in cultured mammalian cells causes induction of YAP/TAZ nuclear localization and target gene expression (Varelas et al. 2010). Moreover, Scrib also positively regulates the Hippo pathway kinases, and downregulation of Scrib leads to YAP/TAZ activation (Cordenonsi et al. 2011). In addition, many cell junction proteins, such as LIN7C, PATJ, MPDZ, PTPN14, angiomotin (AMOT), and α-catenin, have also been identified as interacting partners of core Hippo pathway components (Varelas et al. 2010).

- Angiomotin

AMOT proteins, a family of proteins including AMOT, AMOTL1, and AMOTL2, interact extensively with multiple TJ components and are important for maintaining TJ integrity and epithelial cell polarity (Wells et al. 2006). Three independent studies have recently identified Angiomotin (Amot), Angiomotin-like 1 (AmotL1) and Angiomotin-like 2 (AmotL2) as negative regulators of YAP and TAZ (Zhao B et al. 2011). The AMOT–YAP/TAZ interaction is not dependent on the YAP/TAZ phosphorylation status and is instead mediated by AMOT PPxY motifs and YAP/ TAZ WW domains (Chan SW et al. 2001; Zhao B et al. 2011). AMOT proteins recruit YAP/TAZ to TJs or the actin cytoskeleton, which consequently results in reduced YAP/TAZ nuclear localization and activity. In addition, AMOT proteins also induce YAP/TAZ phosphorylation at Lats target sites; this might be due to a scaffolding function of AMOT on Hippo pathway components such as MST2, Lats2, and YAP (Paramasivam M et al 2011). AMOT proteins can therefore inhibit YAP/TAZ activity by both phosphorylation-dependent and phosphorylation-independent mechanisms. Interestingly, AMOT has been shown to interact with NF2 and is required for tumorigenesis caused by NF2 deficiency (Yi C et al. 2011). Since Amot family proteins are integral components of cell junction complexes, their interaction with YAP and TAZ may play a key role in regulating the Hippo pathway in response to cell contact *in vitro* and organ size control *in vivo*.

– α-catenin

α-catenin is an actin binding protein that bridges the cytoskeleton to cell surface adhesion molecule cadherin. α-catenin is also found to interact with and inhibit YAP and phosphorylation at YAP S127, and subsequent 14-3-3 binding is critical for this association in cells (Schlegelmilch K et al. 2011). In fact α-catenin accomplishes these functions by modulating interaction with 14–3–3 and PP2A phosphatase. Therefore, α-catenin acts in a manner similar to AMOT to inhibit YAP/TAZ. Given the fact that α-catenin is associated with adherent junction and AMOT is associated with tight junction, these two proteins may relay the cell-cell contact signals to inhibit cell growth via modulating YAP/TAZ.

Several other proteins important in establishing or maintaining apical–basal polarity have been shown to modulate the Hippo pathway. These data indicate that cell–cell contact, integrity of cell junctions, and apical–basal polarity are important in regulation of the Hippo pathway. Apical–basal polarity can regulate the Hippo pathway by either recruiting the Hippo pathway kinases to the apical domain for activation or sequestering Yki/YAP/TAZ at cell junctions, both resulting inactivation of YKi/YAP/ TAZ. However, it is worth noting that the cellular localization of YAP is mainly in the cytoplasm and nucleus (Zhao B et al. 2011); the interaction between cell junctional proteins and YAP/TAZ may not result in a predominant localization of YAP/TAZ at the cellular apical domain.

3.1.3 G-protein-coupled receptor (GPCR) signaling

A large number of growth factors regulate cell proliferation by activating membrane receptors and intracellular signaling pathways, so it is reasonable to speculate that the YAP/TAZ oncoproteins are regulated by growth factors. In mammals, the potential identity of extracellular ligands and their cognate

receptors that regulate the Hippo pathway remained elusive until recently. Two independent groups have reported that serum could rapidly activate YAP/TAZ in cultured cells. By extensive biochemical analysis, lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) were identified as the major components in serum responsible for YAP/TAZ activation (Yu FX et al. 2012). Both reports showed that LPA or S1P bound to their corresponding membrane GPCRs and act through Rho GTPases to activate YAP/TAZ. Consistently, another report showed that thrombin, which activates protease-activated receptors (PARs; a GPCR), also stimulated YAP/TAZ activity via Rho GTPases (Mo et al. 2012). These results suggest that YAP/TAZ can be regulated by diffusible extracellular signals and cell surface receptors. Yu et al. further showed that YAP/TAZ is regulated by many GPCRs and their cognate ligands and established a general function of GPCR in YAP/TAZ regulation. GPCRs usually activate downstream signaling through heterotrimeric G proteins. Ga12/13-, Gaq/11-, or Gai/o-coupled signals induce YAP/TAZ activity, whereas Gas-coupled signals repress YAP/TAZ activity.

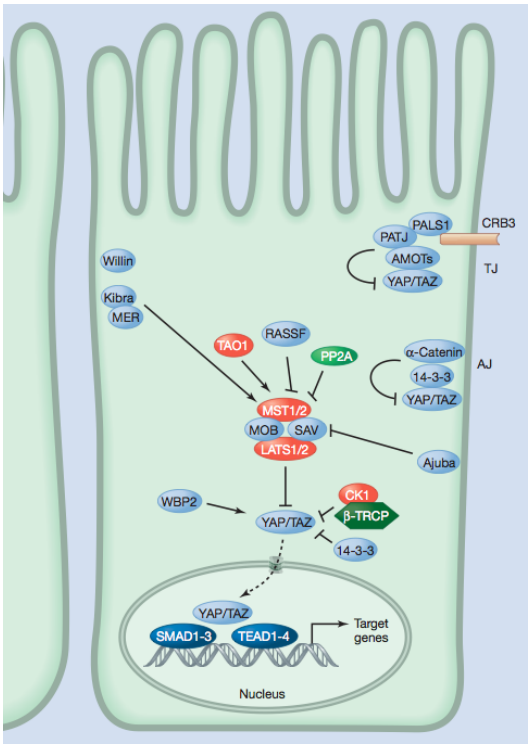


Figure 7. The role of cell contact in YAP regulation. Many Hippo pathway proteins reside, at least partially, at the sub-apical region, adherens junction (AJ) or thigh junctions. Examples of such junctional proteins include, in mammals, angiomotin and α-catenin _ *The Hippo pathway. Kieran F et al. Cold Spring Harb Perspect Biol doi: 10.1101/cshperspect.a011288.*

3.2 POST TRANSLATIONAL MODIFICATIONS

3.2.1 Phosphorylation

The functions of YAP are regulated by multiple post-translational modifications (PTMs). The Hippo pathway can negatively regulate TAZ and YAP by two different mechanisms through cytoplasmic sequestration and proteasomal degradation (Figure 8). Upon activation, the core components of the Hippo pathway phosphorylate TAZ and YAP at multiple sites harboring the HXRXXS motif. When phosphorylated, phospho-Ser89 of TAZ and phospho-Ser127 of YAP serve as the binding sites for 14-3-3 proteins. Accordingly, S89A and S127A mutants are largely refractory to this inhibition and are mainly distributed in the nucleus (Lei QY et al. 2008; Zhao B et al 2009; Zhao B et al. 2007). Other studies also revealed that the Hippo pathway can regulate the stability of YAP and TAZ (Liu CY et al. 2010; Zhao B et al. 2010). Phosphorylation of Ser381 of YAP and Ser314 of TAZ by the Hippo pathway primes YAP and TAZ, respectively, for subsequent phosphorylation by casein kinase 1, leading to their ubiquitination and proteasomal degradation. Chan et al. presented another novel mechanism of regulation of TAZ and YAP function through direct interaction with Amot and AmotL1, leading to their cytoplasmic sequestration that is independent of Hippo pathway-regulated interaction with 14-3-3 proteins (Chan W et al. 2011). The general working model is that Amot and AmotL1 (likely also AmotL2) are primary cytosolic proteins that interact with TAZ and YAP through the first PPXY motif of Amot and AmotL1 and the WW domain of TAZ and YAP. This interaction causes cytoplasmic sequestration of TAZ and YAP in a manner similar to but independent of TAZ-YAP interaction with 14-3-3 proteins. In the case of YAP, it is intriguing to note that first but not the second WW domain is important for interaction with Amot and AmotL1. Because Amot has been shown to be distributed to the junctional complexes of polarized epithelial cells (Wells CD et al. 2006), it is also possible that Amot and AmotL1 may mediate localization of TAZ and YAP to the cell junction in polarized

epithelial cells (described above).

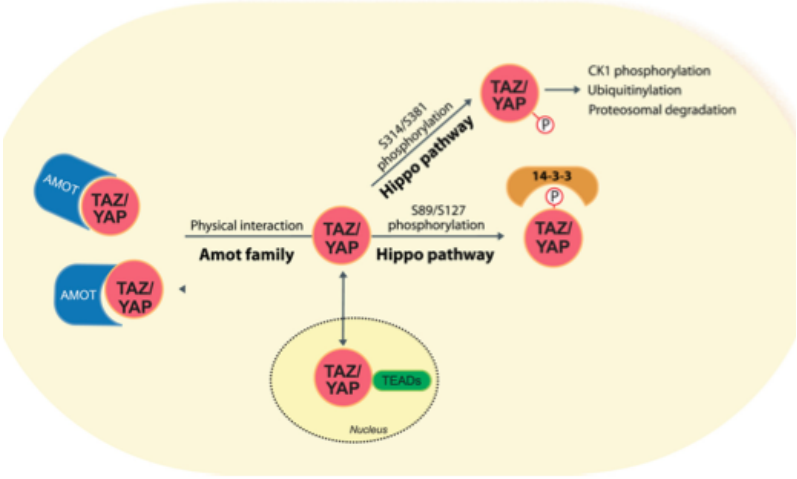


Figure 8. A working model for diverse regulatory mechanisms for TAZ and YAP. The Hippo pathway causes cytoplasmic sequestration of TAZ and YAP through phosphorylation of Ser89 and Ser127, respectively. Furthermore, Hippo pathway-mediated phosphorylation of Ser314 and Ser381 leads to further phosphorylation, ubiquitination, and proteasomal degradation of TAZ and YAP, respectively. Interaction with TEADs is important for nuclear accumulation and transcriptional outcome of TAZ and YAP. Amot and AmotL1 (likely also AmotL2) function as negative regulators of TAZ and YAP through direct interaction with the WW domain of TAZ and the first WW domain of YAP via the first PPXY motif of Amot and AmotL1 that is also conserved in AmotL2, leading to their cytoplasmic retention _ *Hippo Pathway-independent Restriction of TAZ and YAP by Angiomotin. Siew Wee Chan et al. J. Biol. Chem. 2011, 286:7018-7026.*

3.2.2 Acetylation

Hata and co-workers recently reported a novel cycle of acetylation/deacetylation of nuclear YAP induced in response to SN2 alkylating agents (Figure 9). These agents but not other DNA-damaging stimuli are able to reduce YAP S127 phosphorylation mediated by Hippo pathway, leading to nuclear translocation of YAP and its acetylation (Hata S et al. 2012). The nuclear acetyltransferases CBP and p300 are reasonable of YAP acetylation that specifically occurs on specific and highly conserved C-terminal lysine residues: K494 and K497.In previous reports, CBP and p300 were shown to directly acetylate a variety of proteins at a consensus motif [KX1-2(X/K)K] (Wang C et al. 2001). Notably, hYAP contains this consensus motif (K494LDK497), and the YAP acetylation sites identified are consistent with the positions of two of the lysine residues present in this motif. Interestingly, this consensus motif is not found in either Yki

or in TAZ (Zhao B et al. 2001b), thus the CBP/p300-dependent acetylation appears to be a modification specific to vertebrate YAP. Conversely, the nuclear deacetylase SIRT1 is responsible for YAP deacetylation. Importantly, acetylation of YAP influences both its transcriptional co-activator activity and the sensitivity of YAP-expressing cells to MMS-induced DNA damage. However authors indicate that mere nuclear accumulation of hYAP is not enough to induce its acetylation, and other factors must play an important role in this reaction.

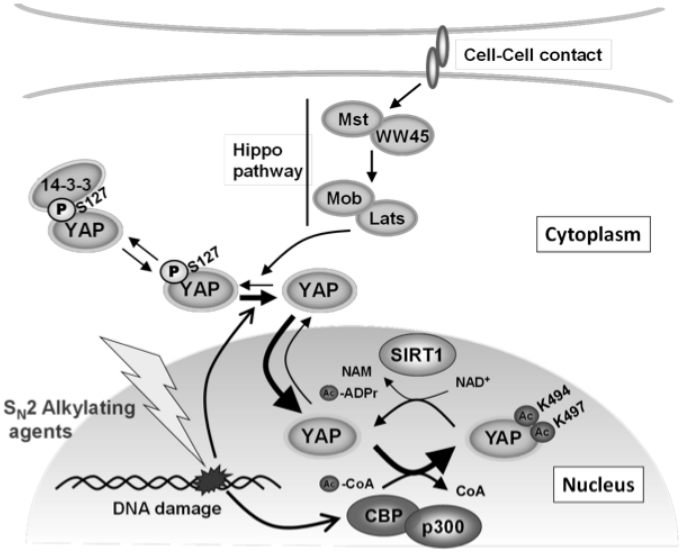


Figure 9. A proposed model for hYAP regulation in response to SN2 alkylating agents. Cell-cell contact triggers Hippo pathway signaling that up-regulates S127 phosphorylation of hYAP and thus its cytoplasmic retention via binding to 14-3-3 protein. SN2 alkylating agents damage DNA, leading to a reduction in S127 phosphorylation of hYAP. Non-phosphorylated hYAP is released from the cytoplasmic 14-3-3 complex and translocates into the nucleus. Within the nucleus, CBP and/or p300 activated by SN2 alkylating agent-mediated DNA damage induce hYAP acetylation at K494 and K497. Concurrently, deacetylation of these residues is mediated by SIRT1. Thus, tight regulation governs the state of hYAP acetylation induced in response to SN2 alkylating agents _ *A novel acetylation cycle of the transcription co-activator Yes-associated protein that is downstream of the Hippo pathway is triggered in response to SN2 alkylating agents. Hata S et al. J Biol Chem 2012;287(26):22089-98.*

3.2.3 Ubiquitination

Ubiquitination is mostly catalyzed by the sequential action of three enzymes namely, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzymes),

and E3 (ubiquitin ligase). E3 determines substrate specificity, and its substrate binding is the primary event controlling the stability of a particular protein (Pickart CM 2001). The adaptor protein Amot130 is shown to scaffold a complex containing overexpressed atrophin-1 interacting protein 4 (AIP4) and YAP. Consequently, Amot130 promotes the ubiquitination of YAP by AIP4 and prevents AIP4 from binding to Lats1, thus reducing YAP stability and inhibiting cell growth (Adler et al. 2013).

3.3 DUAL ROLE OF YAP

It is extremely difficult to include the biological consequences of YAP activity into a unique category. Since its ability to activate apoptosis YAP can be considered a tumor suppressor gene. On the other hand, a huge amount of data suggest the possibility that YAP may indeed be an oncogene, thus supporting the findings that come from *Drosophila* for the conservation of the Hippo pathway. Finally, YAP is defined as a key component of the molecular machinery that drives cell differentiation and regulates organ size. All its conflicting functions support the idea that YAP is placed at the crossroads of many signalling pathways, where it plays a role depending on the upstream stimuli and the binding to its multiple targets. The context appears to be crucial, for example the origin of the cell and the kind of cell line (primary, immortalized, or fully cancerous). In any case, the importance of PTMs in the regulation of YAP is clear (described above). More work therefore has to be done if we want to clarify the role that this protein plays during normal cell life and on the onset of cancer.

3.3.1 Yap as a tumor suppressor gene

Among all proteins found to interact with YAP, the group of Giovanni Blandino found YAP1 to be able to bind through its WW domain to a PY motif present on the C-terminal region of p73. The binding to p73 causes an increment in the transcriptional ability of p73 supporting the possible involvement of YAP in the induction of apoptosis (Strano S et al. 2001). Further works showed how, following DNA damage, p73 and YAP1 associate into the nucleus with p300 and PML, forming a proapoptptic autoregulatory feedback loop. PML is responsible for the sub-nuclear localization of the p73-YAP1 complex in the PML bodies, a dot-looking arrangement of proteins and DNA that appears to be involved in transcriptional regulation. The binding between YAP and p73 is negatively regulated by Akt-mediated YAP phosphorylation (Basu S et al. 2003) and enhanced by DNA damage (Strano S et al. 2005). Upon DNA damage this complex recruits the acetyl transferase p300 and together sit onto the regulatory regions of pro-apoptotic genes and upregulate their transcription (Lapi E et al 2008). When YAP1 associates with p73 it increases the stability of the latter, by competing with the ITCH E3-ligase, thus preventing p73 disposal by proteasome degradation and thus propelling further transcription of pro-apoptotic genes. Collectively, these observations do not classify YAP as a real tumor suppressor, but as a transcriptional co-activator, able to directly or indirectly regulate different tumor suppressor pathways (for example p53 family or PML). Conversely, when YAP promotes the activation of transcription factors whose gene targets encode for oncogenic proteins, its effects are anti-apoptotic and pro-proliferative.

3.3.2 Yap as an oncogene

Despite the evidences underlining YAP as a tumor suppressor (Yuan M et al. 2008), YAP is also a potent growth promoter. Functions of YAP in organ size regulation and tumorigenesis have been confirmed in mammals, using transgenic mouse models (Camargo FD et al. 2007; Dong J et al. 2007). Human colorectal cancers and other types of cancer expressing higher levels of YAP1 share molecular aspects with YAP1-induced dysplastic growth in the

mouse (Camargo FD et al. 2007). Moreover, YAP overexpression in MCF10A cells induces epithelial-mesenchymal transition (EMT), which is often associated with cancer metastasis (Overholtzer M et al. 2006); YAP also cooperates with myc oncogene to stimulate tumor growth in nude mice (Zender L et al. 2006); and more interestingly, transgenic mice with liver-specific YAP overexpression show a dramatic increase in liver size and eventually develop tumors (Camargo FD et al. 2007; Dong J et al. 2007). The oncogenic function of YAP is further supported by the tumor suppressor function of its inhibitory upstream Hippo pathway components. For example, Lats1 knockout leads to soft-tissue sarcoma and ovarian tumor development (St John MA et al). Loss-of-function mutation of WW45 has been observed in several human cancer cell lines (Tapon N et al. 2002).

3.3.3 Organ size

The role of YAP in controlling cell proliferation, and thus organ size, has been reported also at the physiological level (George NM et al 2012). The mammalian pancreas is a dual-function organ critical for the regulation of basic metabolism. In the mouse, development of the pancreas is divided into two stages. Hippo signaling becomes functionally active during the secondary transition, characterized by robust proliferation and differentiation, and plays a crucial role in determining overall pancreas architecture, likely through downregulation of YAP-dependent cell proliferation. Very early in pancreas development, expression of YAP is turned off following specification of endocrine fate, potentially explaining the poor proliferation capacity displayed by these cells (Pictet RL et al. 1972). Hippo pathway components are important in controlling organ size, through YAP regulation. For example, Mst1 and Mst2 double knockout in liver has been reported to largely abolish YAP phosphorylation, and induced an enlarged liver phenotype strikingly similar to YAP overexpression (Zhou D et al. 2009; Lu L et al. 2010). However, this effect on organ size is more dramatic in some organs, such as liver and stomach, than

in others, such as kidney and limb, which do not show an increase in size (Song H et al. 2010). This is possibly due to an organ-specific contribution of impaired cell differentiation versus cell number on the overall size of the organ. Furthermore, the function of Mst1/2 in the Hippo pathway is likely to be cell context-dependent, since Mst1/2 are not required for Lats1/2 phosphorylation and cell density-induced YAP nuclear–cytoplasmic translocation in mouse embryonic fibroblast (MEF) cells (Zhou D et al. 2009), or YAP phosphorylation in early embryos.

3.3.4 Senescence and cell proliferation

On the other hand YAP has been demonstrated to play an important role in the maintenance of cell proliferation and resistance to cellular senescence. The YAP-TEAD signalling pathway has been reported to partially depend on p16 and p53 and to regulate CDK6 expression (Xie Q et al. 2013). Moreover YAP expression is closely correlated with senescence. In particular, YAP down-regulation increased senescence in a colorectal carcinoma HCT116 cell line in a p53 and p21 dependent manner (Vigneron AM and Vousden KH 2012).

3.4 INTERACTORS

Depending on the partner bound, YAP can exert different functions, in different tissue and/or under different conditions (Bertini E et al. 2009; Liu AM et al. 2010) (Figure 10). Despite a major role for TEADs in YAP/TAZ function (described below), other transcription factors containing PPXY motifs are known to interact with the WW domains of YAP/TAZ. These include Smad1, RUNX, ErbB4 and p73 for YAP (Strano,S. et al. 2001; Komuro A et al. 2003; Alarcon C et al. 2009). YAP, TAZ and Yki also induce many other genes directly or

indirectly. In mammals, YAP and TAZ induce the expression of AREG118 and FGF1 (Zhang J et al. 2009), which may mediate non-cell-autonomous functions of the Hippo pathway. However, the mechanisms underlying the induction of these genes, including the responsible transcription factors, are mostly unclear.

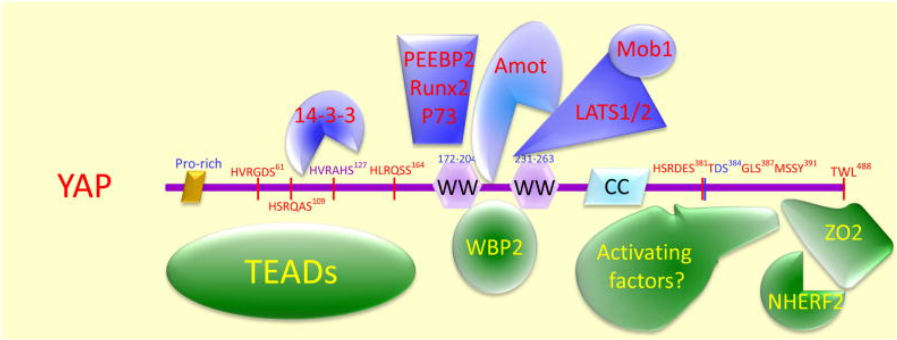


Figure 10. Summary of proteins shown to interact with YAP/TAZ/Yki. Amot family proteins are present only in mammalian cells but the drosophila expanded has been shown to interact with WW domain of fly Yki. The potential factors that interact with the C-terminal transactivation domain to promote transcriptional program are yet to be defined. The C-terminal PZD-binding motif is specific for YAP and TAZ but not present in Yki and has been shown to interact with ZO2 and NHERF2. Not all proteins interact with YAP at the same time. In fact, some interaction may be mutually exclusive. For example, the 14–3–3 associated YAP would be in cytoplasm whereas the TEAD associated YAP should be nuclear _ *The YAP and TAZ transcription coactivators: key downstream effectors of the mammalian Hippo pathway. Hong W and Guan KL. Semin Cell Dev Biol 2012; 23(7):785-93.*

3.4.1 TEAD

The TEAD family transcription factors were found to be critical partners of YAP and TAZ in the regulation of gene expression (the *Drosophila* TEAD homologue Scalloped (Sd) is partner of Yki) (Zhao B et al. 2008; Goulev Y et al. 2008) (Figure 11).An interesting twist in the study of YAP/TAZ/Yki cellular function was the identification of YAP as an interacting protein for TEAD2 transcriptional factor, which does not have the PPXY motif found in other YAP interacting transcription factors (Vassilev A et al. 2001). The C-terminal half of TEAD2 was mapped to be responsible for interaction with YAP. In view of the fact that TEAD2, together with TEAD1, TEAD3 and TEAD4 form a homologous TEAD family, YAP was shown to interact also with TEAD1, TEAD3 and TEAD4

(Vassilev A et al. 2001). Importantly, TEAD-mediated transcription was dependent on YAP and YAP-TEAD complex can bind DNA containing TEAD-binding elements. Knockdown of TEADs or disruption of the YAP–TEAD interaction abolishes YAP-dependent gene transcription and largely diminishes YAP-induced cell proliferation, oncogenic transformation and the epithelial-to-mesenchymal transition (EMT) (Zhao B et al. 2008; Zhang H et al. 2009).S94 in YAP is important for interaction with TEADs and mutation of this residue abolished most if not all property of YAP in promoting cellular transformation and mediating the transcriptional outcome, suggesting that interaction with TEADs is the major functional pathway of YAP. Congruent with this, YAP and TEAD bind to a common set of genomic targets. Several direct target genes of YAP–/TAZ–TEAD and Yki–Sd have been identified, including CTGF and Cyr61 in mammalian cells (Zhao B et al. 2008; Lai D et al. 2011).

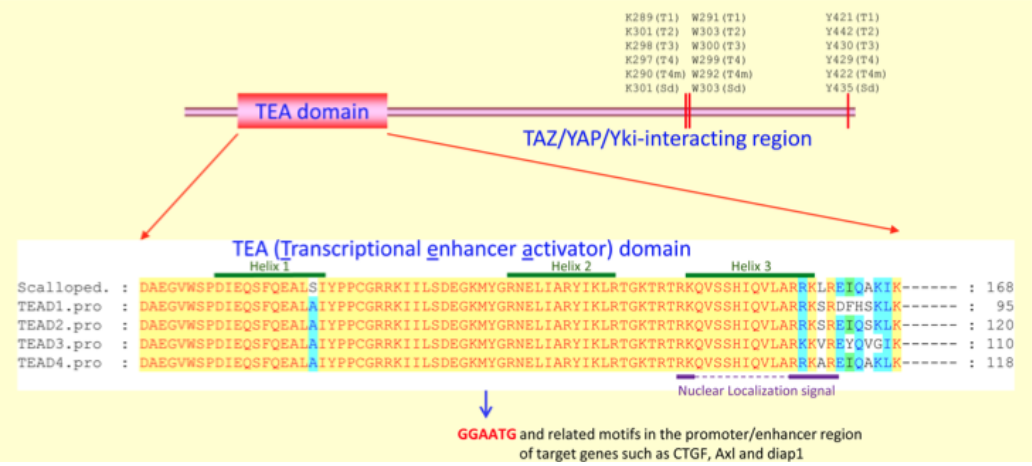


Figure 11. Domain organization of TEADs.The N-terminal region of TEADs contains the highly conserved TEA domain responsible for interaction with DNA elements such as GGAATG in the promoter region of target genes (such as CTGF). The amino acid alignment of the TEA domain of TEAD1–4 is also shown. The TEA domain forms a 3-helix bundle and the regions for the three helices are indicated on top. The nuclear localization signal is indicated below the alignment. The C-terminal region of TEADs is responsible for interaction with YAP/TAZ/Yki. Three residues conserved in TEADs that were experimentally demonstrated (using mouse TEAD4 in the study) to be important for interaction with YAP/TAZ/Yki (using mouse YAP as an interacting partner) are indicated (T1–T4 for TEAD1–4; T4m for mouse TEAD4) _ *The YAP and TAZ transcription coactivators: key downstream effectors of the mammalian Hippo pathway. Hong W and Guan KL. Semin Cell Dev Biol 2012; 23(7):785-93.*

3.5 YAP TARGET GENE: CTGF

The connective tissue growth factor CTGF is a member of the CCN (CTGF, Cyr61, and Nov) family of proteins that promote angiogenesis, cell migration, and cell adhesion (Brigstock DR 1999). CCN proteins are now recognized as matricellular proteins which function as integral but non-structural components of the extracellular matrix (ECM) that modulate cellular responses to other molecular cues in the pericellular environment by interacting with integrins, HSPG or other receptors on the cell surface such as LRP or the TrkA neurotrophin receptor (Rachfal AW and Brigstock DR 2005). A role for CTGF in pancreatic development and morphogenesis was first suggested by its detection in the mouse pancreas at E14.5 (Surveyor GA and Brigstock DR 1999). The pancreas has both endocrine and exocrine functions. The endocrine component is organized as the islets of Langerhans which comprise 1–2% of the pancreatic mass and contain several cell types that secrete distinct products (Prado CL et al. 2004). CTGF has been shown to regulate islet morphogenesis and function during embryogenesis, and it is both required and sufficient to induce proliferation of embryonic b cells (Crawford LA et al. 2009). Several evidences revealed a role of CTGF in pancreatic cancer. In one approach, CTGF has been studied as a product of the tumor cells which are characterized by activation mutations in the K-RAS proto-oncogene during a pre-invasive state termed pancreatic intraepithelial neoplasia (PanIN) and, in invasive PDAC. In another approach, studies have focused on the contribution of CTGF to the desmoplastic reaction which involves the intense production of an interstitial stroma that can contribute up to 80% of the tumor mass and comprises fibroblasts, myofibroblasts, inflammatory cells and PSC that are associated with a highly expanded ECM. This reaction is considered a reservoir of ECM-associated growth factors that functions dynamically to sustain tumor growth and increase its invasive potential (Korc M 2007). Clinical specimens of

pancreatic cancer express elevated levels of CTGF mRNA that are 40–60-fold greater than normal pancreas and well correlated with the extent and intensity of desmoplasia (Wenger C et al. 1999). Studies ofCTGF expression and promoter activity have established that MEK/ERK drives basal CTGF production in Panc-1 pancreatic tumor cells in a Smad-independent manner as well as in other pancreatic cancer cell lines that have defective Smad signaling. On the other hand, in pancreatic tumor cell lines that are TGF-β-responsive, CTGF production is dependent on the type I TGF-β receptor and MEK/ERK (Pickles M and Leask A 2007; Kwon S et al. 2007). Other pathways of CTGF gene transcription have been described in PDAC, including their induction as immediate early genes in human pancreatic tumor cells in response to epidermal growth factor or transforming growth factor-alpha (Wenger C et al. 1999). Moreover CTGF has been also identified in a metastasis-related gene cluster in PDAC neoplastic epithelium (Ryu B et al. 2001). Incubation of Panc-1 cells *in vitro* with recombinant CTGF stimulates cell proliferation in monolayer culture as well invasion in a Matrigel assay (Aikawa T et al. 2006). Knock-down of CTGF mRNA expression has shown to inhibit anchorage-independent growth of pancreatic tumor cells *in vitro* (Bennewith KL, et al. 2009) and, conversely, transfection of the cells with human CTGF cDNA is able to enhance it (Dornhofer N et al. 2006). Notably, CTGF-expressing tumor cells are present in hypoxic regions of experimental tumors *in vivo* or in clinical samples and this has been attributed to the ability of CTGF to confer protection against hypoxia-mediated apoptosis (Bennewith KL, et al. 2009). Taken together these evidences underline an important role of CTGF in pancreatic cancer onset and development.

CTGF has been identified as a direct target gene of YAP-TEAD in mammals cell and several GGAATG motifs for TEAD-binding at the promoter region of CTGF gene have been reported (Zhao B. et al. 2008). CTGF mediates the growth-stimulating and oncogenic function of YAP-TEAD complex. Although CTGF is a known target of both TGFβ and the Hippo signaling pathway, the transcriptional mechanism that converges on CTGF expression involved in the crosstalk between these pathways has been described for the first time in malignant mesothelioma cells (Fujii M et al. 2012). Several studies have

reported the interactions between Smad and YAP/TAZ. Varelas et al. showed that YAP/TAZ controls the nuclear-cytoplasmic shuttling of Smad2/3–4 complexes and regulates the nuclear accumulation of Smad complexes in mouse embryonic stem cell (Varelas X et al. 2008).Likewise Fujii et al. demonstrated a crosstalk between YAP and SMAD3 (Fujii et al. 2012) (Figure 12). Since the weakness of this binding other components were hypothesized to take part of the complex and p300 was found, as well as TEAD (Wu S et al. 2008; Zhang H et al. 2009). TGF-β is a powerful cytokine produced by many different cell types, with effects on multiple cell types. TGF-β can induce extremely variable responses, mainly through the Smad2/3-dependent pathway. For example, TGF-β induces growth arrest and apoptosis in epithelial cells and it can also activate fibroblasts. Subsequent studies further revealed that TGF-β acts as a tumor suppressor in premalignant cells as well as cells progressing through the early stages of carcinogenesis; furthermore, it exerts prooncogenic effects in metastatic tumors (Roberts AB and Wakefield LM 2003). Upon TGF-β stimulation, Smad2 and Smad3 form complexes with Smad4 and accumulate in the nucleus (Massagué J et al. 2005).p300, a transcriptional co-activator, binds with Smad3 and Smad2 and enhances Smad-induced transactivation of target genes (Nishihara A et al. 1998). Recruitment of p300 frequently plays a core role not only in enhancing transactivation but also in binding other proteins to stabilize protein complexes (Fujii M et al. 2006). As previously described, tumor cell–derived CTGF plays an important role in the proliferation of breast cancer cells (Zhao B et al. 2008) and growth of pancreatic tumors (Bennewith KL et al. 2009). In addition CTGF also affects vascularization, migration, and epithelial-mesenchymal transition in the context of oncogenic properties (Wahab NA and Mason RM 2006). In the transformed mesothelium, the activation of the Hippo pathway synergizes with the TGF-β pathway signaling to increase CTGF production and thereby amplify the profibrotic and colony-stimulating effects of TGF-β and potentially inducing other pro-tumorigenic effects in the microenvironment (Fujii et al 2012). In conclusion, determining the proper way to regulate CTGF expression, by directly target CTGF, is critical for clinical applications. The mechanism that allows CTGF to exert its effects has not yet been clarified. Further research regarding CTGF expression and its functions might lead to the discovery of new targets that could be used to regulate CTGF

expression.

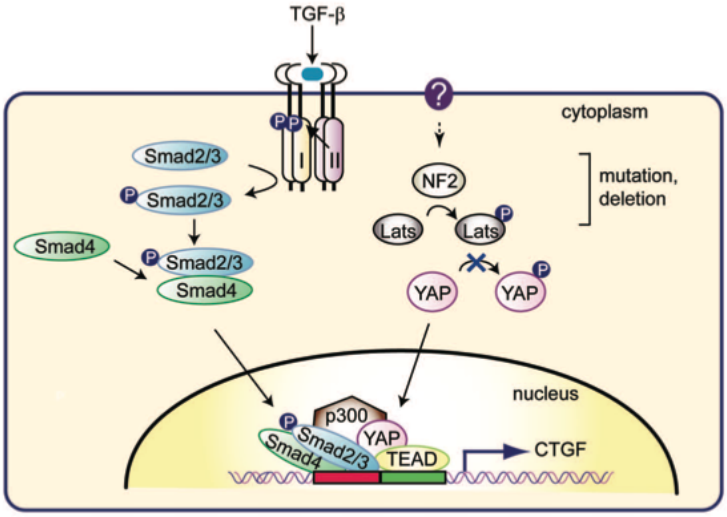


Figure 12. Schematic model of CTGF promoter activation through TGFb/Smad signaling and disturbance of the NF2/Hippo pathways. Because of the genetic disturbance in NF2 and/or Lats2, Yap was dephosphorylated and constitutively translocated to the nucleus. On the other hand, upon TGFb stimulation, Smad2/3 and Smad4 associate, move to the nucleus, make a complex with YAP/TEAD, and recruit p300 to the promoter to activate CTGF expression _ *Convergent signaling in the regulation of connective tissue growth factor in malignant mesothelioma. Fujii M et al. Cell Cycle 2012; 11(18):3373-9.*

4. THE BISINDOLYLMALEIDE I (GF 109203X)

The bisindolylmaleimides, derivatives of staurosporine, are widely used as specific inhibitors of protein kinase C (PKC) isoforms (Figure 13). Bisindolylmaleimide I (GF 109203X) and IX (Ro 31-8220) are the most commonly used PKC inhibitors (Toullec D et al. 1991; Wilkinson SE 1993; Davis PD et al. 1989). GF 109203X is a competitive inhibitor with respect to ATP (Ki = 14 ± 3 nM) and displayed high selectivity for PKCα compared to five different

protein kinases. Toullec et al. studied GF 109203X in two cellular models: human platelets and Swiss 3T3 fibroblasts (Toullec D et al. 1991). In these systems, the role of PKC in signal transduction has been extensively investigated (Rozengurt E 1986). GF 109203X resulted as efficient as staurosporine on PKC (IC50 = 10 nM) and was much less active against cAMP-dependent protein kinase (IC50 = 2 μM) phosphorylase kinase (IC50 = 0.7 μM). GF 109203X was also inactive against 3 tyrosine kinases, namely EGF, platelet-derived growth factor, and insulin receptors (IC50 > 50 μM). Finally, GF 109203X inhibited myosin light chain kinase in human platelets at higher concentrations than those required to suppress PKC activity in those cells. Beltman et al. (Beltman J et al. 1996) used five different bisinolylmaleides to evaluate targeted PKC isoforms. They found that Ro-31-8220 was selective inhibitor of calcium-, diterpine-, and phorbol ester-activable PKC isozymes α and ε, while GF 109203X was an equally effective inhibitor of basal PKC activity composed of α, ε and ξ of activated PKC. Based on several analyses, Beltman et al. concluded, that GF 109203X is less selective for particular PKC isozymes than Ro-31-8220, although the molecular reasons for these observations were not explained. A group from Leicester in 1996 compared staurosporine with its analogues (including GF 109203X) for the following properties: their effects on the cell cycle at equicytostatic concentrations, the time dependency and reversibility associated with their cytostatic properties, and their acute cytotoxic potential (Courage C et al. 1996). They reported that GF 109203X induces a potent G2/M arrest in synchronised cells, but by day 4 this phenomenon had been replaced by a weak G0/1 block. The primary cell cycle target of the staurosporine analogues is probably G2/M. If inhibition of PKC activity is important for their antiproliferation elicited, one might suppose that agents of similar high specificity for PKC would exert similar effects on the cell cycle. Conversely PKC did not appear to play an important role in these mechanisms. In fact results from this publication suggest that inhibition of PKC per se is not the primary arbiter of the growth arrest caused by these compounds. This interpretation is consistent with another investigation which demonstrated that inhibition of PKC activity could not be directly related to cell growth arrest induced by these compounds (Courage C et al. 1995). As PKC is not the prime determinant of the antiproliferative effect of these agents, among the major

targets, the prime candidates are the cyclin-dependent kinases (cdks), which, in concert with cyclins, are the essential components of the cell cycle machinery (Norbury C and Nurse P. 1992). Additional studies demonstrated that staurosporine blocks the progression of human lymphocytes through G0/1 between the cyclin D and cyclin E restriction points and markedly suppresses phytohaemagglutinin-stimulated cyclin E expression (Gong J et al. 1994). It remains to be elucidated whether the bisindolylmaleimides affect cyclins and/or cdks. Finally, all five compounds have reported to inhibit cell growth more effectively after exposure for a longer time period and their cytostatic ability diminishes by drug removal after one day. These observations indicate that to achieve therapeutic efficacy dose, schedules might have to be chosen such that they yield significant drug levels over long periods of time. One advantage of cancer treatment with these modulators of signal transduction pathways might be the possibility that they exert cytostasis and not cytotoxicity, perhaps thus minimizing undue toxicity to the host.

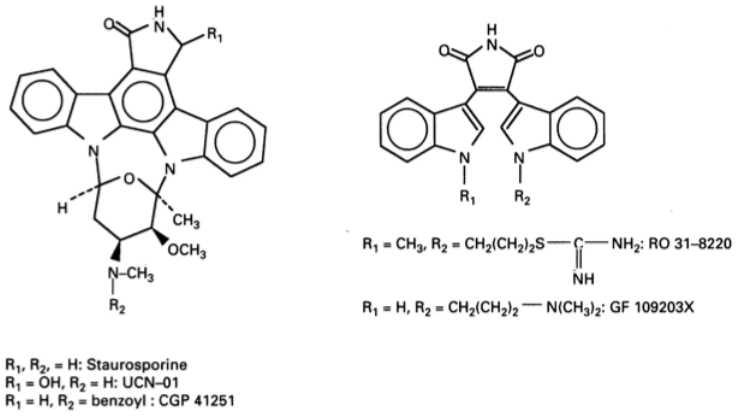


Figure 13. Structures of staurosporine and its four analogues.*Differential effects of staurosporine analogues on cell cycle, growth and viability in A549 cells. Courage C et al. Br J Cancer 1996; 74(8):1199-205.*

4.1 GF 109203X effects on different pathways

Several additional pharmacological effects of bisindolylmaleimides have been reported. For example, Hers et al. reported that GSK-3, a key kinase in insulin-induced activation of glycogen synthase, is potently and directly inhibited by GF 109203X and Ro-31-8220 (Hers I et al. 1999). They reported that Ro-31-8220 is the more potent inhibitor of GSK-3 activity, with an approximately 100 times lower IC50 value than GF 109203X. While, GF 109203X has little effect on insulin-induced inhibition of GSK-3, under conditions where it would be expected to potently inhibit PKC activity (Toullec D et al. 1991). This strongly suggests that PKC, although it has previously been implicated in GSK-3 regulation (Goode N et al. 1992), is not involved in this process.Another example that demonstrates the wide range of bisindolylmaleimides interactions is reported in 2008. The Wnt/β-catenin signaling pathway plays important roles in cell differentiation. Activation of this pathway has been shown to inhibit adipogenesis in cultured 3T3-L1 preadipocytes and mice. Cho et al. reported that GF 109203X inhibits adipogenesis by increasing the stability of β-catenin protein in 3T3-L1 preadipocyte cells. In particular it acts inhibiting adipocyte differentiation through activation of the Wnt/β-catenin signaling pathway (Cho M et al. 2008). Moreover GF 109203X increases β-catenin responsive transcription and up-regulates intracellular β-catenin levels in HEK293 cells and 3T3-L1 preadipocytes. Finally, GF 109203X significantly decreased intracellular lipid accumulation and reduced expression of important adipocyte marker genes including peroxisome-proliferator-activated receptorγ (PPAR γ) and CAATT enhancer-binding protein α (C/EBPα) in 3T3-L1 preadipocytes. Alessi et al. have also shown that Ro 318220 and GF 109203X are not selective inhibitors of the mixed PKC isoforms (Alessi DR 1993). They demonstrated that both MAPKAP-Kl and p70 S6 kinase are inhibited with similar potency by Ro 318220 and GF 109203X. This is due to the similarity in the amino acid sequence of the catalytic domain of p70 S6 kinase and the N-terminal kinase domain of MAPKAP-Kl to the catalytic domain of PKCα.

First report concerning the modulation of multidrug resistance (MDR) proteins by bisindolylmaleimide derivatives were published by Gekeler et al (Gekeler V et al 1995). Using Human leukemia cell line HL60 and its non-P-gp MDR subline, they demonstrated that GF 109203X affected transport function is most

likely associated with MRP/P-gp protein (the main representative of MDR proteins family). Furthermore, they observed that GF 109203X does not significantly alter the expression of the mrp/P-gp gene at the mRNA level. The authors supposed that, either a PKC dependent function could be disturbed by GF 109203X, i.e. phosphorylation of MRP/P-gp, or/and the compound interacts directly with the drug transporter. A year later the same group published results from similar studies performed on MDR-1/P-gp-transfected cells (Gekeler V et al. 1999) At up to 1μM final concentrations of GF 109203X, at which many PKC isozymes should be blocked substantially, no cytotoxic or MDR-reversing effects were seen. Thus, similarly to previous results, Gekeler et al. concluded that GF 109203X influences MDR mostly via direct binding to P-gp, but they did not confirm the concept of a major contribution of PKC to P-gp-associated MDR. The role of bisindolylmaleimide derivatives in multidrug resistance of cancer cells was investigated also by Budworth et al. (Budworth J et al. 1996). In contrast to previously observations, they found that neither GF 109203X nor Ro 31-8820 decreased efflux of the P-gp probe rhodamine 123 from MCF-7/Adr cells, where the most potent P-gp inhibitor was apparently staurosporine. It is possible that in MCF-7 breast cancer cells, P-gp activity depends on isozyme of PKC family, which is not inhibited by tested bisindolylmaleimide derivatives, but could be blocked by staurosporine. The possibility that bisindolylmaleimides-mediates MDR modulation is cell-type specific is supported by results published by Merritt et al. regarding Ro 31-8820 (Merritt JE et al. 1999).

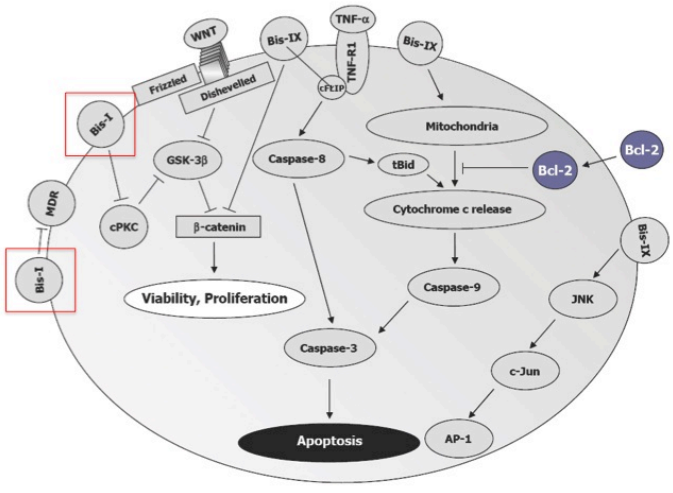


Figure 14. Schematic illustration of bisindolylmaleimides action on intracellular signaling. The pro- and antiapoptotic effects are indicated _ *Bisindolylmaleimides in anti-cancer*

therapy - more than PKC inhibitors. Pajak B et al. Adv Med Sci 2008; 53(1):21-31.

EXPERIMENTAL PROCEDURES

Antibodies siRNAs and primers

Anti-YAP (for ChIP), anti TEF-1, anti IgG, anti pan-Acetyl and pan-Phospho antibodieswere obtained from Santa Cruz Biotechnology. Anti-YAP (for western blotting) and Anti-phospho-YAP (S127) was obtained from Cell Signaling. Alexa Fluor 488-conjugated secondary antibodies were obtained from Invitrogen. SMARTpool ON-TARGETplus YAP1 siRNA and non-targeting siRNA were obtained from Thermo scientific. PKCα siRNA were obtained from Santa Cruz Biotechnology. TaqMan®probes for real time PCR analysis (mRNA expression) were obtained from Applied Biosystem, primers for real time PCR (for ChIP assay) were obtained from MWG/Operon, with the following sequences:

Forward: 5’ TTGGTGCTGGAAATACTGCG 3’

Reverse: 5’ CTCAGCGGGGAAGAGTTGTT 3’

The kinase inhibitory library waspurchased from Enzo Life Sciences.

GF 109203X HYDROCHLORIDE was obtained from Sigma-Aldrich and resuspended in dimethyl sulfoxide (DMSO).

Cell cultures and transfection methods

Human pancreatic carcinoma cell lines (PK-9, MIA PaCa-2, PANC-1) were kindly provided by G. Feldmann (Cell, 2007) and propagated in DMEM high glucouse (LONZA) supplemented with 10% FCS (LONZA), 1X vitamin solution (Sigma), 1X non-essential amino-acid solution (NEAA, Biosource), 1X Sodiumpyruvate (Gibco) and 1X Penicillin-Streptomycin (LONZA). The intermediary cells formed during acinar-to-ductal metaplasia (HPNE) were cultured in DMEM high glucouse (LONZA) supplemented with M3 Base culture medium (Incell), 10% FCS, 25μg/ml Gentamycin (LONZA) and 10ng/ml EGF (R&D Systems). Human YAP siRNA sequences (On-TARGETplusTM SMART pool, Dharmacon RNA Technologies) and Non-targeting Pool (Dharmacon) were transfected with INTERFERinTM siRNA transfection reagent (Polyplus transfection, 50nM) following the manufacturer's recommendations in PK-9 cells. Cells were treated with GF 109203X (1μM) for 24 or 48 hours, adding the compound directly into cell culture medium.

Immunofluorescence staining and drug screening

The experimental procedure used in the drug screening has been extensively optimized in pilot experiments before running the screening experiment. Cell density was the first parameter we tested because of the possible interference with YAP localization. Moreover, the cell confluence level is critical for discriminating the boundary of single cells by the software and thus making a correct analysis. Likewise, the incubation times and the concentrations of the primary and secondary antibodies are important to obtain an image of enough good quality to be used for quantitative purposes. At the end of pilot experiments, PK-9 cells were seeded on OptiPlate-96 Black, Black Opaque 96-well Microplate (PerkinElmer) at a density of 5000 cells/well, to obtain a 70% confluence at the end of the assay. The library of known kinase inhibitors was resuspended in DMSO and diluted in PBS 1X. The compounds were administrated at the final concentration of 1μM, directly in the culture medium 24 hours after the cell seeding and incubated for 24 hours. This temporal range allowed us to observe changes in YAP localization, without extensive citotoxic phenomena. Cells were fixed with 3.7% paraformaldehyde (PFA) for 15 minutes at room temperature. Cells were then treated for 10 minutes with permeabilization buffer (300 mM sucrose, 0.2% Triton X-100) and then for 15 min with blocking buffer (2% Bovine Serum Albumin in PBS). Primary antibody against YAP (1:500) and secondary fluorophore conjugated (Alexa 488) antibody (1:10000) were diluted in PBS + BSA 0.2%. DAPI (1.5 µg/ml) in PBS + BSA 0.2% was used to detect nuclei. PerkinElmer image plate reader Operetta was used for imaging and evaluation. The ratio between Nuclear and Cytoplasmic signal represents the mean of single cells for every well and it were normalized to untreated control. The Z score was calculated to evaluate the significance of results from the screening. Z score was calculated as follow: $Z = (X - \mu) / \sigma$. X = normalized sample ratio, μ = mean, σ = standard deviation.

Western Blot analysis

Whole cell extracts were obtained using Laemmli buffer, afterwards separated on 10% SDS-PAGE gels and transferred to PDVF membranes. Membranes were incubated with the following primary antibodies: anti-YAP (1:500), anti-Phospho-YAP (Ser127) (1:500) (Cell Signalling) and anti-ACTIN (1:1000) (Santa Cruz Biotechnology). The corresponding peroxidase-labeled secondary antibody (Santa Cruz Biotechnology)was detected using ECL western blotting reagents (GE Healthcare).

2 Dimensional Electrophoresis

Proteins were extracted using a lysis buffer (8 M urea, 4% CHAPS, 50 mM dithioerythritol and 0,0002% Bromophenol blue) and rehydrated with 8 M urea, 2% CHAPS, 20 mM dithioerythritol, 0.8% IPG buffer, carrier ampholytes pH 6-11 linear. The first dimension isoelectric focusing (IEF) was performed inimmobiline dry strips (GE) with a pH range from 7 to 4. IEF was performed on IPGphor (GE) according to the manufacturer recommendations. The gels were then equilibrated in 6 M urea, 3% SDS, 375 mM Tris pH 8.6, 30% glycerol, 2% DTE and then incubated with 3% iodoacetamide (IAA) and traces of bromophenol blue (BBP). The second dimension was performed using an 8% SDS-PAGE gel. Transfer and detection were carried out as previously described.

Immunoprecipitation assay

1mg of cellular extract from PK-9 cells was used as INPUT for each immunoprecipitation reaction. 40μl of protein G Agarose (equilibrated with RIPA buffer: 50mM TRIS HCl pH 7.5, 150mM NaCl, 1% NP-40, 0,5% Nadeoxycholate, 0,1% SDS, 1mM EDTA, 2% glycerol) were added with 10μg of antibody of interest (anti-YAP, anti-TEAD, anti-pan Phosho, anti-pan Ac, or anti-IgG1), in batch for 2 hours at 4°C. Then every sample was loaded with 10μg of cellular extract, in batch 1 hour at 4°C. To remove nonspecific linking proteins three washes with RIPA buffer were performed. To elute antibody-antigen complexes for Western Blot analyses 40μl of 2x Leammli solution were added; samples were boiled 5min and microcentrifuged at 14000 rpm for 2 minutes. Samples were immediately immunoblotted to detect immunoprecipitated proteins.

RT-qPCR analysis

Primers to be used in RT-qPCR experiments after ChIP assay were designed with Primer3 software. The selected sequences were tested against public databases (NBLAST) to confirm the identity of the genes. For mRNA expression analysis, PK-9 cell cultures were collected and lysed by addition of RP1 buffer (Agilent total RNA isolation mini kit) and total RNA was isolated. 1μg of total RNA was reverse-transcribed in 20μl using RevertAid^{TM} first strand cDNA

synthesis kit (Fermentas life sciences), according to manufacturer's instructions. Real-time PCR were performed with TaqMan® gene expression assays. For ChIP assay, DNA was extracted by phenol/chloroform/isoamyl alcohol, ethanol precipitated and resuspended in water. Real-time PCR were performed with SYBR GREEN master mix. The reactions were carried out on a CFX96^{TM} real-time system (BIO-RAD). Data were analyzed by averaging technical triplicates. Experiments were run at least four times. In mRNA expression experiments, Ct values of every gene were normalized to the housekeeping GAPDH, while in ChIP assays, the normalization were calculated by the following formula: (X–IgG) / INPUT, where X is the Ct of the sequence of interest derived from the immunoprecipitated DNA bound to the protein of interest; IgG is the Ct of the same sequence derived from the DNA immunoprecipitated with an irrelevant antibody and INPUT is the Ct derived from the total DNA before immunoprecipitation. Experiments were run at least four times.

Toxicity assay

PK-9 cells were seeded as previously described and treated at 10 different concentrations. 48 hours after the treatment, the cells were incubated with Cell proliferation reagent WST-1 (Roche) according to the manufacturer recommendations. Absorbance-based viability was detected after 4 hours using Infinite® M200 (Tecan) at two time points: the day of compound adding and 48 hours after the treatment. The data were analyzed with GraphPadPrism 5.0 (GraphPad, San Diego, CA) software. The IC50 was determined by fitting the data point with the sigmoidal curve.

Cell proliferation assay

Five thousand PK-9 cells were seeded per well of E-plates (Roche), in triplicates. The cell growth curves were automatically recorded on the xCELLigence System (Roche) in real time. The cell attachment was monitored every 15 minutes by a cell electronic system. The doubling times were calculated according with cell index. The cell index is an arbitrary unit for displaying impedence. siRNAs transfection or treatment with GF 109203X were performed by replacing cell medium, including untreated control to operate at the same working conditions. Since the sensitivity of the system used, an optimization phase was required before starting, in order to evaluate the optimal level of cell seeding. Differences in the number of passages of the same cell line could introduce variability to the cell index curves.

Soft agar assay

PK-9 cells were previously treated with GF 109203X (1μM) for 24 hours or transfected with siRNAs against YAP for 48 hours. One thousand of viable cells (treated, transfected and controls) were seeded per well in 96 multi well in quadruplicates. Soft-agar assay was performed using Cell BiolabsCytoSelectTM 96-well cell transformation assay (Soft Agar Colony Formation), according to the manufacturer recommendations. Ten days after cell seeding, cell counting was performed as follow: agar solubilization solution and lysis buffer were added to every well and after incubation with CyQuant

Working Solution, fluorescence was measured using a 485/520 nm filter set.

Annexin V staining and flow cytometry

PK-9 cells (transfected with siRNAs against YAP and control siRNA) were trypsinized and stained with PE Annexin V and analyzed by flow cytometry using the Annexin V Apoptosis Detection kit (BD Biosciences) following the manufacturer's instructions. Data were collected and analyzed on a BD FACSCanto using FACSDiva software.

Chromatin Immunoprecipitation assay

Chromatin Immunoprecipitation (ChIP) assay was performed using untreated and GF 109203X treated PK-9 cells. The cross-linking was obtained with 1% formaldehyde. Cell pellets were wash with PBS 1X and subsequently resuspended in Lysis buffer and sonicated 10 sec x 3 times, 65% power (Branson 450 SLPe digital probe sonicator). Sonicated chromatin was incubated overnight in batch at 4°C in Dilution buffer with anti-IgG, anti-YAP and anti-TEF-1. 1% of total lysate was used for INPUT control. DNA was extracted by the Phenol/Chloroform/Isoamyl-alcohol, ethanol-precipitated, and resuspended in water. ChIP products were amplified using SYBR GREEN master mix and specific primers (see above).

The crucial aspects to set a good ChIP protocol has been:

- incubation with formaldehyde: more than 10 minutes could cause an irreversible cross-linking;
- sonication: time, number of pulses and amplitude have to be tested before starting and can be different depending on cell type. Since sonication buffer

contains SDS, its precipitation (on ice) can increase the density of the samples, causing bubbles that can alter the quality of the sonication;

- antibodies: we changed different kinds of YAP antibody before choosing the best one. This is particular hard when the protein is not directly bound to DNA but to a transcription factor (as YAP with TEAD). The concentration has also to be evaluated.

RESULTS

1. CRUCIAL ROLE OF YAP IN PDAC

1.1 YAP protein is expressed in multiple PDAC cell lines

Increased YAP levels were detected in breast, colorectal, lung, and prostate cancer (Steinhardt AA, et al. 2008). Moreover, several recent observations point to a role of YAP in PDAC. In addition to the mutations in some Hippo pathway components (Jones S, et al. 2008), amplification of the 11q22 locus, where YAP is located, has also been reported in this form of cancer (Bashyam MD, et al. 2005). To evaluate the presence of YAP in PDAC cell models, we performed western blotting analysis on total cell lysates from different PDAC cell lines (Figure 1.1). YAP was expressed in all of the cell lines analyzed and a high YAP expression was found in 3 out of 7 cell lines: PK-9, PANC-1, BxPC-3, suggesting a relevance of this protein in PDAC cells*in vitro*.

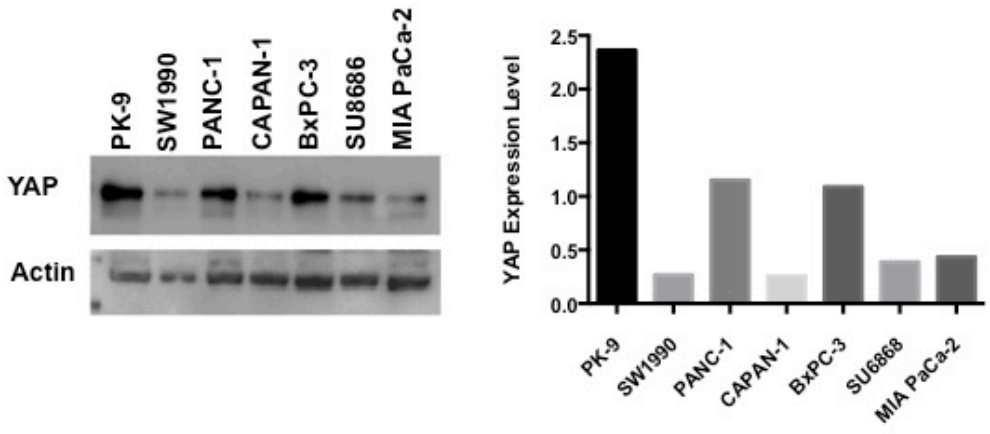


Figure 1.1: YAP expression in different PDAC cell lines. Western blotting analysis on the total cell lysate (left), and relative densiometric analysis (right). Actin was used has housekeeping gene for normalization.

1.2 YAP silencing reduces the anchorage-independent growth of PK-9 cells and leads to a delay in cell proliferation at the exponential phase of growth

Since the highest level of YAP expression was observed in PK-9 cells, and since It has been previously showed that YAP is responsible of the tumorigenic potential of PK-9 cells when xenografted into nude mice (Dong J, et al. 2007), we used this cell line as reference for YAP dependent PDAC cell model. To explore the significance of YAP expression in PDAC cells *in vitro*, we performed transient YAP silencing in PK-9 cells through RNA interference. YAP protein level was reduced to 30% of the original level in YAP-silenced cells compared to controls, as shown by western blotting analysis (Figure 1.2a).

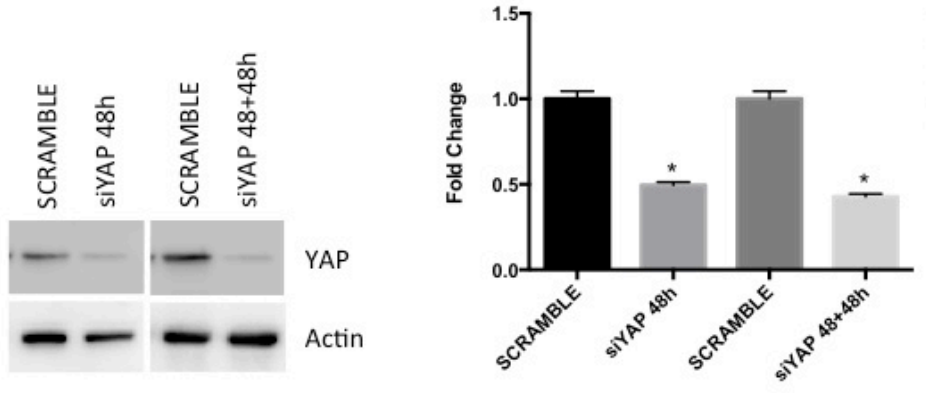


Figure 1.2a: Transient silencing of YAP in PK-9 cell line. YAP expression in PK-9 cells transfected by a control siRNA or with a pool of siRNAs against YAP (left), and relative densiometric analysis (right).

Subsequently, two important parameters have been examined: the anchorage-

independent growth and the cell proliferation. The ability of YAP-silenced cells to grow in anchorage-independent conditions was studied using soft agar colony formation assay (Figure 1.2b). We seeded 1000 cells per well in quadruplicates and PK-9 cells were previously transfected with siRNAs against YAP or control siRNA for 48 hours. Ten days after cell seeding the counting was performed according to the manufacturer recommendations (see materials and methods). The number of colonies was reduced by 20% in YAP-silenced cells compared to controls. These results open the way to further investigation about a putative role of YAP in controlling the cell genetic programing involved in the regulation of anchorage-independent growth in PDAC cell lines and are consistent with the role of YAP in modulating the tumorigenic potential of PK-9 cells *in vivo*.

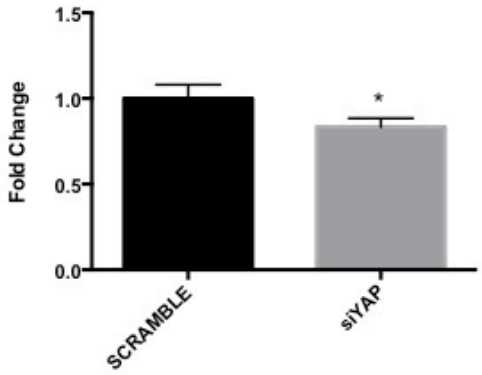


Figure 1.2b: Colony formation assay of PK-9 cells in the absence of YAP. Cells were transfected with siRNAs against YAP, grown for 10 days and colonies counted.

Next we measured YAP dependent cell proliferation by monitoring in real time through xCELLingence system (Figure 1.2c). In this system cell attachment is detected by microelectrode sensors and is directly correlated to cell proliferation.We chose this technology because of the peculiar role of YAP in changing its activation status upon cell confluency. At low density, YAP is predominantly localized in the nuclear compartment, while, YAP translocates to the cytoplasm at high density (Zhao B, et al. 2007). We performed siRNAs transfection 24 and 72 hours after the cell seeding. We reported a significant growth delay 48 hour after the first silencing (time point: 72 hours), indicating

that functional ablation of YAP led to an inhibition of cell proliferation at the exponential phase, and thus a role of YAP during this growth phase, in PK-9 cell lines. This can be explained by the fact that YAP co-transcriptional activity and pro-proliferation effect are prevalent when cells are in the low confluent status. We also observed a relevant reduction in the number of silenced cells after the plateau phase was reached (time point: 140 hours). This could be addressed to a role of YAP in inhibiting apoptosis activation during confluency. However the role of YAP in regulating apoptosis is controversial. It was proposed that this co-transcriptional factor can show oncogenic or tumor suppressor behavior depending on the cellular context, and consequently the choice of its partners, that determine the final outcome (Bertini, E. et al. 2009). In order to investigate whether the reduction in the number of adherent cells was due to apoptotic phenomena we used flow cytometry assay (Figure 1.2d). We collected both untransfected and transfected PK-9 cells at the steady state growth and stained with Annexin V. Then the samples were analyzed by flow cytometry and the percentage of cells at all of the cell cycle phases was reported in graph. We excluded the presence of apoptosis in YAP silenced cells. Nearly all the cells belonged to the viable compartment, while the rate of apoptotic cells is not significantly different in both untransfected and transfected cells. Interestingly, we observed an increased number of cells into the “Q1” compartment in YAP silenced cells compare to control (about 10%). These features were consistent with change in cell morphology. To test this hypothesis, we performed an immunofluorescence assay, by incubating fixed cells with antibody against YAP and with DAPI. An image plate reader was used to visualize and measure the size of control and silenced cells (Figure 1.2e). Changes in cell and nuclear dimension and shape suggested the onset of senescence upon YAP silencing. This hypothesis will be subject of future studies but, in support to our hypothesis, a recent paper (Xie Q, et al. 2013) showed that silencing of YAP induces premature senescence. Therefore, together with previous observation, our results demonstrated a YAP involvement in regulating cell proliferation and anchorage-independent growth, thus identifying YAP protein as a potential target for PDAC treatment.

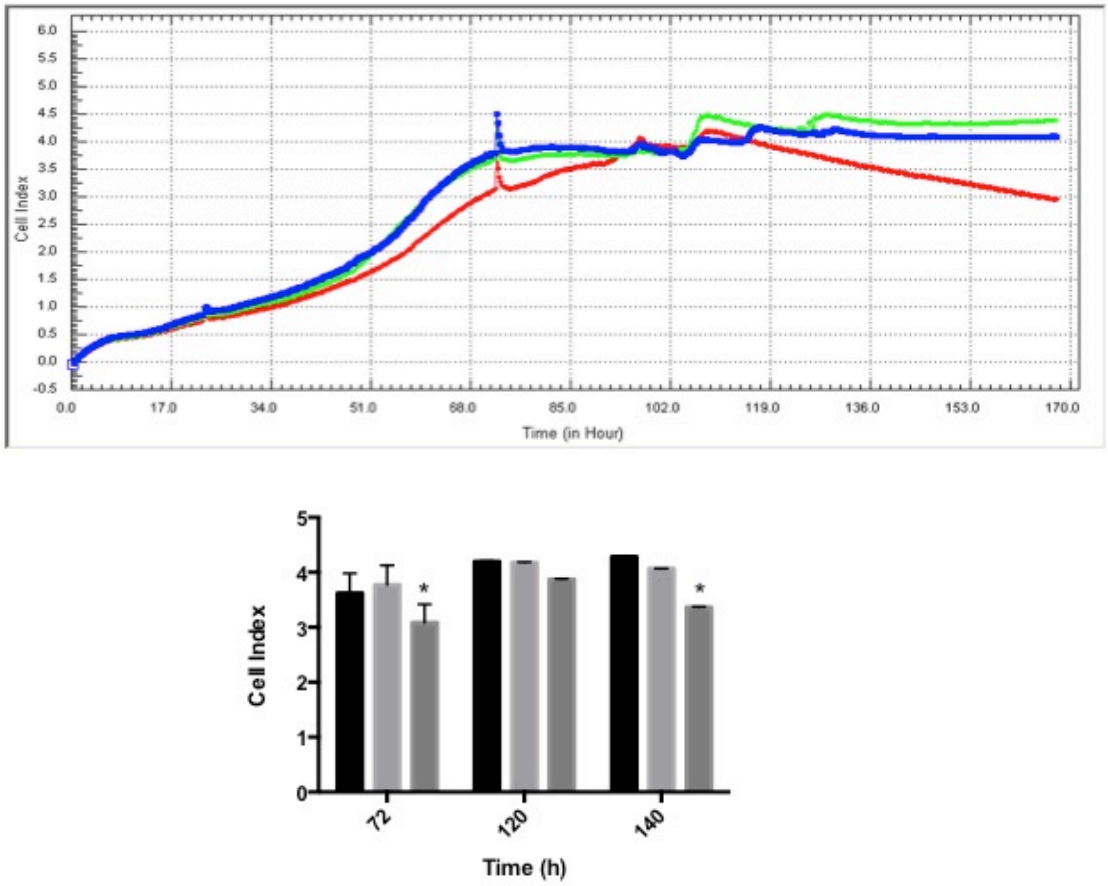


Figure 1.2c: Proliferation of PK-9 cells after siRNAs inhibition of YAP expression. Five thousand cells were seeded per well of E-plate in triplicate and trasfected with siRNAs against YAP (red line/siYAP), control siRNA (green line/scramble) or without siRNA (blue line/mock), after 24 and 72 hours. Cell attachment was monitored every 15 minutes by a real-time cell electronic system. The cell index is an arbitrary unit for displaying impedence. In the upper part of the panel Cell growth curves are reported;the quantification of cell proliferation 72, 120 and 140 hours after the cell seeding are reported in the lower part.

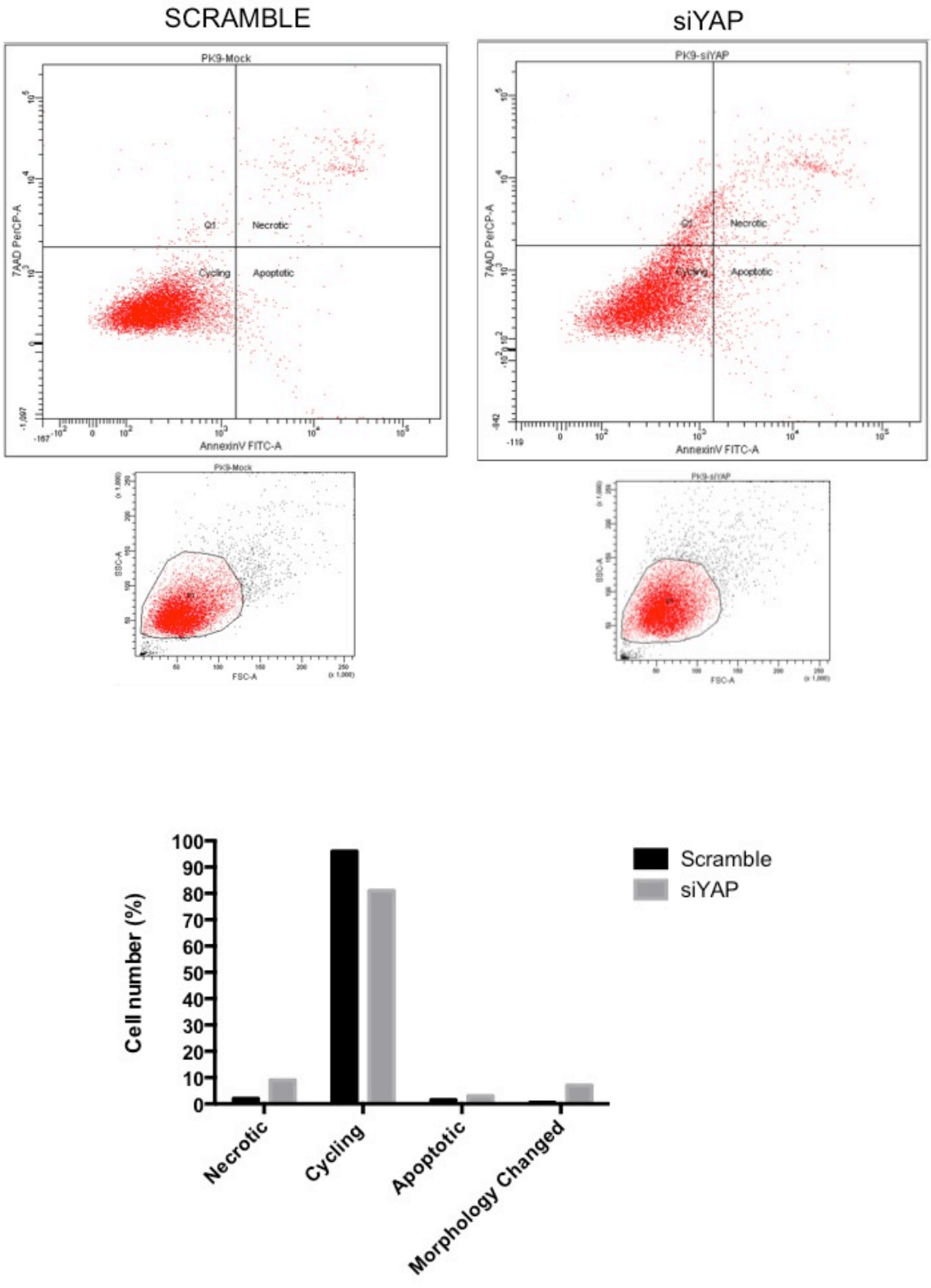


Figure 1.2d: Knockdown of YAP does not induce apoptosis at the steady state growth.PK-9 cells were transfected with control siRNA or siRNAs targeting YAP 24 and 72 hours after the cell seeding. Cells were collected and stained with PE Annexin V and analyzed by FACS. The rates of all cell cycle phases were quantified and reported in graph.

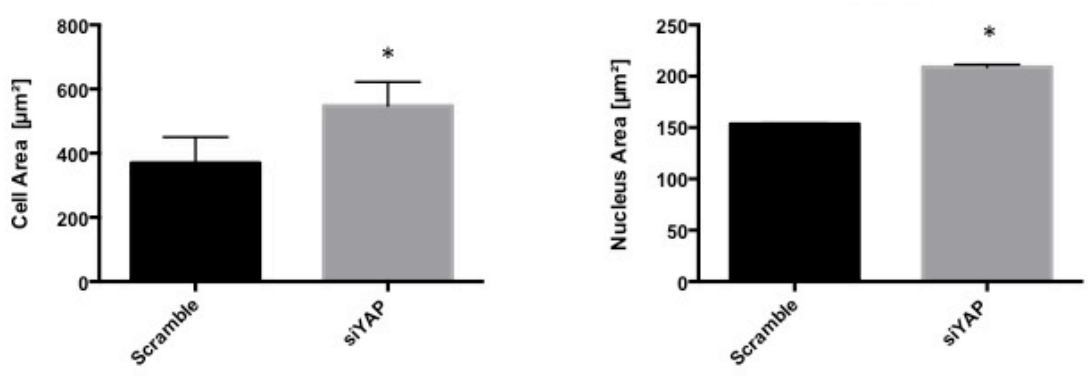


Figure 1.2e: Increased cell size after YAP silencing in PK-9 cells. PK-9 cells were transfected with control siRNA (Scramble) or siRNAs targeting YAP (siYAP) 24 and 72 hours after the cell seeding. Fixed cells were incubated with with antibody against YAP and DAPI staining. Cellular and nuclear areas were measured through the PerkinElmer microplate image reader, Operetta and reported in graph.

2. IDENTIFICATION OF A KINASE INHIBITOR, GF 109203X, ABLE TO INDUCE YAP NUCLEAR RETENTION BY AFFECTING YAP POST TRANSLATIONAL MODIFICATIONS

2.1 Development of a high content assay for the identification of molecules that interfere with YAP subcellular localization

YAP is an attractive therapeutic target, but no inhibitors of YAP activity are currently available as therapeutic options. The identification of molecular entities targeting the effects of YAP overexpression in PDAC could pave the way to the identification of novel therapeutics for this lethal malignancy. YAP cytoplasmic accumulation is accompanied by phosphorylation on S127 from the Hippo pathway component Lats1/2, and consequent protein inactivity and degradation by 14-3-3 (Hao Y, et al. 2008; Zhao, B. et al. 2007). On the other hand, nuclear localization is a prerequisite for its co-transcriptional activity on specific gene promoters (Zhao B, et al. 2007). However a second level of post-translational regulation, i.e. acetylations on K494 and K497, occurs on YAP and finely drives its activation independently of the nuclear localization of the protein (Hata S, et al. 2012). We exploited the nucleo-cytosplasmic properties of YAP to set up a high content assay that quantified the protein localization inside PK-9 cells by mean of immunocytochemistry. We administered a library of 80 characterized kinase inhibitors (purchased from Enzo Life Sciences) for 24 hours at the final concentration of 1 μM, with the aim to find a molecule able to regulate YAP localization. Fixed cells were incubated with antibody against YAP and DAPI staining. Intracellular localization of endogenous YAP protein was visualized through the PerkinElmer microplate image reader, Operetta (non confocal mode). The ratio between nuclear and cytoplasmic regions was calculated and normalized to untreated control. The Z-score of this value is reported in figure 2.1a. Most of the molecules did not have an effect on YAP localization being the ratio of the intensity of nuclear/cytoplasm close to controls. Only two

compounds lead YAP accumulation into the nucleus: GF 109203X and Ro 31-8220 (Figure 2.1b). GF 109203X, also called Bisindolylmaleimide I, is known as a cell-permeable and reversible protein kinase C (PKC) inhibitor. It acts as a competitive inhibitor for the ATP binding site of PKC and shows high selectivity for the PKC family members with specific affinity for PKCα-, β1-, β2-, γ-, δ-, and ε-isozymes. The Ki value is 14 ± 3 nM being most affine for the PKCα isoform. Moreover it can also directly target multiple other proteins (see introduction). Further immunofluorescence confirmation assays showed higher reproducibility of the nuclear retention effect for GF 109203X (Figure 2.1c) while Ro 31-8220 gave more variable results. While YAP results spread within the two subcellular compartments in untreated cells at our experimental conditions, we can see a significant nuclear accumulation after GF 109203X treatment.

Therefore, since the high structural homology of the two compounds we chose to use only GF 109203X for further experiments. In conclusion, we developed a high content assay to screen libraries of compounds and successively identified molecules able to interfere with the protein subcellular localization. Through this assay we identified a molecule that is able to retain YAP protein within the nuclear compartment.

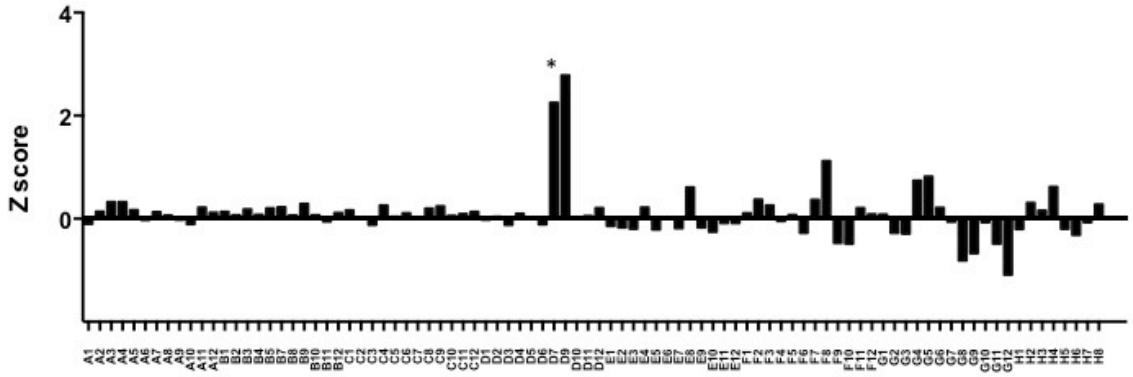


Figure 2.1a: Screening assay development. A kinase inhibitor library was administrated to PK-9 cells using a high-throughput approach (1 μM, 24hours). The ratio between nuclear and cytoplasmic regions was calculated and normalized to untreated control. The Z-score was reported in graph.

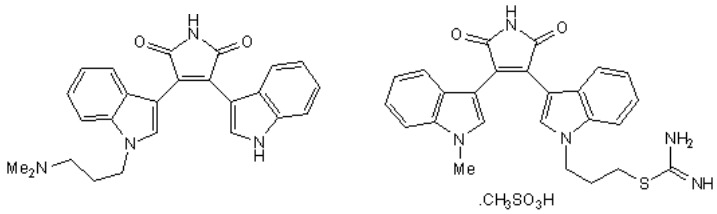


Figure 2.1b: GF 109203X and Ro 31-8220. Molecular structure of the two identified compounds.

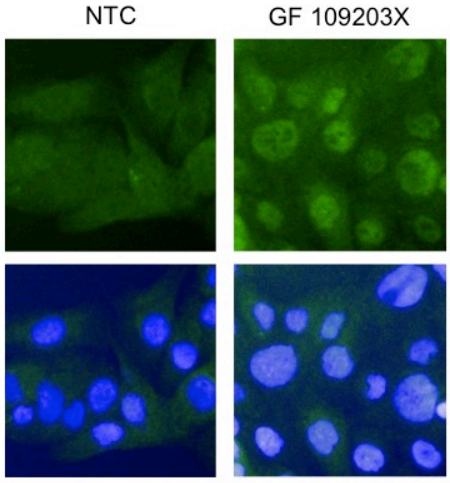


Figure 2.1c: Validation of one significant hit, GF 109203X, by immunofluorescence images. PK-9 cells were treated with GF 109203X 1 µM for 24 hours and compared with untreated cells (NTC). Fixed cells were incubated with antibody against YAP (green) and DAPI staining (blue). Intracellular localization of endogenous YAP protein was visualized through the PerkinElmer microplate image reader, Operetta (non confocal mode).

2.2 GF 109203X enhances YAP phosphorylation and acetylation, without affecting phosphorylation at Serine 127

As already stated, YAP nuclear localization is a necessary, but not sufficient, condition to allow its transcriptional activity. Therefore we thought that GF 109203X-induced nuclear accumulation could not necessarily lead to YAP activation but could depend on the level of post-translational modifications occurring on YAP protein and eventually lead to YAP inhibition. We searched if GF 109203X caused an alteration of the phosphorylation and acetylation pattern of YAP in standard serum conditions. By two dimensional western blotting we observed that GF 109203X induced a higher number of migratory species at different pI than those observed during standard serum conditions (Figure 2.2a). The appearance of migratory bands towards lower pH is compatible with the onset of phosphorylations on free hydroxyl groups or acetylation and methylation of aminogroups. When we treated the cell lysate with calf intestinal alkaline phosphatase (CIAP) many bands, but not all, disappeared showing that GF 109203X induces a complex cellular answer comprising phosphorylation events. However, and consistently with YAP nuclear localization after the treatment, GF 109203X does not induce YAP phosphorylation on S127 and it has no effect on YAP Ser127-P localization. As internal positive control, we used cell lysates obtained from confluent cells in which the condition of S127 is phosphorylated (Zhao, B. et al. 2007). These results are in line with western blotting analysis on total cell lysates from treated and untreated PK-9 cells (Figure 2.2b). Immunoblotting with antibodies against total YAP or YAP Ser127-P showed that the level of both YAP proteins does not undergo any change after the treatment.

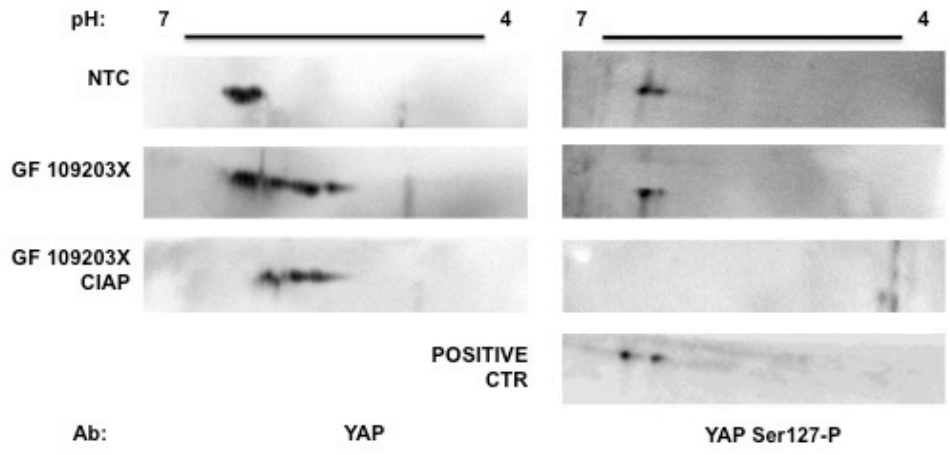


Figure 2.2a: 2D western blotting on whole cell lysates. Filters were blotted with antibodies against YAP and YAP Ser127-P. As negative control for phosphorylation the treated sample was incubated with calf intestinal alkaline phosphatase (CIAP). As positive control for phosphorylation at Ser127, cell lysate from high density cell culture was used.

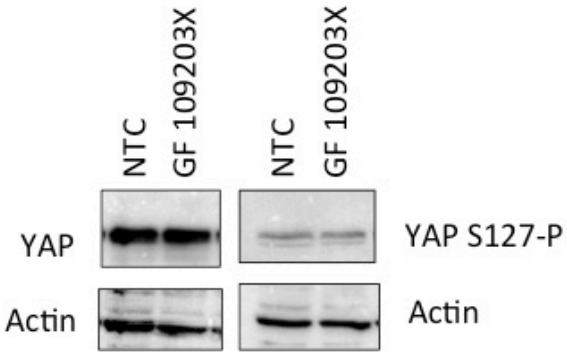


Figure 2.2b: western blotting analysis on total cell lysates. YAP expression level in untreated (NTC) and GF 109203X-treated (1μM) PK-9 cells. Filters werehybridized with antibodies against total YAP and YAP Ser127-P.

In order to confirm the results obtained in 2D experiments we immunoprecipitated YAP protein and blotted using antibodies against pan phosphorylation and pan acetylation residues (Figure 2.2c). In YAP immunoprecipitated samples we observed increase of both acetylations and phosphorylations on YAP protein. Therefore GF 109203X deeply changes the post-translational status of YAP favoring the appearance of phosphorylations, with the exception P-S127, and of acetylations. These results suggest that GF 109203X mechanism of action could be Hippo pathway-independent. Moreover the increased level of YAP acetylation after the treatment can explain the GF 109203X-induced YAP nuclear accumulation.Anexample of YAP regulation depending on its acetylation status within the nuclear compartment, has been already described (Hata S et al. 2012). We speculated that the putative Hippo pathway-independent regulation of YAP and changes in PTMs, in particular on acetylation, after GF 109203X treatment could be indications of a new mechanism for YAP regulation, not tightly dependent on its subcellular localization.

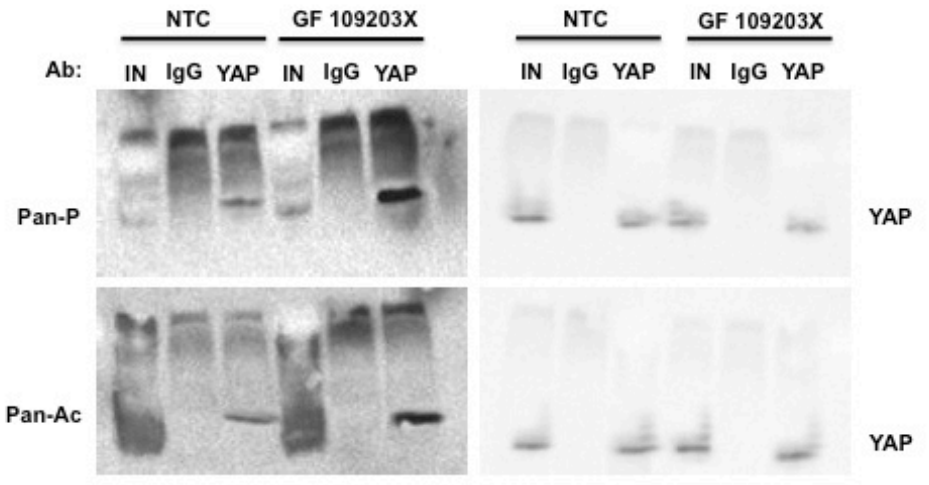


Figure 2.2c: YAP immunoprecipitation on whole cell lysates. Total extracts were immunoprecipitated with antibody against YAP, pan phospho (pan-P) and pan acetylation (pan-Ac).

3. GF 109203X TREATMENT RESULTS IN A DOWNREGULATION OF YAP TARGET GENE, CTGF, LEADING TO A CELL GROWTH DELAY AT THE EXPONENTIAL PHASE AND A REDUCED ANCHORAGE-INDEPENDENT GROWTH

3.1 GF 109203X mimics YAP silencing effects on anchorage-independent growth and cell proliferation at the exponential phase of growth

We previously showed the phenotypic effects of YAP functional ablation on cell proliferation and anchorage-independent growth. In order to investigate whether the identified compound could induce similar consequences through its ability to affect YAP subcellular localization, we repeated the assays above in PK-9 cells after GF 109203X treatment. We evaluated the cells capability to grow in anchorage-independent conditions by seeding PK-9 cells in soft agar (Figure 3.1a). The first set of experiments was performed treating cells with GF 109203X 1μM only at the moment of cell seeding. The number of colonies, counted ten days later, although we observed a clear decrease, was not significantly changed in treated cells compare to untreated control. In order to potentiate the effect of the compound, we decided to pretreat PK-9 cells with the same conditions used in the screening, before starting the colony formation assay, and then seed the same number of viable cells as in the previous experiment with a second dose of the compound. Interestingly we observed a marked reduction in the number of colonies in treated cells compare to control, concluding that after GF 109203X treatment PK-9 cells are not clonogenic anymore. Thus we identified a compound able to induce YAP relocalization that

leads to the same effect then YAP silencing in cell capability to growth in anchorage-independent conditions.

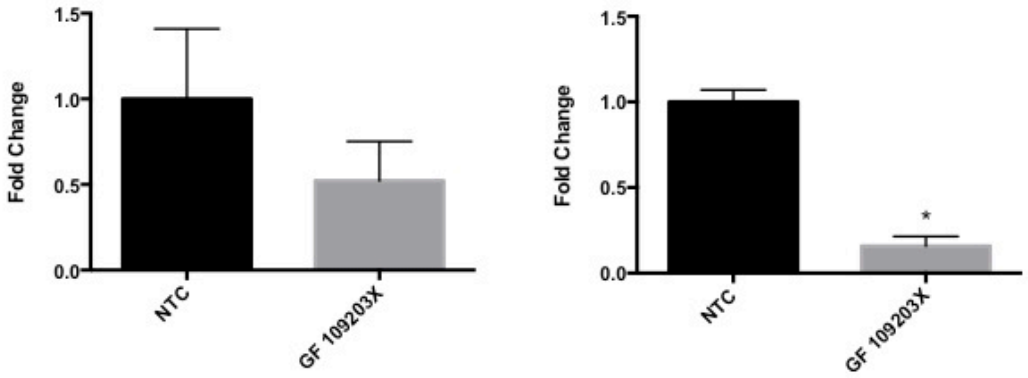


Figure 3.1a: Colony formation assay of PK-9 cells after GF 109203X treatment. Cells were treated with GF 109203X at 1 μM after the seeding in soft agar medium. Cells were previously cultured in PL medium without the compound (left) or with GF 109203X at 1 μM for 24 hours (right). The number of colonies was counted after 10 days.

Cell proliferation was observed in real time through xCELLingence system as previously described (Figure 3.1b). We treated PK-9 cells with GF 109203X 1μM 24 and 72 hours after the cell seeding. In order to compare the curve trend with the growth of YAP silenced cells, we also performed siRNAs transfections at the same time points. As already reported for YAP silencing, GF 109203X treatment induced a growth delay at the exponential phase of growth, i.e. 24 hours after the second treatment. Furthermore the presence of GF 109203X in YAP silenced cells did not lead to an additive effect of the treatment. Taken together these data shown that GF 109203X treatment mimics the effects observed in YAP silenced cells on PK-9 cell growth, suggesting that GF 109203X action is YAP-dependent. Since we reported no change in YAP expression level after the treatment, GF 109203X mechanism of action seems to be more related with YAP activity. Moreover, these results confirmed the relevance of cell growth conditions, especially cell density, in YAP study. These effects, complies with a YAP-dependent anti cancer action of the identified compound, have encouraged us to further investigate the role of GF 109203X as a YAP inhibitor.

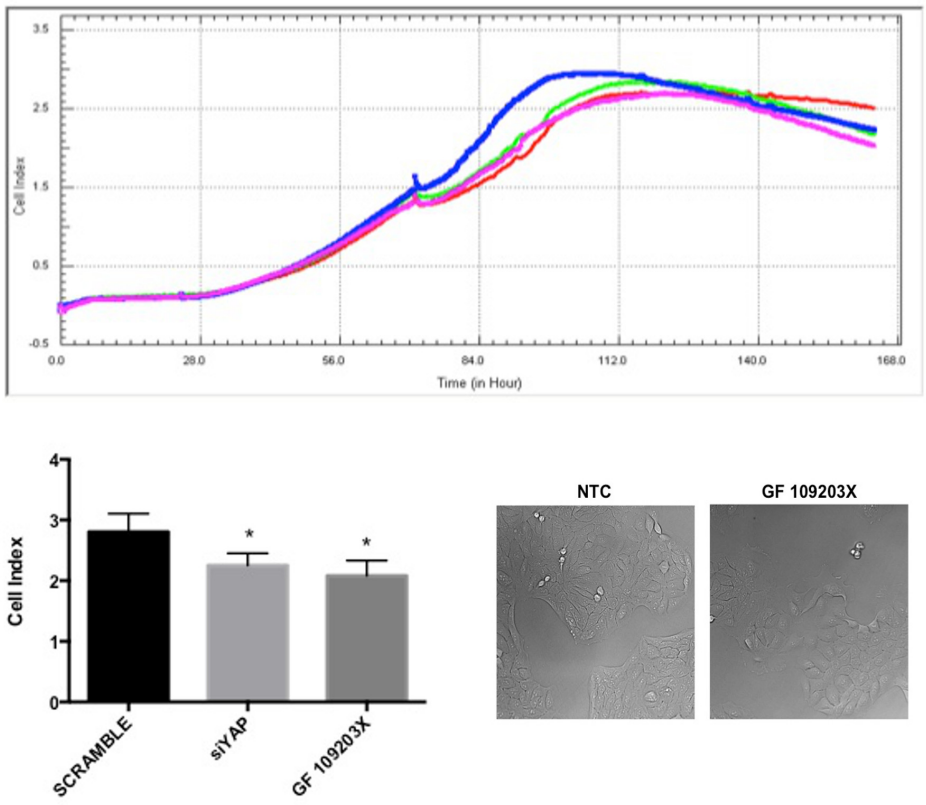


Figure 3.1b: Proliferation of PK-9 cells after GF 109203X treatment. Five thousand cells were seeded per well of E-plate in triplicate and treated with: siRNAs against YAP (red line), control siRNA (blue line), GF 109203X 1 μM (purple line), GF 109203X 1 μM plus siRNAs against YAP (green line) 24 and 72 hours after the ell seeding. Cell attachment was calculated as described previously. Cell index was quantified 96 hours after the cell seeding. In the lower part: the quantification of cell index 96 hours after the cell seeding (left) and a bright-field microscopy image of untreated (NTC) and treated (GF 109203X) PK-9 cells at 72 hours (right) are reported.

3.2 GF 109203X reduces CTGF expression level in PK-9 cell line

The connective tissue growth factor (CTGF) has been identified as a direct target gene of YAP and the transcription factor TEAD. Functional ablation of CTGF has been demonstrated to block YAP-stimulated cell growth and significantly reduces YAP-induced colony formation in soft agar (Zhao B, et al. 2008). In order to investigate if the phenotypic effects described after GF 109203X treatment can be caused by an alteration in CTGF expression, we performed quantitative real time PCR on mRNA from untreated and treated PK-9 cells (Figure 3.2). Despite an unchanged level of YAP expression, both at the protein (Figure 2.2b) and mRNA level, in GF 109203X treated cells, we observed a substantial decrease of CTGF expression compared to control. We also examined CTGF expression level in YAP silenced cells. The expected reduction of YAP mRNA level after YAP silencing was associated with a moderate downregulation of CTGF expression. These results demonstrated that GF 109203X treatment negative regulates CTGF expression and suggested that this effect is not dependent on YAP expression level, but again, on the modulation of YAP activity at the post-translational level. Moreover, CTGF downregulation is able to explain the decrease of cell proliferation and anchorage-independent growth reported after GF 109203X treatment.

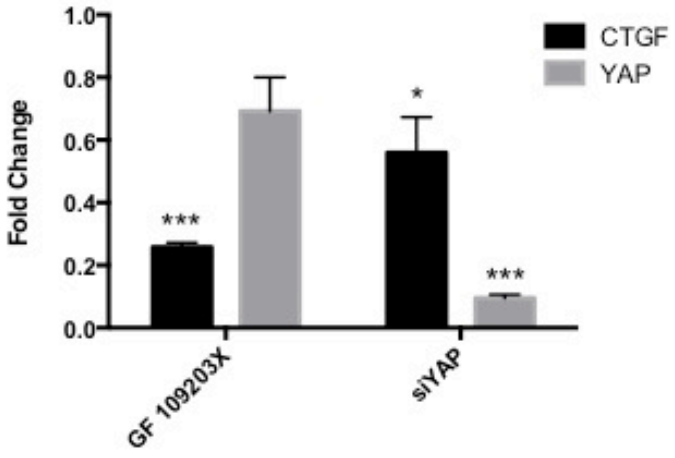


Figure 3.2: CTGF expression decreases after GF 109203X treatment in PK-9 cell line. Cells were treated with GF 109203X 1 µM for 24 hours. The level of CTGF and YAP mRNA was determined by Q-RT-PCR, using GAPDH as reference gene and normalized to untreated/untransfected control. Mean values and standard deviations were calculated from triplicates of three independent repeats.

3.3 GF 109203X effect on CTGF expression is PKCα-independent

Since GF 109203X was defined as a PKCα inhibitor (Toullec D, et al. 1991), we studied the role of this protein kinase in our model. In particular, we explored whether CTGF regulation by GF 109203X was dependent on PKCα expression level. We performed PKCα silencing through RNA interference in PK-9 cell line and evaluated CTGF mRNA levels by quantitative real time PCR (Figure 3.3). We observed that functional ablation of PKCα is not associated with change in YAP expression level, while CTGF transcript resulted upregulated. This result indicates that PKCα is clearly implicated in the regulation of CTGF mRNA level, however since its functional ablations leading to opposite results than GF 109203X administration on the expression level of CTGF, this molecule is triggering more complex pathways than simply inhibit PKCα. Due to the difficulties in identifying the GF 109203X target responsible of these effects we decided to better investigate the nuclear activity of YAP after GF 109203X treatment on the CTGF promoter.

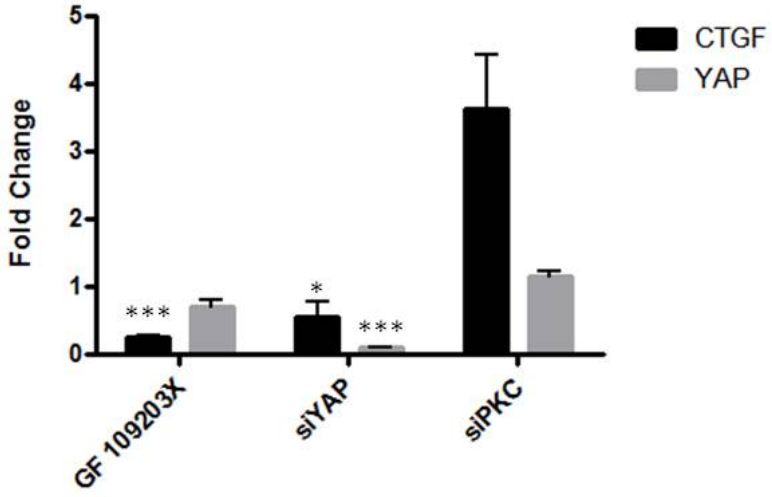


Figure 3.3: CTGF and YAP expression in PK-9 cells after GF 109203X treatment and YAP or PKCαsilencing. Cells were treated with GF 109203X 1 μM for 24 hours or transfected with siRNAs against YAP or PKCα for 48 hours. The level of CTGF and YAP mRNA was determined by Q-RT-PCR, using GAPDH as reference gene and normalized to untreated/untransfected control. Mean values and standard deviations were calculated from triplicates of three independent repeats.

4. GF 109203X DISPLACES YAP PROTEIN FROM CTGF PROMOTER

TEAD is the major partner for functional activity of YAP (Vassilev A, et al. 2001). YAP/TEAD cooperate to activate the transcription of a number of genes by the physical and functional co-presence on their promoter regions and CTGF (connective tissue growth factor) is considered one of the main, although not exclusive, representative target. We therefore chose to investigate the co-presence of YAP and TEAD at the promoter of CTGF by chromatin immunoprecipitation in the region containing the putative TEAD-binding sites (Figure 4a) identified by Zhang H and coworkers and important for CTGF promoter activity (Zhang H, et al. 2009). DNA from Chromatin immunoprecipitation was extract and analyzed by Real time PCR (Figure 4b). The TEAD1/3/4 transcription factors have been demonstrated to play a critical role in the expression of YAP-dependent genes, in particular data from (ChIP)-on-chip experiments shown that YAP and TEAD1 co-occupy >80% of the promoters pulled down by either of them in MCF10A cells (Zhao B, et al. 2008). We explored the presence of three members of TEAD family, using antibodies against TEF-1 (TEAD1), TEF-3 (TEAD4) and TEF-4 (TEAD2). Interestingly, we obtained more reproducible data only from TEAD1 immunoprecipitated material, suggesting that in our model this member is the most involved in binding the investigated CTGF promoter region. As shown in figure 4, YAP and TEAD1 are associated on the CTGF promoter region during exponential growth phase (untreated sample, NTC), while GF 109203X can displace YAP protein from CTGF promoter. On the other hand, TEAD1 binding seemed to be not affected by treatment, and it can be considered an internal positive control. These data indicate that GF 109203X can break the interaction between YAP and TEAD at the chromatin level on the CTGF promoter, likely causing a decrease in the expression level of CTGF mRNA, but this effect does not necessarily inhibit TEAD binding to DNA. YAP displacement is consistent with the reported reduction of CTGF mRNA level during GF 109203X administration. Furthermore these results underlined that YAP functionality and activity, being this protein a

co-transcriptional activator,primary dependent on its capability to interact with its targets, i.e. multiple transcription factors, more than its simple localization. This could explain how a compound able to lead a nuclear accumulation of YAP, can be its inhibitor at the same time.

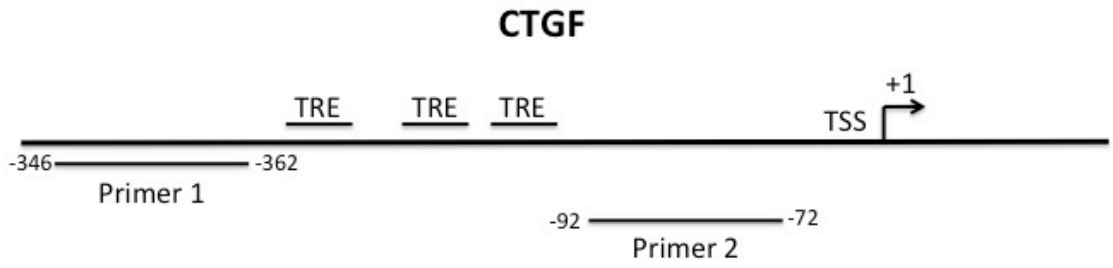


Figure4a: Map of CTGF promoter region. The position of the two primers used for ChIP analysis is indicated. The amplified sequence includes the previously identified TEAD responsive elements (TRE). TSS indicates the transcription start site.

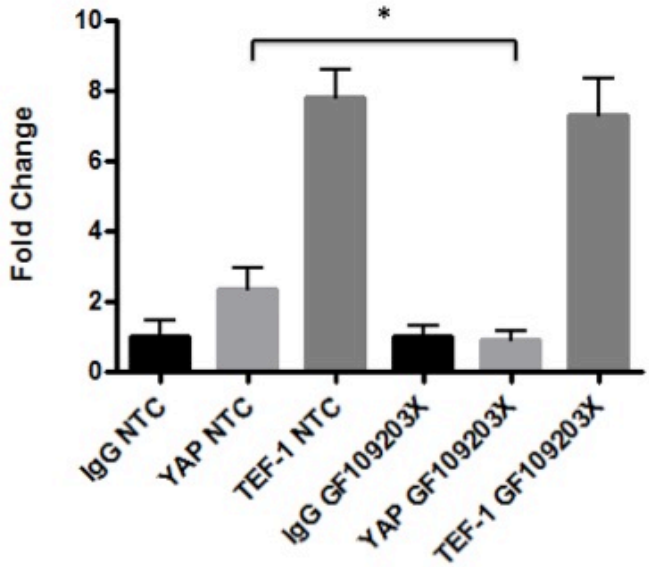
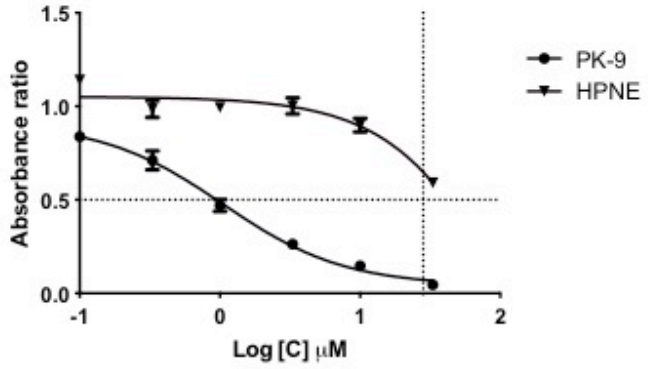


Figure 4b: Chromatin immunoprecipitation of YAP and TEF-1 at CTGF promoter. ChIP was performed using antibodies against YAP, TEF-1 and IgG. After DNA extraction, Real time PCR amplification was performed. The results were analyzed using IgG as a negative control and compared to not immunoprecipitated sample (INPUT).

5. GF 109203X TOXICITY ON PK-9 AND HPNE CELLS

5.1 GF 109203X exhibits IC50 value significantly higher on PK-9 cells then on HPNE immortalized cell line

Since the relevance of the identified compound in regulating YAP as a potential drug target, we decided to investigate whether GF 109203X could induce toxicity in PDAC cells. We performed a cell viability assay by incubating cells with WST. Figure 5.1 shows the toxicity curves on PK-9 and HPNE cells exposed to increasing doses of GF 109203X for 48 hours. The hTERT-HPNE cell line was developed from human pancreatic duct by transduction with a retroviral expression vector (pBABEpuro) containing the hTERT gene. hTERT-HPNE cells have properties of the intermediary cells formed during acinar-to-ductal metaplasia, which included their undifferentiated phenotype (Lee KM, et al. Biochem Biophys Res Commun 2003; 301(4):1038-44). The comparison between cancer cells and immortalized cells gave us a preliminary indication about the selectivity of the compound in affecting only cancer cells. Gemcitabine is the only drug used in PDAC treatment at the moment. It has an IC50 value of about 60nM in our cell line (data not shown). As reported in table, IC50 value of GF 109203X in PK-9 cells is in the low micromolar range, which can be considered an index of low toxicity compared to gemcitabine. Interestingly, the administration of GF 109203X did not cause toxic effect in HPNE cells at tested concentrations, underling chemical features exploitable for drug development



	PK-9	HPNE
IC50	1.051	163.0

	PK-9	HPNE
R square	0.9970	0.9217

Figure5.1: GF 109203X-induced cell toxicity. PK-9 and HPNE cells were treated with increasing dose of GF 109203X (from 0,1 to 33μM) for 48 hours.The cell viability assay was performed by incubating cells with WST for 4 hours. The absorbance ratio is the signal from treated cells normalized to the untreated control. The IC50 value was calculated by nonlinear regression, using Graphpad Prism software.

5.2 GF 109203X shows differential toxic effects on different PDAC cell lines

In order to have an overview of GF 109203X effect on PDAC cell lines, we extend the cell viability assay to MIA PaCa-2 and PANC-1 cells, as previously described. We reported in graph the values of cell viability after 48 hours treatment with GF 109203X 10μM (Figure 5.2a). We observed toxicity only in PK-9 cells that have shown the highest YAP expression, suggesting that GF 109203X mechanism of action could depend on the presence of YAP within cells. However this hypothesis has to be further explored, taking into account the genetic backgroundof the analyzed cell lines. Table 5.2b summarizes the most common mutated genes in PDAC and their state in PK-9 MIA PaCa-2 and PANC-1 cells, as reported in literature (Kobari M, et al. 1986; Deer EL, et al. 2010). The behavior of these cell lines is currently on study in our laboratory, in order to elucidate the cell specific response to GF 109203X treatment.

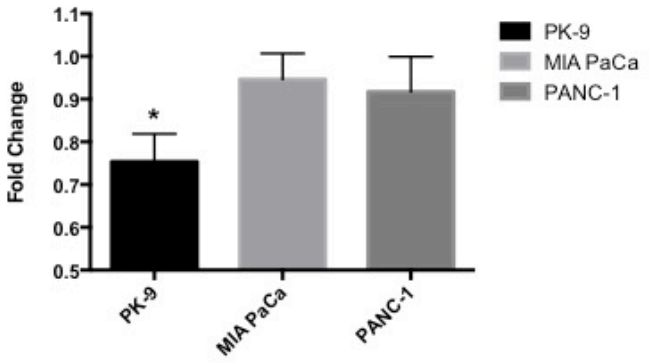


Figure 5.2a: Cell viability assay on different PDAC cell lines. The analyzed cell lines express YAP at differential levels: high (PK-9), low (MIA PaCa-2) and intermediate (PANC-1).Cells were treated with GF 109203X (10μM) for 48 hours and subsequently incubated with WST for 4 hours.

PDAC cell lines	KRAS	CDKN2A	TP53	SMAD4	YAP exp
PK-9	G12D	HD exons 1-3	R213X	HD exons1-11	++++
MIA PACA-2	G12D	HD	248W	WT	+
PANC-1	G12D	HD	273H	WT	++

Figure 5.2b: PDAC cell lines genotype. Summary ofthe mutations present in analyzed cell lines of the most representative genes in PDAC, and schematic representation of YAP expression.

DISCUSSION

This study allowed the identification of an inhibitor of YAP functionality, a small molecule called GF 10923X, an already known kinase inhibitor preferentially targeting PKCα. We characterized its molecular mechanism leading to YAP inhibition and this molecule, endowed with a brand new property, can now be thought as a potential lead to be used in diseases where YAP deregulation is observed, as may be pancreatic ductal adenocarcinoma. Here we reported lines of evidence demonstrating a new role of this compound in regulating YAP protein localization, thereby arresting YAP oncogenic activity in PDAC, *in vitro*. We developed a high throughput approach by which we identified this compound able to reduce YAP-induced proliferation and clonogenicity, despite it leads to increased YAP nuclear levels. The main inhibitory pathway of YAP protein is the Hippo pathway, that phosphorylates YAP at Serine127, causing its cytoplasmic accumulation and degradation (Zhao B et al. 2007). Together with the "classical" regulators, novel mechanisms have been recently reported. A novel cycle of YAP acetylation within the nucleus has been demonstrated following MMS-induced DNA damage (Hata S et al. 2012).GF 109203X-induced YAP nuclear accumulation is in line with the increased protein acetylation we observed after the treatment, further underlining the importance of post translational modifications (PTMs) in YAP localization and regulation. On the other hand we deviate from the simplistic association between YAP localization and its functionality, suggesting that its PTMs are crucial for the binding to its targets and thus for its regulation.

The best characterized genetic lesions in evolving pancreatic adenocarcinoma include mutations of K-RAS, TP53, CDKN2A, SMAD4 and BRCA2. Our model, PK-9 cell line, shows a peculiar genotype: point mutations in K-RAS (G12D) and P53 (R213X) genes and homozygous deletions at SMAD4 and CDKN2A. In particular K-RAS plays an important role in pancreatic cancer etiology and development (Sugio K, et al. 1997; Luttges J, et al. 1999);the p53 tumor suppressor gene is inactivated in 40% to 75% of pancreatic cancers (Pellegata NS, et al. 1994) and cooperates with K-RAS in tumorigenesis (Kalthoff H, et al. 1993). Likewise, DPC4 inactivation is always accompanied by inactivation of p16 (Cowgill SM and Muscarella P 2003), as observed in PK-9 cells. While loss of SMAD4/DPC4 interferes with the intracellular signaling cascades downstream from TGF-β and activin, resulting in decreased growth inhibition via

loss of proapoptotic signaling or inappropriate G1/S transition(Massague, J et al. 2000). In addition to these driving forces, the relevance of YAP in pancreatic cancer was suggested by J. Guo and colleagues, and confirmed in a recent publication by C. H. Diep (Guo J et al. 2006; Diep CH et al. 2012) in which they reported that YAP expression level results increased in PDAC in comparison to levels observed in chronic pancreatitis samples or in normal pancreatic tissues. Moreover YAP overexpression leads to immediate organomegaly followed by tumor formation in mouse model(Dong J, et al. 2007). Two distinct patterns of YAP distribution in tumor cells have been observed —with or without nuclear accumulation— implying different mechanisms by which Hippo signaling may be deregulated in cancer cells. Based on the mechanism of Yki/YAP inactivation by Hippo signaling, authors suggest that the former pattern could result from inactivation of tumor suppressors upstream of YAP, mutation of the S127 phosphorylation site, or perturbation of the nuclear-cytoplasmic shuttling machinery, whereas the latter pattern could be caused by YAP overabundance, either via gene amplification, increased transcription, or protein stabilization. In line with these findings,we reported YAP expression in all of the analyzed PDAC cell lines and YAP overexpression in 3 out of 7 cell lines. The stable presence of YAP is consistent with an important role of this protein in PDAC. Confirming this data, we observed that the functional ablation of YAP leads to an inhibition of cell proliferation and cell growth in anchorage-independent conditions at the exponential phase of growth in PK-9 cells. The controversial view of YAP as oncogene or tumor suppressor is underlined by its dual role in inhibiting apoptosis and promoting cell proliferation, and on the other hand in increasing the ability of p73 in inducing apoptosis (Blandino G et al. 2009). In our experimental conditions, we excluded the onset of apoptosis in YAP silenced cells after the confluence stage was reached. These data confirm cell density as a crucial parameter in YAP functionality and underline that reduction in cell proliferation is not necessary related with apoptosis.Additionally, the change in cellular shape and size suggested the induction of senescence, in line with the new finding that identified YAP as a regulator of this mechanism, through transcriptionally upregulating CDK6 expression (Xie Q et al. 2013). We are currently investigating this hypothesis in our model. The phenotypic effects we described in YAP silenced cells were observed also after GF 109203X

treatment and are tightly dependent on cellular confluence level. We reported a delay in cell proliferation and an inhibition of anchorage-independent growth only at the exponential phase of growth and at a cell density starting from fifty percent. The maintaining of these conditions, especially for screening development, has been crucial for evaluating the effects not dependent on changes in cell density.

The potential interaction with a number of genes that displays deregulated expression in PDAC point to an important role of YAP in modulating aberrant signaling pathways that contribute to the pathogenesis of this disease (Guo J et al. 2006). YAP is located within a network of multiple pathways acting upstream and downstream our protein. For example, YAP has been reported to bind to p53 binding protein-2 (p53BP-2), an important regulator of the apoptotic activity of p53(Strano S et al. 2001). In addition, other transcription factors could potentially be co-activated by YAP, including: AP2, C/EBPα, c-Jun, Krox-20, Krox-24, MEF2B, NF-E2, Oct-4 and p73 (see introduction).Finally, YAP can also interact with the transforming growth factor-beta (TGF-β)/Smad signaling pathway(Ferrigno O et al. 2002),mediating the nuclear accumulation of SMADs in response to TGF-β responsiveness to cell density(Varelas et al. 2008 and 2010). The capability of YAP to regulate a wide range of factors that are important in PDAC onset and development, makes it a critical factor in pancreatic cancer etiology. TEAD is the most potent YAP target, able to mediated YAP-induced cell growth and EMT in mammals. The connective tissue growth factor (CTGF) is a direct target gene of YAP and TEAD, playing an important role in growth-promoting function of YAP and its activity level is commonly used to evaluate YAP functionality (Zhao B et al. 2008). We reported that GF 109203X displaces YAP from the CTGF promoter region comprising the previously identified TEAD/YAP response elements (Zhang H et al. 2009), leading to a downregulation of CTGF expression. GF 109203X is a kinase inhibitor with specific affinity for PKC family members. In an attempt to identify the molecular target of GF 1093203X responsible of its effects on YAP functionality we silenced PKCα and evaluated CTGF expression level. The transient silencing of PKCα does not lead to a downregulation of CTGF expression, but preliminary data from real time PCR analysis shows that the silencing of PKCα correlates with an increase of CTGF expression, without

affecting YAP level. These results shows an involvement of PKCα in the modulation of CTGF expression level however they do not suggest that PKCα is the primary target of GF 109203X in the modulation of CTGF. The presence of a functional relationship between PKCα and YAP protein is still an open question. Future perspectives could be planned for further investigating the target of GF 109203X. For example an affinity chromatography linking GF 109203X to a resin could be useful for identifying the kinase able to bind our compound. The subsequent silencing of the target or of the putative targets in our cell model could be used in order to explore its role in affecting YAP PTMs and/or binding to the promoter regions. Since we have not observed any alterations in YAP phosphorylation at Serine127 in GF 109203X treated cells, we discarded the hypothesis of a regulation mediated by the Hippo pathway. Chromatin immunoprecipitation experiments allowed us to demonstrate that the identified compound interferes with YAP binding to CTGF promoter, without affecting the presence of TEAD at the same region. In summary, in the model proposed GF 109203X acts most likely affecting differential signaling pathways than the Hippo pathway to modulate YAP functionality. However it causes an alteration in YAP PTMs, in particular phosphorylation and acetylation, the latter leads to its nuclear retention. In this mechanism, the YAP activity is not correlated with its nuclear localization, but with its capacity to participate in the transcriptional complex formation at promoter regions. GF 109203X treatment prevents the binding of YAP to the CTGF promoter, thereby inhibiting CTGF expression. Biological effects of CTGF include stimulation of proliferation, angiogenesis, migration, ECM production, cell attachment, cell survival and in some cell types apoptosis (Perbal B et al. 2001). Moreover different studies have shown that CCN genes are involved in tumorigenesis, and that CTGF is overexpressed in pancreatic cancers (Wenger C et al. 1999) and melanomas (Kubo M et al. 1998), so understanding mechanisms of CCN gene inhibition may be useful in new strategies for cancer therapy. In this study we reported that the GF 109203X-mediated downregulation of CTGF, through YAP binding inhibition, leads to a delay in cell proliferation and a reduced anchorage-independent growth of pancreatic cancer cells at the exponential phase. Among the further investigations needed, a chemical optimization of the identified compound would be desirable in order to increase the potency of the compound

in decreasing CTGF expression levels. This would be important also to evaluate if the differential toxicity that we observed in between pancreatic cancer cells and immortalized HPNE cell lines is dependent on the inhibition of YAP functionality or rely alternative or nonspecific mechanisms. Taken all together, these data make GF 109203X a potential lead compound to be used in the drug development for YAP deregulated PDAC treatment.

In conclusion, in my PhD project we identified for the first time to our knowledge a compound able to displace the co-transcription factor YAP from the promoter region of the CTGF gene, an important YAP target gene, leading to an inhibition of cell proliferation and of the capability of PK-9 cells to grow in anchorage-independent conditions.

NOTES

In our laboratory, another PhD student is focused on testing the effect of GF 109203X and of three more chemical analogues on different PDAC cell lines. Her results regarding YAP nuclear retention, CTGF expression and cell toxicity are described, together with the data presented in this thesis, in an article recently submitted.

OTHER PROJECTS

In addition to my thesis project, I collaborated with other members of my laboratory in projects regarding the role of the RNA binding protein, HuR, in breast cancer cell lines. Article number one has been submitted (in attach), article number two is currently in preparation. The second article will be reported as attachment in the final thesis. Here I describe my specific contribution to both projects.

Article 1: Loss of PKCδ/HuR interaction is necessary to doxorubicin resistance in breast cancer cell lines

Elisa Latorre*[#], Ilaria Castiglioni*, Pamela Gatto[°], Stephana Carelli[#] Alessandro Quattrone[+], Alessandro Provenzani*

* Laboratory of Genomic Screening, Centre for Integrative Biology, University of Trento, Trento, Italy

[+] Laboratory of Translational Genomics, Centre for Integrative Biology, University of Trento, Italy

[#] Pharmacology Laboratory, Health Sciences Dept, University of Milan, Italy

[°] High Throughput Screening Facility, Centre for Integrative Biology, University of Trento, Italy

Here we demonstrated that the genotoxic stimulus induced by doxorubicin triggers PKCδ interaction with HuR and leads to HuR phosphorylation on serine 221 and 318 and cytoplasmic translocation. This series of events is crucial to elicit the death pathway triggered by doxorubicin and is also necessary to promote HuR function in post-transcriptional regulation of gene expression, since genetic ablation of PKCδ brought to HuR inability to bind its target mRNAs. PKCδ and HuR proteins were coordinately downregulated together with the HuR-regulated TOP2A mRNA, a doxorubicin target, in *in vitro* selected doxorubicin resistant human breast cancer cell lines upregulating the multidrug resistance marker ABCG2. Therefore, we show here that PKCδ, HuR and

TOP2A constitute a network mediating doxorubicin efficacy in breast cancer cells.

I participated evaluating the differential expression of the proteins of interest between wild type and doxorubicin resistant breast cancer cell lines, specifically I evaluated HuR, ABCG2, PKCδ, TOP2A proteins. Western blotting analysis was performed with a SDS-PAGE Electrophoresis System (Biorad) in two populations of doxorubicin-resistant breast cancer cells we previously developed (Latorre et al. 2012), MCF-7/doxoR and MDA-MD231/doxoR. I observed a downregulation of PKCδ in doxoR populations with respect to the parental doxorubicin-sensitive cells, as was HuR, while it did not occur in the SK-BR3/no-doxoR population used as negative control. Interestingly, TOP2A protein level decreased significantly in breast cancer resistant cells that showed low HuR expression level consistently with what we observed in the stably HuR silenced MCF-7 cells. Moreover, the ABCG2 transporter, a marker for the doxorubicin resistance phenotype, was found to be overexpressed, compared to parental cells, in the MCF-7/doxoR and in the MDA-MD231/doxoR but not in the SK-BR3/no-doxoR strain.

Article 2: HuR and MALAT1: a new ribonucleic complex regulating cancer staminality in human breast tumor

Elisa Latorre PhD[§#], Stephana Carelli[*#], Ilaria Castiglioni[*§], Vito D'Agostino PhD[§], Giacomina Moro MD°, Andrea Luciani MD PhD[#], Giorgio Ghilardi MD[#], Annamaria Di Giulio PhD[#], Alfredo Gorio PhD[#] and Alessandro Provenzani PhD[§]

§ University of Trento, Center for Integrative Biology, Genome screening laboratory - Trento (ITALY)

University of Milan, Department of Health Sciences, Laboratory of Pharmacology - Milan (ITALY)

° San Paolo Hospital - Milan (ITALY)

(*)These authors contributed equally to this work

In the second project, we showed a new gene expression regulatory mechanism in which the lincRNA, MALAT1, cooperates with the RNA binding protein, HuR, and the histone de-acetylase, HDAC6, into a new ribonucleoprotein complex playing an important role in gene expression of cancer stem cells (CSCs) markers. In order to identify where and if HuR was bound to MALAT1 and to the chromatin, I screened different chromatin regions in the lincRNA regulated genes by chromatin immunoprecipitation assay (ChIP) looking for regions where HuR was bound. I identified, within the CD133 locus, a strong HuR binding signal, we called D-region. HDAC6 is a well known de-acetylase that possesses histone deacetylase activity and represses transcription in particular of CD133 gene (Pellacani et al. 2011 Mol. Cancer 10; 94-108). I also confirmed that in our cellular model both HuR and HDAC6 are present on the CD133 D-region (Figure 1). Finally I performed a ChIP experiment in the presence or absence of RNase, concluding that the HuR binding to the chromatin is dependent on the presence of RNA (figure 2).

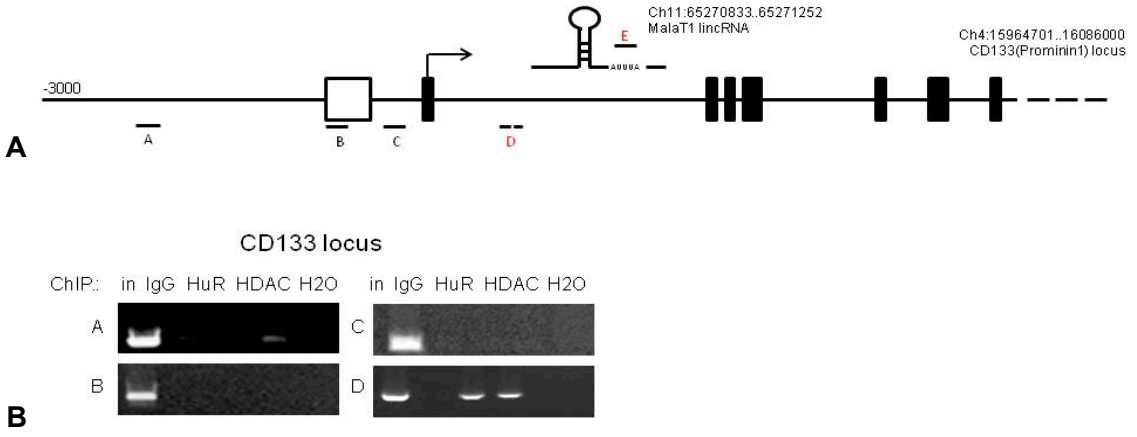
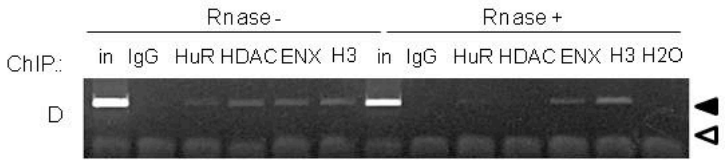


Figure 1. Ribonucleoprotein complex HuR-HDAC6 binds to Prominin (CD133) gene intronic regulatory region in mammospheres. A. Map of prominin 1 locus organization**.** Black and white boxes indicate exons. The small black bars named from A to D are the regions previously investigated by ChIP. The RNA hairpin is representative of MALAT1 lincRNA and the small black bar named E is the regions investigated by RIP for the binding of HuR and HDAC6. **B.** ChIP analysis on the Prominin1 locus in mammospheres. The results are reported as semiquantitative PCR on samples derived from immuneprecipitation with anti-HuR or anti-HDAC6 antibodies. Input is the positive control, IgG is the negative one. The different primer pairs are named with letters (A-D) matching the map in panel A. A strong positive signal is reported on the D region for both anti-HuR or anti-HDAC6 antibodies.



Fugure 2. RNA depended ChIP analysis on the D region of Prominin1 locus. The results are reported as semiquantitative PCR on samples derived from the immuneprecipitation with anti-HuR, anti-HDAC6 or anti-ENX antibodies in presence or absence of RNAase A pretreatment (with + and without -). Input is the PCR positive control, IgG and H3 are respectively the negative and the positive ChIP controls. H2O is the control without DNA. The white harrow indicates the self annealing primers band, the black one points to the PCR positive signal band.

We therefore analyzed in details the proliferative and adhesive capability of mammospheres in the absence of HuR *in vivo* and *in vitro*. I demonstrated that in the absence of anchorage HuR KD mammosphers showed impaired growth, as they formed less colonies if cultured in soft agar (Figure 3a) and I observed less proliferative behavior of HuR KD mammospheres after 8 passages (Figure 3b).

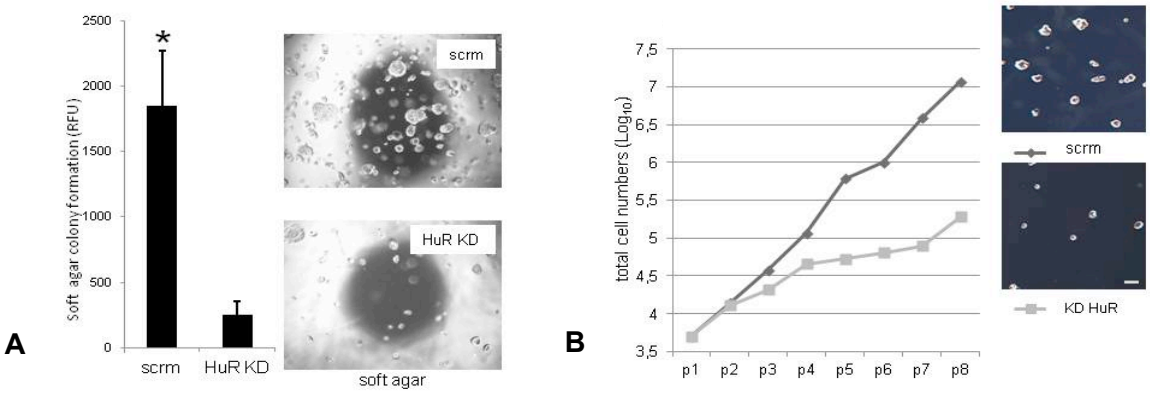


Figure 3.HuR knocked down mammospheres cellular characterization.A. Colony formation in anchorage independent conditions. Scramble (scrm) and HuR KD mammospheres were seeded in soft agar. The graph on the left shows the quantification of the relative fluorescent units (RFU). Pictures on the right show the colonies ten days after the cell seeding. **B.**Cell growth curves of scrambled (scrm) and HuR silenced (HuR KD) mammospheres, reported as total cell number counted at each culture passage (p1-p8). Pictures on the right show scrambled and HuR KD mammospheres from p2 culture passage in direct light. Scale bar correspond to 100um.

REFERENCES

Abrams, R. A. (2012). Radiotherapy in the adjuvant management of pancreatic adenocarcinoma: is it helpful? *Expert review of gastroenterology & hepatology*, *6*(2), 149–61. doi:10.1586/egh.12.3

Adler, J. J., Heller, B. L., Bringman, L. R., Ranahan, W. P., Cocklin, R. R., Goebl, M. G., … Wells, C. D. (2013). Amot130 adapts atrophin-1 interacting protein 4 to inhibit yes-associated protein signaling and cell growth. *The Journal of biological chemistry*, *288*(21), 15181–93. doi:10.1074/jbc.M112.446534

Aikawa, T., Gunn, J., Spong, S. M., Klaus, S. J., & Korc, M. (2006). Connective tissue growth factor-specific antibody attenuates tumor growth, metastasis, and angiogenesis in an orthotopic mouse model of pancreatic cancer. *Molecular cancer therapeutics*, *5*(5), 1108–16. doi:10.1158/1535-7163.MCT-05-0516

Alarcón, C., Zaromytidou, A.-I., Xi, Q., Gao, S., Yu, J., Fujisawa, S., … Massagué, J. (2009). Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways. *Cell*, *139*(4), 757–69.doi:10.1016/j.cell.2009.09.035

Alessi, D. R. (1997). The protein kinase C inhibitors Ro 318220 and GF 109203X are equally potent inhibitors of MAPKAP kinase-1beta (Rsk-2) and p70 S6 kinase. *FEBS letters*, *402*(2-3), 121–3. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9037179

Anderson K, Potter JD, Mack TM. Pancreatic cancer. In: Schottenfeld D, Fraumeni JF, editors. Cancer Epidemiology and Prevention. New York: Oxford University Press, 2006:721-62

Arslan, A. A., Helzlsouer, K. J., Kooperberg, C., Shu, X.-O., Steplowski, E., Bueno-de-Mesquita, H. B., … Lacroix, A. Z. (2010). Anthropometric measures, body mass index, and pancreatic cancer: a pooled analysis from the Pancreatic Cancer Cohort Consortium (PanScan). *Archives of internal medicine*, *170*(9), 791–802. doi:10.1001/archinternmed.2010.63

Bao, Y., Nakagawa, K., Yang, Z., Ikeda, M., Withanage, K., Ishigami-Yuasa, M., … Hata, Y. (2011).A cell-based assay to screen stimulators of the Hippo pathway reveals the inhibitory effect of dobutamine on the YAP-dependent gene transcription. *Journal of biochemistry*, *150*(2), 199–208. doi:10.1093/jb/mvr063

Bashyam, M. D., Bair, R., Kim, Y. H., Wang, P., Hernandez-Boussard, T., Karikari, C. A., … Pollack, J. R. (2005).Array-based comparative genomic hybridization identifies localized DNA amplifications and homozygous deletions in pancreatic cancer. *Neoplasia (New York, N.Y.)*, *7*(6), 556–62. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1501288&tool=pmcentrez&rendertype=abstract

Basu, S., Totty, N. F., Irwin, M. S., Sudol, M., & Downward, J. (2003). Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. *Molecular cell*, *11*(1), 11–23. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12535517

Beltman, J., McCormick, F., & Cook, S. J. (1996). The selective protein kinase C inhibitor, Ro-31-8220, inhibits mitogen-activated protein kinase phosphatase-1 (MKP-1) expression, induces c-Jun expression, and activates Jun N-terminal kinase. *The Journal of biological chemistry*, *271*(43), 27018–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8900190

Bennewith, K. L., Huang, X., Ham, C. M., Graves, E. E., Erler, J. T., Kambham, N., … Giaccia, A. J. (2009). The role of tumor cell-derived connective tissue growth factor (CTGF/CCN2) in pancreatic tumor growth.*Cancer research*, *69*(3), 775–84. doi:10.1158/0008-5472.CAN-08-0987

Berman, D. B., Costalas, J., Schultz, D. C., Grana, G., Daly, M., & Godwin, A. K. (1996). A common mutation in BRCA2 that predisposes to a variety of cancers is found in both Jewish Ashkenazi and non-Jewish individuals. *Cancer research*, *56*(15), 3409–14. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8758903

Berrington de Gonzalez, A., Sweetland, S., & Spencer, E. (2003).A meta-analysis of obesity and the risk of pancreatic cancer.*British journal of cancer*, *89*(3), 519–23. doi:10.1038/sj.bjc.6601140

Bertini, E., Oka, T., Sudol, M., Strano, S., & Blandino, G. (2009). YAP: at the crossroad between transformation and tumor suppression.*Cell cycle (Georgetown, Tex.)*, *8*(1), 49–57. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19106601

Bosetti, C., Lucenteforte, E., Silverman, D. T., Petersen, G., Bracci, P. M., Ji, B. T., … La Vecchia, C. (2012). Cigarette smoking and pancreatic cancer: an analysis from the International Pancreatic Cancer Case-Control Consortium (Panc4). *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*, *23*(7), 1880–8. doi:10.1093/annonc/mdr541

Brigstock, D. R. (1999). The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. *Endocrine reviews*, *20*(2), 189–206. doi:10.1210/edrv.20.2.0360

Budworth, J., Davies, R., Malkhandi, J., Gant, T. W., Ferry, D. R., & Gescher, A. (1996). Comparison of staurosporine and four analogues: their effects on growth, rhodamine 123 retention and binding to P-glycoprotein in multidrug-resistant MCF-7/Adr cells. *British journal of cancer*, *73*(9), 1063–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2074394&tool=pmcentrez&rendertype=abstract

Caldas, C., Hahn, S. A., Hruban, R. H., Redston, M. S., Yeo, C. J., & Kern, S. E. (1994). Detection of K-ras mutations in the stool of patients with pancreatic adenocarcinoma and pancreatic ductal hyperplasia.*Cancer research*, *54*(13), 3568–73. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8012983

Camargo, F. D., Gokhale, S., Johnnidis, J. B., Fu, D., Bell, G. W., Jaenisch, R., & Brummelkamp, T. R. (2007). YAP1 increases organ size and expands undifferentiated progenitor cells. *Current biology : CB*, *17*(23), 2054–60. doi:10.1016/j.cub.2007.10.039

Chan, S. W., Lim, C. J., Chong, Y. F., Pobbati, A. V, Huang, C., & Hong, W. (2011). Hippo pathway-independent restriction of TAZ and YAP by angiomotin.*The Journal of biological chemistry*, *286*(9), 7018–26. doi:10.1074/jbc.C110.212621

Cho, M., Park, S., Gwak, J., Kim, D.-E., Yea, S. S., Shin, J.-G., & Oh, S. (2008). Bisindoylmaleimide I suppresses adipocyte differentiation through stabilization of intracellular beta-catenin protein. *Biochemical and biophysical research communications*, *367*(1), 195–200. doi:10.1016/j.bbrc.2007.12.147

Cordenonsi, M., Zanconato, F., Azzolin, L., Forcato, M., Rosato, A., Frasson, C., … Piccolo, S. (2011).The Hippo transducer TAZ confers cancer stem cell-related traits on breast cancer cells. *Cell*, *147*(4), 759–72.doi:10.1016/j.cell.2011.09.048

Courage, C., Budworth, J., & Gescher, A. (1995).Comparison of ability of protein kinase C inhibitors to arrest cell growth and to alter cellular protein kinase C localisation.*British journal of cancer*, *71*(4), 697–704. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2033742&tool=pmcentrez&rendertype=abstract

Courage, C., Snowden, R., & Gescher, A. (1996).Differential effects of staurosporine analogues on cell cycle, growth and viability in A549 cells.*British journal of cancer*, *74*(8), 1199–205. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2075912&tool=pmcentrez&rendertype=abstract

Crane, C. H., Varadhachary, G. R., Yordy, J. S., Staerkel, G. A., Javle, M. M., Safran, H., … Wolff, R. A. (2011). Phase II trial of cetuximab, gemcitabine, and oxaliplatin followed by chemoradiation with cetuximab for locally advanced (T4) pancreatic adenocarcinoma: correlation of Smad4(Dpc4) immunostaining with pattern of disease progression. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, *29*(22), 3037–43. doi:10.1200/JCO.2010.33.8038

Crawford, L. A., Guney, M. A., Oh, Y. A., Deyoung, R. A., Valenzuela, D. M., Murphy, A. J., … Gannon, M. (2009). Connective tissue growth factor (CTGF) inactivation leads to defects in islet cell lineage allocation and beta-cell proliferation during embryogenesis. *Molecular endocrinology (Baltimore, Md.)*, *23*(3), 324–36. doi:10.1210/me.2008-0045

Cubilla, A. L., & Fitzgerald, P. J. (1976). Morphological lesions associated with human primary invasive nonendocrine pancreas cancer. *Cancer research*, *36*(7 PT 2), 2690–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1277176

Davis, P. D., Hill, C. H., Keech, E., Lawton, G., Nixon, J. S., Sedgwick, A. D., … Wilkinson, S. E. (1989). Potent selective inhibitors of protein kinase C. *FEBS letters*, *259*(1), 61–3.Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2532156

Deer, E. L., González-Hernández, J., Coursen, J. D., Shea, J. E., Ngatia, J., Scaife, C. L., … Mulvihill, S. J. (2010). Phenotype and genotype of pancreatic cancer cell lines. *Pancreas*, *39*(4), 425–35.doi:10.1097/MPA.0b013e3181c15963

Diep, C. H., Zucker, K. M., Hostetter, G., Watanabe, A., Hu, C., Munoz, R. M., … Han, H. (2012). Down-regulation of Yes Associated Protein 1 expression reduces cell proliferation and clonogenicity of pancreatic cancer cells. *PloS one*, *7*(3), e32783. doi:10.1371/journal.pone.0032783

Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S. A., … Pan, D. (2007). Elucidation of a universal size-control mechanism in Drosophila and mammals.*Cell*, *130*(6), 1120–33. doi:10.1016/j.cell.2007.07.019

Dornhöfer, N., Spong, S., Bennewith, K., Salim, A., Klaus, S., Kambham, N., … Giaccia, A. (2006).Connective tissue growth factor-specific monoclonal antibody therapy inhibits pancreatic tumor growth and metastasis. *Cancer research*, *66*(11), 5816–27. doi:10.1158/0008-5472.CAN-06-0081

Eagle, H., & Levine, E. M. (1967).Growth regulatory effects of cellular interaction.*Nature*, *213*(5081), 1102–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6029791

Eheman, C., Henley, S. J., Ballard-Barbash, R., Jacobs, E. J., Schymura, M. J., Noone, A.-M., … Edwards, B. K. (2012). Annual Report to the Nation on the status of cancer, 1975-2008, featuring cancers associated with excess weight and lack of sufficient physical activity. *Cancer*, *118*(9), 2338–66.doi:10.1002/cncr.27514

Ferlay, J., Shin, H.-R., Bray, F., Forman, D., Mathers, C., & Parkin, D. M. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International journal of cancer. Journal international du cancer*, *127*(12), 2893–917. doi:10.1002/ijc.25516

Ferrigno, O., Lallemand, F., Verrecchia, F., L'Hoste, S., Camonis, J., Atfi, A., & Mauviel, A. (2002). Yes-associated protein (YAP65) interacts with Smad7 and potentiates its inhibitory activity against TGF-beta/Smad signaling. *Oncogene*, *21*(32), 4879–84.doi:10.1038/sj.onc.1205623

Fujii, M., Lyakh, L. A., Bracken, C. P., Fukuoka, J., Hayakawa, M., Tsukiyama, T., … Roberts, A. B. (2006).SNIP1 is a candidate modifier of the transcriptional activity of c-Myc on E box-dependent target genes. *Molecular cell*, *24*(5), 771–83. doi:10.1016/j.molcel.2006.11.006

Fujii, M., Nakanishi, H., Toyoda, T., Tanaka, I., Kondo, Y., Osada, H., & Sekido, Y. (2012).Convergent signaling in the regulation of connective tissue growth factor in malignant mesothelioma: TGFβ signaling and defects in the Hippo signaling cascade. *Cell cycle (Georgetown, Tex.)*, *11*(18), 3373–9.doi:10.4161/cc.21397

Fujii, M., Toyoda, T., Nakanishi, H., Yatabe, Y., Sato, A., Matsudaira, Y., … Sekido, Y. (2012).TGF-β synergizes with defects in the Hippo pathway to stimulate human malignant mesothelioma growth. *The Journal of experimental medicine*, *209*(3), 479–94. doi:10.1084/jem.20111653

Gekeler, V., Boer, R., Ise, W., Sanders, K. H., Schächtele, C., & Beck, J. (1995). The specific bisindolylmaleimide PKC-inhibitor GF 109203X efficiently modulates MRP-associated multiple drug resistance. *Biochemical and biophysical research communications*, *206*(1), 119–26. doi:10.1006/bbrc.1995.1017

Gekeler, V., Boer, R., Uberall, F., Ise, W., Schubert, C., Utz, I., … Grunicke, H. (1996).Effects of the selective bisindolylmaleimide protein kinase C inhibitor GF 109203X on P-glycoprotein-mediated multidrug resistance. *British journal of cancer*, *74*(6), 897–905. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2074754&tool=pmcentrez&rendertype=abstract

Genevet, A., Wehr, M. C., Brain, R., Thompson, B. J., & Tapon, N. (2010). Kibra is a regulator of the Salvador/Warts/Hippo signaling network. *Developmental cell*, *18*(2), 300–8. doi:10.1016/j.devcel.2009.12.011

George, N. M., Day, C. E., Boerner, B. P., Johnson, R. L., & Sarvetnick, N. E. (2012). Hippo signaling regulates pancreas development through inactivation of Yap.*Molecular and cellular biology*, *32*(24), 5116–28. doi:10.1128/MCB.01034-12

Gong, J., Traganos, F., & Darzynkiewicz, Z. (1994). Staurosporine blocks cell progression through G1 between the cyclin D and cyclin E restriction points. *Cancer research*, *54*(12), 3136–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8205531

Goode, N., Hughes, K., Woodgett, J. R., & Parker, P. J. (1992). Differential regulation of glycogen synthase kinase-3 beta by protein kinase C isotypes.*The Journal of biological chemistry*, *267*(24), 16878–82. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1324914

Goulev, Y., Fauny, J. D., Gonzalez-Marti, B., Flagiello, D., Silber, J., & Zider, A. (2008). SCALLOPED interacts with YORKIE, the nuclear effector of the hippo tumor-suppressor pathway in Drosophila. *Current biology : CB*, *18*(6), 435–41. doi:10.1016/j.cub.2008.02.034

Greenhalf, W., Grocock, C., Harcus, M., & Neoptolemos, J. (2009). Screening of high-risk families for pancreatic cancer. *Pancreatology : official journal of the International Association of Pancreatology (IAP) ... [et al.]*, *9*(3), 215–22. doi:10.1159/000210262

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646–74.doi:10.1016/j.cell.2011.02.013

Hao, Y., Chun, A., Cheung, K., Rashidi, B., & Yang, X. (2008). Tumor suppressor LATS1 is a negative regulator of oncogene YAP. *The Journal of biological chemistry*, *283*(9), 5496–509. doi:10.1074/jbc.M709037200

Hata, S., Hirayama, J., Kajiho, H., Nakagawa, K., Hata, Y., Katada, T., … Nishina, H. (2012).A novel acetylation cycle of transcription co-activator Yes-associated protein that is downstream of Hippo pathway is triggered in response to SN2 alkylating agents. *The Journal of biological chemistry*, *287*(26), 22089–98. doi:10.1074/jbc.M111.334714

Heldin, C. H., Miyazono, K., & ten Dijke, P. (1997).TGF-beta signalling from cell membrane to nucleus through SMAD proteins.*Nature*, *390*(6659), 465–71.doi:10.1038/37284

Hers, I., Tavaré, J. M., & Denton, R. M. (1999). The protein kinase C inhibitors bisindolylmaleimide I (GF 109203x) and IX (Ro 31-8220) are potent inhibitors of glycogen synthase kinase-3 activity. *FEBS letters*, *460*(3), 433–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10556511

Hoshino, M., Qi, M., Yoshimura, N., Miyashita, T., Tagawa, K., Wada, Y., … Okazawa, H. (2006). Transcriptional repression induces a slowly progressive atypical neuronal death associated with changes of YAP isoforms and p73. *The Journal of cell biology*, *172*(4), 589–604. doi:10.1083/jcb.200509132

Hruban, R. H., Adsay, N. V, Albores-Saavedra, J., Compton, C., Garrett, E. S., Goodman, S. N., … Offerhaus, G. J. (2001). Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. *The American journal of surgical pathology*, *25*(5), 579–86. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11342768

Huang, J., Wu, S., Barrera, J., Matthews, K., & Pan, D. (2005). The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP.*Cell*, *122*(3), 421–34. doi:10.1016/j.cell.2005.06.007

Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M., & Miyazono, K. (1997). Smad6 inhibits signalling by the TGF-beta superfamily. *Nature*, *389*(6651), 622–6.doi:10.1038/39355

Iodice, S., Gandini, S., Maisonneuve, P., & Lowenfels, A. B. (2008). Tobacco and the risk of pancreatic cancer: a review and meta-analysis. *Langenbeck's archives of surgery / Deutsche Gesellschaft für Chirurgie*, *393*(4), 535–45. doi:10.1007/s00423-007-0266-2

Jones, S., Zhang, X., Parsons, D. W., Lin, J. C., Leary, R. J., Angenendt, P., … Kinzler, K. W. (2010). NIH Public Access, *321*(5897), 1801–1806. doi:10.1126/science.1164368.Core

Kalthoff, H., Schmiegel, W., Roeder, C., Kasche, D., Schmidt, A., Lauer, G., … Riethmüller, G. (1993).p53 and K-RAS alterations in pancreatic epithelial cell lesions. *Oncogene*, *8*(2), 289–98. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8426738

Klein, W. M., Hruban, R. H., Klein-Szanto, A. J. P., & Wilentz, R. E. (2002). Direct correlation between proliferative activity and dysplasia in pancreatic intraepithelial neoplasia (PanIN): additional evidence for a recently proposed model of progression. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*, *15*(4), 441–7. doi:10.1038/modpathol.3880544

Kobari, M., Hisano, H., Matsuno, S., Sato, T., Kan, M., & Tachibana, T. (1986). Establishment of six human pancreatic cancer cell lines and their sensitivities to anti-tumor drugs.*The Tohoku journal of experimental medicine*, *150*(3), 231–48. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3547771

Komuro, A., Nagai, M., Navin, N. E., & Sudol, M. (2003).WW domain-containing protein YAP associates with ErbB-4 and acts as a co-transcriptional activator for the carboxyl-terminal fragment of ErbB-4 that translocates to the nucleus. *The Journal of biological chemistry*, *278*(35), 33334–41. doi:10.1074/jbc.M305597200

Koorstra, J.-B. M., Hustinx, S. R., Offerhaus, G. J. A., & Maitra, A. (2008). Pancreatic carcinogenesis. *Pancreatology : official journal of the International Association of Pancreatology (IAP) ... [et al.]*, *8*(2), 110–25. doi:10.1159/000123838

Korc, M. (2007). Pancreatic cancer-associated stroma production. *American journal of surgery*, *194*(4 Suppl), S84–6. doi:10.1016/j.amjsurg.2007.05.004

Kubo, M., Kikuchi, K., Nashiro, K., Kakinuma, T., Hayashi, N., Nanko, H., & Tamaki, K. (1998).Expression of fibrogenic cytokines in desmoplastic malignant melanoma.*The British journal of dermatology*, *139*(2), 192–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9767231

Kwon, S., Munroe, X., Crawley, S. C., Lee, H. Y., Spong, S., Bradham, D., … Kim, Y. S. (2007). Expression of connective tissue growth factor in pancreatic cancer cell lines.*International journal of oncology*, *31*(4), 693–703. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17786299

Lai, D., Ho, K. C., Hao, Y., & Yang, X. (2011).Taxol resistance in breast cancer cells is mediated by the hippo pathway component TAZ and its downstream transcriptional targets Cyr61 and CTGF. *Cancer research*, *71*(7), 2728–38. doi:10.1158/0008-5472.CAN-10-2711

Lapi, E., Di Agostino, S., Donzelli, S., Gal, H., Domany, E., Rechavi, G., … Blandino, G. (2008). PML, YAP, and p73 are components of a proapoptotic autoregulatory feedback loop. *Molecular cell*, *32*(6), 803–14. doi:10.1016/j.molcel.2008.11.019

Lee, K. M., Nguyen, C., Ulrich, A. B., Pour, P. M., & Ouellette, M. M. (2003). Immortalization with telomerase of the Nestin-positive cells of the human pancreas.*Biochemical and biophysical research communications*, *301*(4), 1038–44. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12589817

Lei, Q.-Y., Zhang, H., Zhao, B., Zha, Z.-Y., Bai, F., Pei, X.-H., … Guan, K.-L. (2008). TAZ promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway. *Molecular and cellular biology*, *28*(7), 2426–36. doi:10.1128/MCB.01874-07

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell*, *88*(3), 323–31. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9039259

Liu, A. M., Xu, M. Z., Chen, J., Poon, R. T., & Luk, J. M. (2010). Targeting YAP and Hippo signaling pathway in liver cancer. *Expert opinion on therapeutic targets*, *14*(8), 855–68. doi:10.1517/14728222.2010.499361

Liu, C.-Y., Zha, Z.-Y., Zhou, X., Zhang, H., Huang, W., Zhao, D., … Guan, K.-L. (2010). The hippo tumor pathway promotes TAZ degradation by phosphorylating a phosphodegron and recruiting the SCF{beta}-TrCP E3 ligase. *The Journal of biological chemistry*, *285*(48), 37159–69. doi:10.1074/jbc.M110.152942

Lu, L., Li, Y., Kim, S. M., Bossuyt, W., Liu, P., Qiu, Q., … Johnson, R. L. (2010).Hippo signaling is a potent in vivo growth and tumor suppressor pathway in the mammalian liver. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(4), 1437–42. doi:10.1073/pnas.0911427107

Lüttges, J., Schlehe, B., Menke, M. A., Vogel, I., Henne-Bruns, D., & Klöppel, G. (1999). The K-ras mutation pattern in pancreatic ductal adenocarcinoma usually is identical to that in associated normal, hyperplastic, and metaplastic ductal epithelium. *Cancer*, *85*(8), 1703–10. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10223563

Lynch, H. T., Smyrk, T., Kern, S. E., Hruban, R. H., Lightdale, C. J., Lemon, S. J., … Ghadirian, P. (1996). Familial pancreatic cancer: a review. *Seminars in oncology*, *23*(2), 251–75. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8623061

Martin-Belmonte, F., & Perez-Moreno, M. (2012). Epithelial cell polarity, stem cells and cancer.*Nature reviews. Cancer*, *12*(1), 23–38. doi:10.1038/nrc3169

Massagué, J., Seoane, J., & Wotton, D. (2005). Smad transcription factors.*Genes& development*, *19*(23), 2783–810. doi:10.1101/gad.1350705

McCormick, F. (1995). Ras-related proteins in signal transduction and growth control.*Molecular reproduction and development*, *42*(4), 500–6. doi:10.1002/mrd.1080420419

Merritt, J. E., Sullivan, J. A., Drew, L., Khan, A., Wilson, K., Mulqueen, M., … Warr, R. (1999). The bisindolylmaleimide protein kinase C inhibitor, Ro 32-2241, reverses multidrug resistance in KB tumour cells. *Cancer chemotherapy and pharmacology*, *43*(5), 371–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10100591

Meszoely, I. M., Means, A. L., Scoggins, C. R., & Leach, S. D. (n.d.). Developmental aspects of early pancreatic cancer.*Cancer journal (Sudbury, Mass.)*, *7*(4), 242–50. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11561600

Mo, J.-S., Yu, F.-X., Gong, R., Brown, J. H., & Guan, K.-L. (2012). Regulation of the Hippo-YAP pathway by protease-activated receptors (PARs).*Genes& development*, *26*(19), 2138–43. doi:10.1101/gad.197582.112

Morin-Kensicki, E. M., Boone, B. N., Howell, M., Stonebraker, J. R., Teed, J., Alb, J. G., … Milgram, S. L. (2006). Defects in yolk sac vasculogenesis, chorioallantoic fusion, and embryonic axis elongation in mice with targeted disruption of Yap65. *Molecular and cellular biology*, *26*(1), 77–87. doi:10.1128/MCB.26.1.77-87.2006

Moskaluk, C. A., Hruban, R. H., & Kern, S. E. (1997). p16 and K-ras gene mutations in the intraductal precursors of human pancreatic adenocarcinoma. *Cancer research*, *57*(11), 2140–3. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9187111

Naderi, A., & Couch, F. J. (2002). BRCA2 and pancreatic cancer.*International journal of gastrointestinal cancer*, *31*(1-3), 99–106. doi:10.1385/IJGC:31:1-3:99

Neoptolemos, J. P. (2011). Adjuvant treatment of pancreatic cancer.*European journal of cancer (Oxford, England : 1990)*, *47 Suppl 3*, S378–80. doi:10.1016/S0959-8049(11)70210-6

Nishihara, A., Hanai, J. I., Okamoto, N., Yanagisawa, J., Kato, S., Miyazono, K., & Kawabata, M. (1998).Role of p300, a transcriptional coactivator, in signalling of TGF-beta.*Genes to cells : devoted to molecular & cellular mechanisms*, *3*(9), 613–23. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9813111

Norbury, C., & Nurse, P. (1992). Animal cell cycles and their control.*Annual review of biochemistry*, *61*, 441–70. doi:10.1146/annurev.bi.61.070192.002301

Okada, T., Lopez-Lago, M., & Giancotti, F. G. (2005). Merlin/NF-2 mediates contact inhibition of growth by suppressing recruitment of Rac to the plasma membrane. *The Journal of cell biology*, *171*(2), 361–71. doi:10.1083/jcb.200503165

Overholtzer, M., Zhang, J., Smolen, G. A., Muir, B., Li, W., Sgroi, D. C., … Haber, D. A. (2006). Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(33), 12405–10.doi:10.1073/pnas.0605579103

Paramasivam, M., Sarkeshik, A., Yates, J. R., Fernandes, M. J. G., & McCollum, D. (2011). Angiomotin family proteins are novel activators of the LATS2 kinase tumor suppressor. *Molecular biology of the cell*, *22*(19), 3725–33.doi:10.1091/mbc.E11-04-0300

Pellegata, N. S., Sessa, F., Renault, B., Bonato, M., Leone, B. E., Solcia, E., & Ranzani, G. N. (1994). K-ras and p53 gene mutations in pancreatic cancer: ductal and nonductal tumors progress through different genetic lesions. *Cancer research*, *54*(6), 1556–60. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8137263

Perbal, B. (2001). The CCN family of genes: a brief history. *Molecular pathology : MP*, *54*(2), 103–4. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1187011&tool=pmcentrez&rendertype=abstract

Pickart, C. M. (2001).Mechanisms underlying ubiquitination.*Annual review of biochemistry*, *70*, 503–33. doi:10.1146/annurev.biochem.70.1.503

Pickles, M., & Leask, A. (2007). Analysis of CCN2 promoter activity in PANC-1 cells: regulation by ras/MEK/ERK. *Journal of cell communication and signaling*, *1*(2), 85–90. doi:10.1007/s12079-007-0008-9

Pictet, R. L., Clark, W. R., Williams, R. H., & Rutter, W. J. (1972). An ultrastructural analysis of the developing embryonic pancreas.*Developmental biology*, *29*(4), 436–67. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4570759

Prado, C. L., Pugh-Bernard, A. E., Elghazi, L., Sosa-Pineda, B., & Sussel, L. (2004). Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(9), 2924–9. doi:10.1073/pnas.0308604100

Rachfal, A. W., & Brigstock, D. R. (2005). Structural and functional properties of CCN proteins.*Vitamins and hormones*, *70*, 69–103. doi:10.1016/S0083-6729(05)70003-0

Roberts, A. B., & Wakefield, L. M. (2003). The two faces of transforming growth factor beta in carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(15), 8621–3.doi:10.1073/pnas.1633291100

Roberts, P. J., & Der, C. J. (2007). Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene*, *26*(22), 3291–310.doi:10.1038/sj.onc.1210422

Rozengurt, E. (1986). Early signals in the mitogenic response.*Science (New York, N.Y.)*, *234*(4773), 161–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3018928

Ryu, B., Jones, J., Hollingsworth, M. A., Hruban, R. H., & Kern, S. E. (2001). Invasion-specific genes in malignancy: serial analysis of gene expression comparisons of primary and passaged cancers. *Cancer research*, *61*(5), 1833–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11280733

Schlegelmilch, K., Mohseni, M., Kirak, O., Pruszak, J., Rodriguez, J. R., Zhou, D., … Camargo, F. D. (2011).Yap1 acts downstream of α-catenin to control epidermal proliferation.*Cell*, *144*(5), 782–95. doi:10.1016/j.cell.2011.02.031

Schutte, M., Hruban, R. H., Geradts, J., Maynard, R., Hilgers, W., Rabindran, S. K., … Herman, J. G. (1997).Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas.*Cancer research*, *57*(15), 3126–30. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9242437

Sellers, W. R., Rodgers, J. W., & Kaelin, W. G. (1995). A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(25), 11544–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=40438&tool=pmcentrez&rendertype=abstract

Shin, E. J., & Canto, M. I. (2012).Pancreatic cancer screening.*Gastroenterology clinics of North America*, *41*(1), 143–57. doi:10.1016/j.gtc.2011.12.001

Song, H., Mak, K. K., Topol, L., Yun, K., Hu, J., Garrett, L., … Yang, Y. (2010). Mammalian Mst1 and Mst2 kinases play essential roles in organ size control and tumor suppression. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(4), 1431–6. doi:10.1073/pnas.0911409107

St John, M. A., Tao, W., Fei, X., Fukumoto, R., Carcangiu, M. L., Brownstein, D. G., … Xu, T. (1999). Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. *Nature genetics*, *21*(2), 182–6. doi:10.1038/5965

Steinhardt, A. A., Gayyed, M. F., Klein, A. P., Dong, J., Maitra, A., Pan, D., … Anders, R. A. (2008).Expression of Yes-associated protein in common solid tumors.*Human pathology*, *39*(11), 1582–9. doi:10.1016/j.humpath.2008.04.012

Strano, S, Munarriz, E., Rossi, M., Castagnoli, L., Shaul, Y., Sacchi, A., … Blandino, G. (2001). Physical interaction with Yes-associated protein enhances p73 transcriptional activity. *The Journal of biological chemistry*, *276*(18), 15164–73. doi:10.1074/jbc.M010484200

Strano, Sabrina, Monti, O., Pediconi, N., Baccarini, A., Fontemaggi, G., Lapi, E., … Blandino, G. (2005). The transcriptional coactivator Yes-associated protein drives p73 gene-target specificity in response to DNA Damage. *Molecular cell*, *18*(4), 447–59. doi:10.1016/j.molcel.2005.04.008

Sudol, M. (1994). Yes-associated protein (YAP65) is a proline-rich phosphoprotein that binds to the SH3 domain of the Yes proto-oncogene product. *Oncogene*, *9*(8), 2145–52. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8035999

Sudol, M., Chen, H. I., Bougeret, C., Einbond, A., & Bork, P. (1995).Characterization of a novel protein-binding module--the WW domain.*FEBS letters*, *369*(1), 67–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7641887

Sudol, M., & Hunter, T. (2000).NeW wrinkles for an old domain.*Cell*, *103*(7), 1001–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11163176

Sugio, K., Molberg, K., Albores-Saavedra, J., Virmani, A. K., Kishimoto, Y., & Gazdar, A. F. (1997). K-ras mutations and allelic loss at 5q and 18q in the development of human pancreatic cancers.*International journal of pancreatology : official journal of the International Association of Pancreatology*, *21*(3), 205–17. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9322119

Surveyor, G. A., & Brigstock, D. R. (1999).Immunohistochemical localization of connective tissue growth factor (CTGF) in the mouse embryo between days 7.5 and 14.5 of gestation.*Growth factors (Chur, Switzerland)*, *17*(2), 115–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10595311

Tapon, N., Harvey, K. F., Bell, D. W., Wahrer, D. C. R., Schiripo, T. A., Haber, D. A., & Hariharan, I. K. (2002). salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell*, *110*(4), 467–78. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12202036

Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., … Loriolle, F. (1991). The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *The Journal of biological chemistry*, *266*(24), 15771–81. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1874734

Van der Heijden, M. S., Brody, J. R., Dezentje, D. A., Gallmeier, E., Cunningham, S. C., Swartz, M. J., … Kern, S. E. (2005). In vivo therapeutic responses contingent on Fanconi anemia/BRCA2 status of the tumor.*Clinical cancer research : an official journal of the American Association for Cancer Research*, *11*(20), 7508–15. doi:10.1158/1078-0432.CCR-05-1048

Varelas, X., Samavarchi-Tehrani, P., Narimatsu, M., Weiss, A., Cockburn, K., Larsen, B. G., … Wrana, J. L. (2010). The Crumbs complex couples cell density sensing to Hippo-dependent control of the TGF-β-SMAD pathway. *Developmental cell*, *19*(6), 831–44. doi:10.1016/j.devcel.2010.11.012

Vassilev, A., Kaneko, K. J., Shu, H., Zhao, Y., & DePamphilis, M. L. (2001). TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. *Genes & development*, *15*(10), 1229–41. doi:10.1101/gad.888601

Vigneron, A. M., & Vousden, K. H. (2012). An indirect role for ASPP1 in limiting p53-dependent p21 expression and cellular senescence.*The EMBO journal*, *31*(2), 471–80. doi:10.1038/emboj.2011.402

Von Hoff, D. D., Ervin, T., Arena, F. P., Chiorean, E. G., Infante, J., Moore, M., … Renschler, M. F. (2013).Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine.*The New England journal of medicine*, *369*(18), 1691–703. doi:10.1056/NEJMoa1304369

Wahab, N. A., & Mason, R. M. (2006). A critical look at growth factors and epithelial-to-mesenchymal transition in the adult kidney.Interrelationships between growth factors that regulate EMT in the adult kidney.*Nephron. Experimental nephrology*, *104*(4), e129–34. doi:10.1159/000094963

Wang, C., Fu, M., Angeletti, R. H., Siconolfi-Baez, L., Reutens, A. T., Albanese, C., … Pestell, R. G. (2001). Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transactivation and hormone sensitivity. *The Journal of biological chemistry*, *276*(21), 18375–83. doi:10.1074/jbc.M100800200

Wells, C. D., Fawcett, J. P., Traweger, A., Yamanaka, Y., Goudreault, M., Elder, K., … Pawson, T. (2006). A Rich1/Amot complex regulates the Cdc42 GTPase and apical-polarity proteins in epithelial cells. *Cell*, *125*(3), 535–48.doi:10.1016/j.cell.2006.02.045

Wenger, C., Ellenrieder, V., Alber, B., Lacher, U., Menke, A., Hameister, H., … Gress, T. M. (1999). Expression and differential regulation of connective tissue growth factor in pancreatic cancer cells.*Oncogene*, *18*(4), 1073–80. doi:10.1038/sj.onc.1202395

Wilentz, R. E., Iacobuzio-Donahue, C. A., Argani, P., McCarthy, D. M., Parsons, J. L., Yeo, C. J., … Hruban, R. H. (2000). Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. *Cancer research*, *60*(7), 2002–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10766191

Wu, S., Liu, Y., Zheng, Y., Dong, J., & Pan, D. (2008). The TEAD/TEF family protein Scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. *Developmental cell*, *14*(3), 388–98. doi:10.1016/j.devcel.2008.01.007

Xie, Q., Chen, J., Feng, H., Peng, S., Adams, U., Bai, Y., … Yuan, Z. (2013). YAP/TEAD-mediated transcription controls cellular senescence. *Cancer research*, *73*(12), 3615–24. doi:10.1158/0008-5472.CAN-12-3793

Yagi, R., Chen, L. F., Shigesada, K., Murakami, Y., & Ito, Y. (1999). A WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator. *The EMBO journal*, *18*(9), 2551–62. doi:10.1093/emboj/18.9.2551

Yamano, M., Fujii, H., Takagaki, T., Kadowaki, N., Watanabe, H., & Shirai, T. (2000).Genetic progression and divergence in pancreatic carcinoma.*The American journal of pathology*, *156*(6), 2123–33. doi:10.1016/S0002-9440(10)65083-3

Yi, C., Troutman, S., Fera, D., Stemmer-Rachamimov, A., Avila, J. L., Christian, N., … Kissil, J. L. (2011). A tight junction-associated Merlin-angiomotin complex mediates Merlin's regulation of mitogenic signaling and tumor suppressive functions. *Cancer cell*, *19*(4), 527–40. doi:10.1016/j.ccr.2011.02.017

Yu, F.-X., Zhao, B., Panupinthu, N., Jewell, J. L., Lian, I., Wang, L. H., … Guan, K.-L. (2012). Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling.*Cell*, *150*(4), 780–91. doi:10.1016/j.cell.2012.06.037

Yu, J., Zheng, Y., Dong, J., Klusza, S., Deng, W.-M., & Pan, D. (2010). Kibra functions as a tumor suppressor protein that regulates Hippo signaling in conjunction with Merlin and Expanded. *Developmental cell*, *18*(2), 288–99. doi:10.1016/j.devcel.2009.12.012

Yuan, M., Tomlinson, V., Lara, R., Holliday, D., Chelala, C., Harada, T., … Basu, S. (2008). Yes-associated protein (YAP) functions as a tumor suppressor in breast. *Cell death and differentiation*, *15*(11), 1752–9. doi:10.1038/cdd.2008.108

Zender, L., Spector, M. S., Xue, W., Flemming, P., Cordon-Cardo, C., Silke, J., … Lowe, S. W. (2006).Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach.*Cell*, *125*(7), 1253–67.doi:10.1016/j.cell.2006.05.030

Zhang, H., Liu, C.-Y., Zha, Z.-Y., Zhao, B., Yao, J., Zhao, S., … Guan, K.-L. (2009). TEAD transcription factors mediate the function of TAZ in cell growth and epithelial-mesenchymal transition. *The Journal of biological chemistry*, *284*(20), 13355–62. doi:10.1074/jbc.M900843200

Zhang, J., Ji, J.-Y., Yu, M., Overholtzer, M., Smolen, G. A., Wang, R., … Haber, D. A. (2009). YAP-dependent induction of amphiregulin identifies a non-cell-autonomous component of the Hippo pathway. *Nature cell biology*, *11*(12), 1444–50. doi:10.1038/ncb1993

Zhang, L., Zhang, X., & Westlund, K. N. (2004).Restoration of spontaneous exploratory behaviors with an intrathecal NMDA receptor antagonist or a PKC inhibitor in rats with acute pancreatitis.*Pharmacology, biochemistry, and behavior*, *77*(1), 145–53. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2768069&tool=pmcentrez&rendertype=abstract

Zhao, B., Li, L., Lei, Q., & Guan, K.-L. (2010). The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. *Genes & development*, *24*(9), 862–74. doi:10.1101/gad.1909210

Zhao, B., Li, L., Lu, Q., Wang, L. H., Liu, C.-Y., Lei, Q., & Guan, K.-L. (2011). Angiomotin is a novel Hippo pathway component that inhibits YAP oncoprotein. *Genes & development*, *25*(1), 51–63. doi:10.1101/gad.2000111

Zhao, B., Tumaneng, K., & Guan, K.-L. (2011). The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal.*Nature cell biology*, *13*(8), 877–83. doi:10.1038/ncb2303

Zhao, B., Wei, X., Li, W., Udan, R. S., Yang, Q., Kim, J., … Guan, K.-L. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes & development*, *21*(21), 2747–61. doi:10.1101/gad.1602907

Zhao, B., Ye, X., Yu, J., Li, L., Li, W., Li, S., … Guan, K.-L. (2008). TEAD mediates YAP-dependent gene induction and growth control. *Genes & development*, *22*(14), 1962–71. doi:10.1101/gad.1664408

Zhou, D., Conrad, C., Xia, F., Park, J.-S., Payer, B., Yin, Y., … Bardeesy, N. (2009). Mst1 and Mst2 maintain hepatocyte quiescence and suppress hepatocellular carcinoma development through inactivation of the Yap1 oncogene. *Cancer cell*, *16*(5), 425–38. doi:10.1016/j.ccr.2009.09.026

Declaration of authorship

I confirm that the thesis titled "Development of drug screening assays for identification of new molecules against pancreatic ductal adenocarcinoma" is my own work and the use of all material from other sources has been properly and fully acknowledged.

ACKNOWLEDGEMENTS

Dr. Alessandro Provenzani and all the members of the Laboratory of Genomic Screening

People who work in CIBIO, in particular Valentina Adami,in charge of the High Throughput Facility

All the members of the PANACREAS project, in particular the coordinator Georg Feldmann MD (Universitäts klinikum Bonn) and Dr. Alessio Nencioni (University of Genoa)

Many thanks to my family and the people who love me

This work has been supported by the European Community's Seventh FrameworkProgramme [FP7-2007-2013] under Grant Agreement No. HEALTH-F2-2011-256986 (A.P., G.F. A.N:)

"You will never know what you can achieve unless you try"