# The role of YAP/Yorkie during compensatory liver regeneration

**Diplomarbeit**

angefertigt an der Fakultät für Biologie

der Albert-Ludwigs-Universität Freiburg

in Zusammenarbeit mit dem toxikologischen Institut

der Universität in Cagliari

Betreuer:

Prof. Dr. K.-G. Collatz

Albert-Ludwigs-Universität Freiburg

Prof. Dr. A. Columbano

Università degli studi di Cagliari

vorgelegt von

Sebastian Schildge

Freiburg, August 2010

# Erklärung

Hiermit erkläre ich, dass ich die vorliegende Diplomarbeit

„The role of YAP/Yorkie during compensatory liver regeneration“

selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen verfasst habe.

Freiburg, den 30.08.2010

Sebastian Schildge

# Danksagung

Besonders möchte ich mich bei meinen Eltern für die Ermöglichung dieses Studiums und ihre großartige Unterstützung bedanken. Ein großes Dankeschön gilt Professor Collatz für seine freundliche und unkomplizierte Hilfe, ohne ihn wäre diese Diplomarbeit nicht möglich gewesen. Weiterhin möchte ich mich bei dem Team der toxikologischen Abteilung der Universität Cagliari bedanken, insbesondere bei Professor Columbano für die Bereitstellung des Arbeitsplatzes und bei Frau Dr. Pibirri für ihre unermüdliche Unterstützung.

Vielen Dank!

# Contents

# 1 Introduction

## 1.1 Background

A still unanswered question in biology is how the size of an organ is determined. Accumulating evidence suggests that a ósize checkpointô exists and operates at the level of the organôs total mass, rather than regulating the size or the number of the constituent cells. Coordination of cell proliferation and cell death is essential not only to attain proper organ size during development, but also for maintaining tissue homeostasis throughout postnatal life. Liver, for example, is an interesting organ which in adult organisms is in a quiescent state but under certain conditions shows a remarkable regenerative capacity. Commonly, regeneration of the liver is studied by performing a surgical removal of 2/3 of the liver mass in rodents (rats and mice), a technique known as 2/3 partial hepatectomy (2/3 PH) (Higgins and Anderson, 1931). In this case, three of the five liver lobes can be removed by an easy surgical procedure, without causing any tissue damage to the residual two lobes. The latter grow in size to restore the original mass of the organ. The process, in rats and mice, is completed within 5-7 days. After the initial growth, no further enlargement of the liver is observed, suggesting the existence of pathways leading to termination of liver regeneration. Moreover, livers from large animals (dogs) transplanted to smaller animals decrease in size, while the opposite is also true (Starzl et al, 1993). These findings have raised the issue of the existence of a ñhepatostatò control system, which ensures that the liver weight is as it should be (and not more or less) for the performance of its homeostatic functions.

While some studies have initially proposed transforming growth factor (TGF-ɓ) as the terminator of regeneration (Michalopoulos, 2007), given its mito-inhibitory properties and its up-regulated expression as regeneration advances, no clear evidence for this has ever been achieved; moreover, how the liver senses this termination signal or what are the mechanism(s) involved is unknown.

The balance between cell proliferation and apoptosis is essential to proper tissue growth, development, and function. In Drosophila, these two processes are orchestrated by the Hippo kinase cascade, a growth suppressive pathway that

ultimately antagonizes the trascriptional co-activator Yorkie (Yki). Components of the Hippo pathway, known to be crucial in control of organ size (Marsman et al, 1988; Zhao et al, 2007), are highly conserved in mammals and results in phosphorylation of the core components WW45, Mob1 and Lats1/2 (Chan et al, 2005; Callus et al, 2006; Praskova et al, 2008). Subsequently, the transcriptional co-activator YAP, the mammalian homolog of Yki, is directly phosphorylated by Lats and retained within the cytoplasm in the inactive state (Steinhardt et al, 2008); this results in organ growth inhibition, being YAP, in its active form, a potent growth promoter. Dysregulation of the Hippo pathway results in YAP activation through loss of the kinase cascade (that inactivates YAP); upon dephosphorylation, YAP translocates into the nucleus where it binds to transcription factor(s) and regulates transcription of the Hippo pathway target genes. It has been reported, using a conditional YAP transgenic mouse model, that YAP induces a dramatic increase in liver size and eventually leads to tumor formation, its overexpression phenocopying the loss of the Hippo pathway components (Zhao et al. 2008a), related to organ size control.

## 1.2 Compensatory regeneration

Hepatocytes usually are in a quiescent state (G0-state of cell cycle), with only 1 out of 1000 cells passing over to G1-state (Court, 2002); nonetheless, in presence of appropriate stimuli they can pass over to G1-state. The best characterized model to study liver regeneration is represented by compensatory regeneration following 2/3 partial hepatectomy. Compensatory regeneration can be triggered by surgical liver removal or by cell loss induced by necrogenic agents, such as hepatotoxins, viruses (hepatitis virus) or chemicals (CCl4) (Michalopulous, 2007). Loss of parenchyma triggers cellular proliferation, which over the course of time reconstitutes the lost liver mass. Emphasis must be laid on the fact that the term regeneration is not meant to be understood in the biological way which is defined as the exact reconstitution of a removed structure (Fausto et al, 2006). In case of compensatory regeneration the removed liver lobes will not grow back, but the remaining lobes will increase in size, thus restoring the lost liver mass. The compensatory regeneration, triggered by loss of tissue, is attended by sequential changes of the genetic response, release of specific cytokines, stimulation of specific growth factors and activation of certain

pathways which allow hepatocytes to re-enter cell cycle (Kontouras, 2001). The process of liver regeneration implies the interaction of numerous factors, though it can be classified into 2 basic states. The first state is called priming-phase and induces the transition of hepatocytes from G0- to G1-state and lasts about 4 hours after PH (Morello 1990, Hsu 1992).

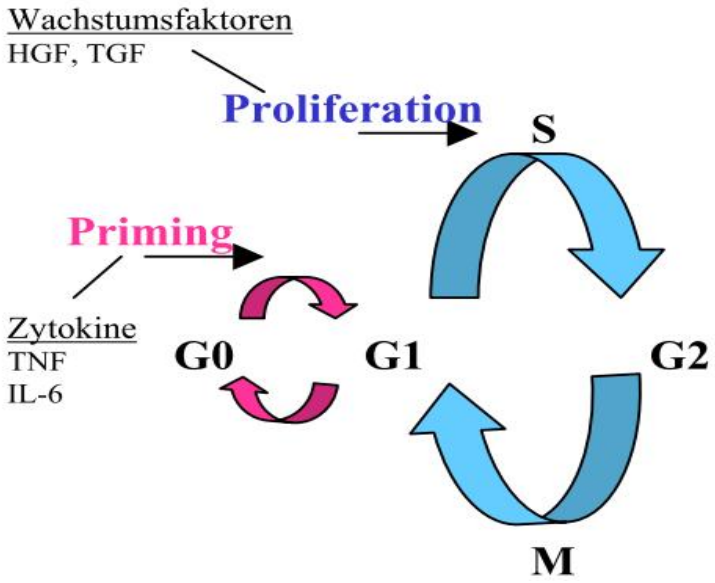



**Image 1**: *The 2 basic states of liver regeneration: priming-phase prepares hepatocytes for reentering cell cycle. In progression-phase growth factors regulate cell cycle upon a certain point in transition G1 to S-phase, where cell cycle becomes independent from them. Since then cyclins control further progression of cell cycle.*

*Source: Denz 2004*

Hepatocytes so are sensitised towards influences of various growth factors, like EGF (Epidermal Growth Factor), TGF-Ŭ (Transforming Growth Factor alpha) and HGF (Hepatocyte Growth Factor) (Koniaris et al 2003). During priming-phase a cytokine network in Kupffer-cells is activated, which finally leads to expression of so called immediate-early-genes in hepatocytes. In more details activated Kupffer cells release the cytokine TNF-α (tumor necrosis factor α). The binding of TNF-α to TNFR1 (TNF-receptor 1) triggers the MAP-kinase cascade, whereby the activation of transcription factor NF-əB (nuclear factor kappa-B) is induced and in turn leads to stimulation of IL-6 (Interleukin 6). IL-6 is secreted by Kupffer-cells and binds to its receptor (IL-6R), which is located on the membrane of hepatocytes. This engenders that STAT3 (Signal transducer and activator of transcription 3) in hepatocytes will be phosphorylated and thereby activated (Fausto, 2006; Kishimoto, 1992). STAT3 is translocated into the nucleus of the hepatocyte and leads through another signal

cascade to activation of the immediate early genes (i.e. c-jun, c-fos, c-myc; IGFBP-1).

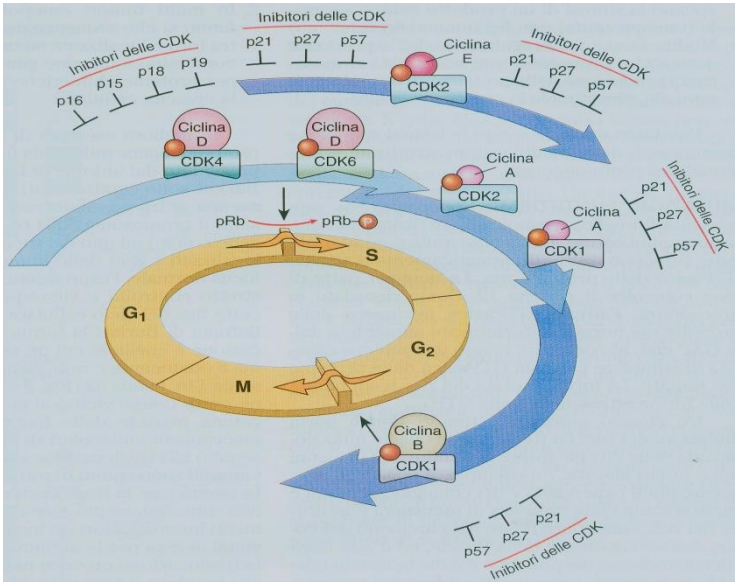



**Image 2**: *Each phase of cell cycle is governed by their corresponding cyclin-CDK complexes and their inhibitors.*

*Source: Columbano Laboratory, University of Cagliari*

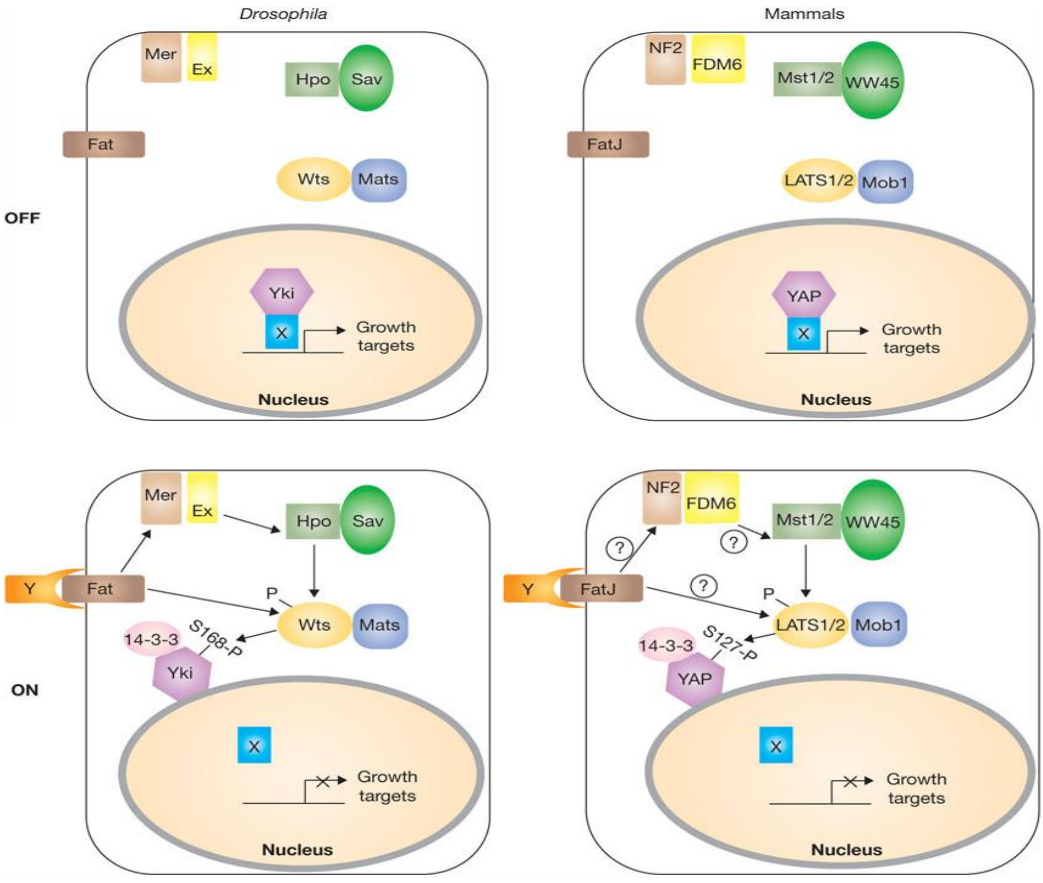
The second phase of gene expression after PH, called progression-phase, starts after exceeding the threshold from G0 to G1 and is still influenced by growth factors upon a certain point in G1-/S-phase transition, since then DNA-replication takes place independently of growth factors (Fausto 2000). During second phase the so called delayed early genes are activated, which encode for various proteins, being involved in the control of cell cycle, for example cyclins, cyclin dependent kinases (CDK) and inhibitors of cyclin-CDK complexes. Within their activation cell cycle progression is independent of growth factors. The above described Cyclin-CDK-complexes mediate intracellular signals to regulate cell cycle at different time points (Sherr 2004). Thereby different cyclins (Cyclin D, -E, -A, -B) are expressed at different time points of cell cycle (Albrecht 1999). G1 state is controlled by the complexes cyclin D1/CDK4 or CDK6 and Cyclin E/CDK2; S-state is dominated by activity of the complexes Cyclin A/CDK1 or CDK2, whereas the transition G2→M is regulated by Cyclin B/CDK1. An important restriction point of cell cycle is the transition G1→S, which is normally blocked by pRb (Retinoblastoma protein), a protein which is phosphorylated in its active state, thus preventing the release of transcription factors of the E2F family, which are responsible for the expression of

genes leading to S-phase (Pines 1998). Inactivation of pRb and the related progression of cell cycle is induced by the cyclin D1/CDK4 and cyclin E/CDK2. Those inactivate pRb through hyperphosphorylation, which results in release of the E2F transcription factors and hence the entrance in S-phase. In rats the peak of DNA synthesis is seen 24 hours after PH, in mice after 40 hours. The Synthesis-phase is followed by the postreplicative G2-phase, in which the cell synthesises molecules, which are required for the following mitosis-state (M-state). After 1 or 2 rounds of replication the hepatocytes leave cell cycle again and re-enter in G0-state. The end of liver regeneration is mediated through inhibitors like i.e. TGF-ß and Activin A, when the functional capacity of the liver has been restored and thus is able to fulfill its metabolic requirements.

It is of interest to note that during the aging process there is a decline in the cellular capacity to respond to proliferative stimuli. Indeed, several studies on the regenerative response of the liver that follows 2/3 PH have shown a striking difference in both the magnitude and the peak response in DNA synthesis and the time at which maximal DNA synthesis occurs between young (8 weeks) and aged (12 months) rats and mice (Bucher et al 1964; Stocker et al 1971; Fry et al 1984). Although the mechanism(s) responsible for the age-related decline in the post-hepatectomy proliferative response are not known, an age-related switch from cyclin-dependent kinase inhibition in repression of E2F transcription factor and/or a decrease in expression of a gene coding for a critical Forkhead Box transcription factor have been correlated with reduced proliferation of regenerating hepatocytes from old rodents (Iakova et al 2003; Wang et al 2001), Notably, it has been shown that hepatocyte proliferation induced by direct mitogens (direct hyperpalsia), ligands of nuclear receptors, is age- independent (Ledda-Columbano et al 2004). Since the early signal transduction pathways involved in nuclear receptor-mediated hepatocyte proliferation are different from those involved in liver regeneration, in that early changes are considered to be essential for liver regeneration after PH (i.e activation of latent transcription factors; increased expression of immediate early genes and release of cytokines) has been not observed (Pibiri et al 2001; Ledda-Columbano et al 1998), this has suggested that hepatocytes retain their proliferative capacity in old age despite impaired liver regeneration (Ledda-Columbano et al 2004).

## 1.3 YAP/Yorkie and the Hippo pathway

As already noted coordination of cell proliferation and cell death is essential to attain proper organ size during development and for maintaining tissue homeostasis throughout postnatal life (Conlon and Raff 1999). Recent genetic studies in Drosophila as well as in mammals delineated the importance of the Hippo (hpo) signalling pathway to play a key role in restricting organ size by controlling both cell proliferation and apoptosis (Edgar 2006; Harvey and Tapon 2007; Pan 2007). Experiments with Drosophila mutants revealed that loss of components of the hippo pathway lead to massive tissue overgrowth and tumorigenesis suggesting the importance of this pathway in the control of organ size.




*Source: Buttitta & Edgar 2007*

**Image 3:** *The Hippo pathway is a kinase cascade shown in activated (on) and deactivated (off) condition; Drosophila on the left, mammals on the right. The corresponding (homolog) components of the pathway are shown in the same colour. In activated condition Yap is phosphorylated by Lats and remains in the cytoplasm. When Hippo pathway is deactivated Yap enters the nucleus and induces via transcription factors growth target genes.*

The Hippo pathway is represented by a kinase cascade leading to regulation of a transcriptional co-activator, known as Yorkie (Yki) in Drosophila and YAP (Yes-associated protein) and TAZ (transcriptional co-activator with PDZ binding motif) in mammals. TAZ is a YAP paralog with 50 % genetic agreement. Components of the Hippo pathway, firstly identified in Drosophila, are highly conserved in mammals, as indicated in Table 1.

| **Drosophila** | **Mammalian homolog** |
|---|---|
| Fat | Fat4 |
| Mer | NF2 |
| Ex | FDM6 |
| Hpo | Mst1/2 |
| Sav | WW45 |
| Wts | LATS1/2 |
| Mats | Mob1 |
| Yorkie | YAP,TAZ |

**Table 1:** *The components of Hippo-pathway in Drosophila and their corresponding homologs in mammals*

This homology was shown in experiments demonstrating the ability of the human components of hippo pathway to substitute the function of their homologs in mutant Drosophila (Edgar, 2006). In mammals, activation of the hpo pathway results in a complex formation between MST1/2 (Mammalian sterile 20 like kinase) and the adaptor protein WW 45. This complex phosphorylates(P) Lats 1/2. (P)Lats1/2 complexes with Mob1 and phosphorylates the S (serine) 127 binding site of YAP. YAP phosphorylation by Lats leads to YAP cytoplasmatic retention, via a 14-3-3 binding site, and, thus, to its inactivation.

The exact involvement of the MST upstream Hippo pathway components (NF2 and FDM6, ezrin/radixin/moesin (ERM) family actin-binding proteins) (McClatchey and Giovannini 2005; Okada et al. 2007) is not clear, although they have been shown to positively regulate the Hippo pathway in Drosophila (Hamaratoglu et al. 2006).

Furthermore, other genetic data suggests Fat, a protocadherin tumor suppressor, as an upstream Hippo pathway component of MST (Bennett and Harvey 2006; Cho et al 2006) which may interact with another protocadherin, known as Dachsous, at the cell surface (Matakatsu and Blair 2004, Halbleib and Nelson 2006). MST (hpo-homolog) plays a key role in the mammalian hpo-pathway as it phosphorylates all three core components of the hippo-pathway (WW45, Mob and Lats) (Zhao et al 2008a). The two splicing forms of MST, MST 1 and MST 2, are 56-60 kDa class 2 GC kinases that share 76 % identity in amino acid sequence (Dan et al, 2001). The loss of hpo function in Drosophila results in a massive tissue overgrowth, due to a cell cycle progression and a failure of developmental apoptosis (Harvey et al, 2003; Udan et al, 2003; Wu et al, 2003). In 2009 Zhou et al examined the role of MST 1/2 regarding the liver in a MST 1/2 deficient mouse model. Dual inactivation of MST 1 and 2 lead to YAP 1 activation which results in liver overgrowth, resistance to Fas-induced apoptosis and rapid hepatocellular carcinoma (HCC) development. Further deactivation of MST1 and 2 lead, beside increased YAP1 activation, to a decrease in Mob1 activity and to induction of the candidate YAP target genes CTGF (connective tissue growth factor) and AFP (alpha fetoprotein) (Zhou et al, 2009), delineating the tumor suppressive activity of MST as mediated by negative regulation of YAP. Interestingly, no significant alteration of Lats1/2 was seen, suggesting the existence of a kinase other than Lats1/2 acting downstream of MST and leading to YAP activation.

The YAP gene is located on chromosome 11q22. The YAP transcript exists in two different splicing forms, YAP1 and YAP2. Most studies referring to YAP actually are focused on YAP2 which is translated into a 65 kDa protein. The difference of the two splicing forms is that YAP2 has two WW domains, whereas YAP1 has only one, as well as TAZ. Regulation of the switch of those two isoforms isn't understood. The YAP gene was found amplified in many human cancers, suggesting a role for YAP as a possible oncogene. Besides the genomic amplification, YAP expression and its nuclear localization was shown to be elevated in several human cancer types (Dong et al 2007) and, more in general, in condition of low cell density (Zhao et al. 2007), while high cell density seems to activate the hippo-pathway to inhibit Yap. Under conditions of YAP over expression human non transformed mammary epithelial cells showed phenotypic alterations typical of cancer cells, like epithelial-to-mesenchymal transition (associated with metastasis), suppression of apoptosis, growth factor-

independent proliferation and anchorage-independent growth in soft agar (Overholtzer et al 2006). Moreover, in the liver of rodents with induced YAP over expression a dramatic increase in size and often tumor formation was noted (Camargo et al 2007).

The growth promoting role of YAP seems to be due to its ability to activate transcription of genes related to induction of cell proliferation and inhibition of apoptosis. In particular, YAP is classified as a transcriptional co-activator because it doesn't have any obvious DNA binding site (Zhao et al 2008b). Thus, YAP must activate other transcription factors to induce target genes leading to increased cell proliferation and decreased apoptosis. Recent studies identified the transcription factor TEAD (Tea domain/transcription enhancer factor) as a key mediator of YAP function (Zhao et al 2008b). TEAD exists in four different splicing forms, but only TEAD1 and 2 seem to interact genetically with YAP in vivo. TEAD 1/2 double knockout mice show a similar phenotype to YAP knockout mice suggesting their close genetic interaction (Sawada et al 2008). The YAP target genes related to cell proliferation, activated by YAP-dependent transcription factors (especially TEAD), are still poorly characterized, but recent works suggest connective tissue growth factor (CTGF) as candidate YAP target gene (Dong et al. 2007; Zhao et al 2008b). Indeed, Zhao et al, 2008 found CTGF as a direct target gene of YAP-TEAD in mammalian cells. Interestingly, elevated CTGF levels have been detected in human cancers (Xie et al. 2001), and anti-CTGF antibody inhibited tumor growth and metastasis (Dornhofer et al. 2006). This supports a possible role of CTGF in mediating the growth-stimulating and oncogenic function of YAP-TEAD. Although CTGF appears to play an important role in YAP-induced cell growth, the same study demonstrated that it may not be required for YAP-induced EMT (Zhao et al, 2008b), suggesting that other genes may be involved in the biological function of YAP. Consistently, the TEAD binding- defective YAP-S94A mutant was found to still induce expression of a fraction of the YAP-regulated genes. These observations indicate that additional transcription factors may be used by YAP/Yki to regulate cell and tissue growth. Beside regulation of growth promoting genes, lack of, or mutation of any factors in the Hpo signalling pathway leading to YAP activation, has been demonstrated to induce genes which codify for proteins acting as inhibitors of apoptosis, as BIRC5/survivin (Dong et al, 2007). Survivin is a new member of the inhibitor of apoptotic protein (IAP) family, and was initially cloned by the cDNA of effector cell

protease receptor-1 in the human genomic library in 1997 (Ambrosini et al 1997). Many investigations have found survivin to be overexpressed in most common tumors, but almost never in normal tissues (Lehner et al, 2002). The overexpression of survivin was closely related to tumorigenesis and progression, and was one of the strongest apoptotic inhibitors identified. In tumors, the massive induction of BIRC5/survivin, can lead to breakdown in the balance of cellular proliferation and apoptosis, increasing the occurrence and development of tumors (Dong et al 2007).

## 1.4 Aim of the study

Mechanisms governing liver growth during compensatory regeneration after 2/3 partial hepatectomy are still not fully understood. In particular, very unclear are the hepatostat mechanisms leading to growth inhibition when the original size of the liver has been regained after partial hepatectomy. Up to now, there are no informations about YAP involvement in liver regeneration after 2/3PH. Indeed, the only study which has analysed Yap expression during liver regeneration, was done in AFP-albumin-ILK-KO animals along with their wildtype (WT)-Cre-negative siblings that were subjected, instead of to classical 70% PH, to a 35%-40% PH (Apte et al 2009). In this study, the authors demonstrated that integrin-linked kinase (ILK) (involved in transmission of the extracellular matrix [ECM] signaling by way of integrin receptors) and/or hepatic adaptations that ensue following ILK hepatocyte-targeted removal were critical for proper termination of liver regeneration. Indeed, ILK-KO livers demonstrated a termination defect resulting in a 58% larger liver than their original pre-PH mass. This increase in post-PH liver mass was due to sustained cell proliferation driven in part by increased signaling through hepatocyte growth factor (HGF), the beta- catenin pathway and Hippo kinase pathways. In particular, regarding to YAP, authors found higher YAP protein in ILK-KO livers at 5, 7, and 14 days after 35%-40% PHx, which was associated to overall lower phosphorylated YAP protein at all timepoints following PH as compared to the control mice, suggesting that ILK signalling contributes to control of the Hippo kinase pathway, and particularly YAP activation, in the liver. Nevertheless, apart from the particular genetic background of animals employed and the unusual 35-40%PH performed, in this study a strict correlation between liver enlargement and YAP activation is not demonstrated, as YAP activation could simply reflect a sustained proliferative response, not allowing to discriminate it from common cell cycle genes. On this basis, we wished to determine in this study, for the first time, the role of YAP in compensatory regeneration after 2/3 PH. In particular, our aims were:

- ***Aim 1: to investigate whether Hippo pathway is involved in control of compensatory regeneration after 2/3 PH in rats and mice*.** In particular, we address the question whether the co-activator YAP, whose induction, following inhibition of the growth suppressive Hippo pathway, has been correlated to enlargement of organs, is activated during liver regeneration when the organ

was reaching its original size and inhibited when the process has been completed (see experimental protocols 1 and 2);

- ***Aim 2: to analyze YAP expression in condition of impaired liver regeneration, namely compensatory regeneration in aged mice.*** As liver regeneration is known to be reduced and delayed in liver of aged rodents (12 months) (Bucher et al, 1964; Fry et al, 1984), in order to possibly show that YAP expression does not mirror cell cycle gene expression, we addressed the question if compensatory regeneration in aged animals could be associated to a prolonged and robust YAP activation, opposite to cell cycle genes. For this evaluation YAP expression after 2/3 PH has been analyzed in aged rodents, using young animals as control (see Experimental Protocol 3);
- ***Aim 3: to analyze YAP expression after partial hepatectomy to be done in an enlarged liver.*** Results obtained from this aim could further support the notion that YAP is a mediator of liver cell proliferation organ size restricted. Previously, it was shown that a single treatment with the hepatic mitogen TCPOBOP, a ligand of the nuclear receptor CAR, induces a doubling of the liver mass and, more important, of the hepatic DNA content within 3 days. When 70% PH was performed in those large livers, a massive liver regeneration (L.I. was approximately 70%) occurred, similar to that observed in mice subjected to PH alone (Columbano et al, 2008). On this base, in order to exclude a YAP regulation merely dependent from signal related cell cycle progression, and to eventually support the notion that, unlike common cell cycle genes, YAP is organ size restricted, we evaluated YAP expression in livers of mice subjected to 2/3 PH after pre-treatment with the mitogen TCPOBOP, according to Experimental Protocol 4. The possible demonstration of a decreased YAP activation in animals subjected to 2/3 PH in an enlarged liver, compared to animals subjected to 2/3 PH only, could strongly support the dependence of YAP activation on liver size.

# 2 Materials and Methods

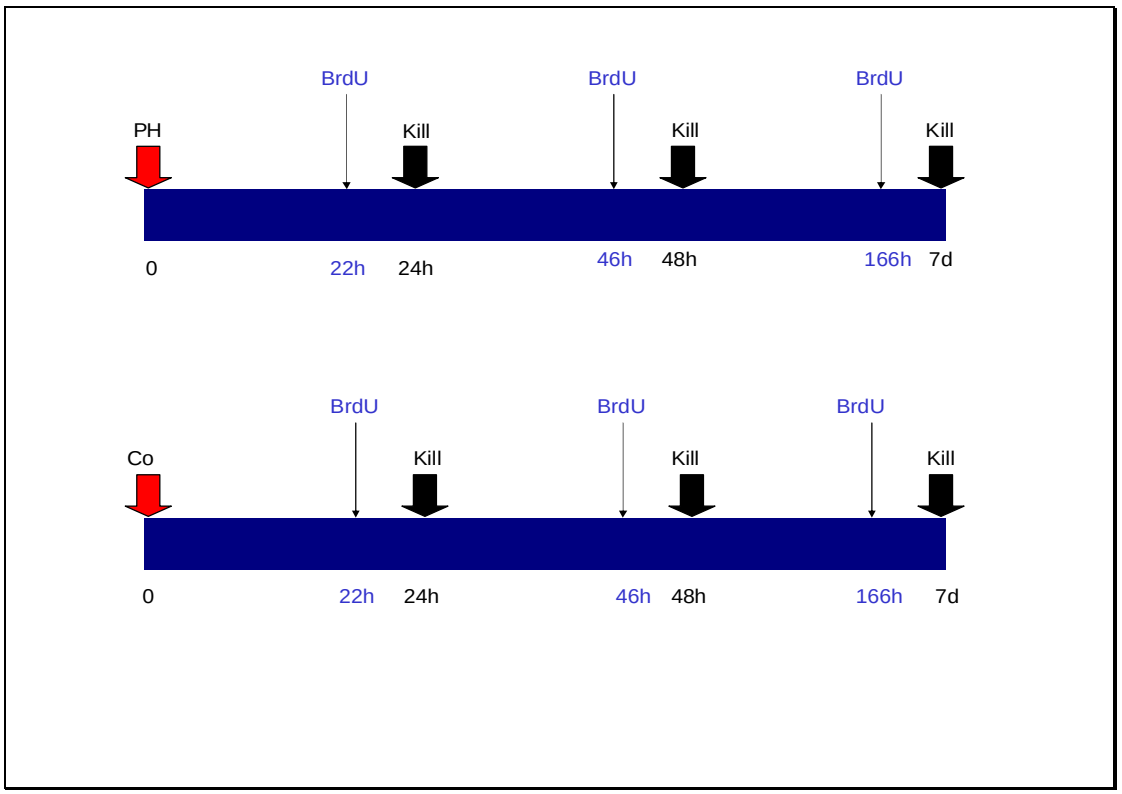
## 2.1 Animal treatment and surgery:

7 week-old male Fisher (F-344) rats , 8 and10 weeks-old and 12 months-old female CD-1 mice purchased from Charles River (Milano, Italy), were maintained on a standard laboratory diet (Standard Diet 4RF21, Mucedola, Settimo Milanese, Italy). The animals were given food and water *ad libitum* with a 12 h light/dark daily schedule at a temperature of 24 degrees and were acclimatized for 1 week before starting the experiments. Temperature and humidity were controlled for the entire period of experimentation. All procedures were performed in accordance with the Universities Federation for Animal Welfare Handbook on the Care and management of Laboratory Animals and the guidelines of the animal ethics committee of the University of Cagliari. Animals were submitted to 2/3 PH according to Experimental Protocols 1, 2, 3 and 4. All treatments were performed between 9 am and 12 pm in order to standardize the effects of diurnal changes. Briefly, animals were given diethyl ether anesthesia in a closed jar. The abdominal area was cleaned with Batticon (povidine iodine) solution after shaving. A median abdominal incision was performed and the left and median liver lobes were exposed with the standard 70% hepatectomy technique *(*Higgins and Anderson*).* The peduncle of the left and median lobe was resected, while the right and caudate lobes of the liver were left in place. The abdominal incision was closed with 2/0 silk continuous sutures as a single layer. All of the operations were performed under clean but not sterile conditions. After hepatectomy, the body weights of the rodents were recorded. Untreated animals served as controls. TCPOBOP (1,4-Bis [2-(3,5 dichloropyridyloxy)])benzene was administered by gavage treatment at a dose of 3 mg/kg of body weight, dissolved in dimethylsulfoxide/corn oil solution.

To label dividing hepatocytes, 5ôBromodesoxyuridine (BrdU), a thymine analog which is incorporated by proliferating cells in S-phase, was injected intraperitoneally, at a dose of 100 mg/Kg body weight, 2 hours prior to sacrifice or given continuously in drinking water, at a concentration of 1 mg/ml.

# 2.2 Experimental protocols

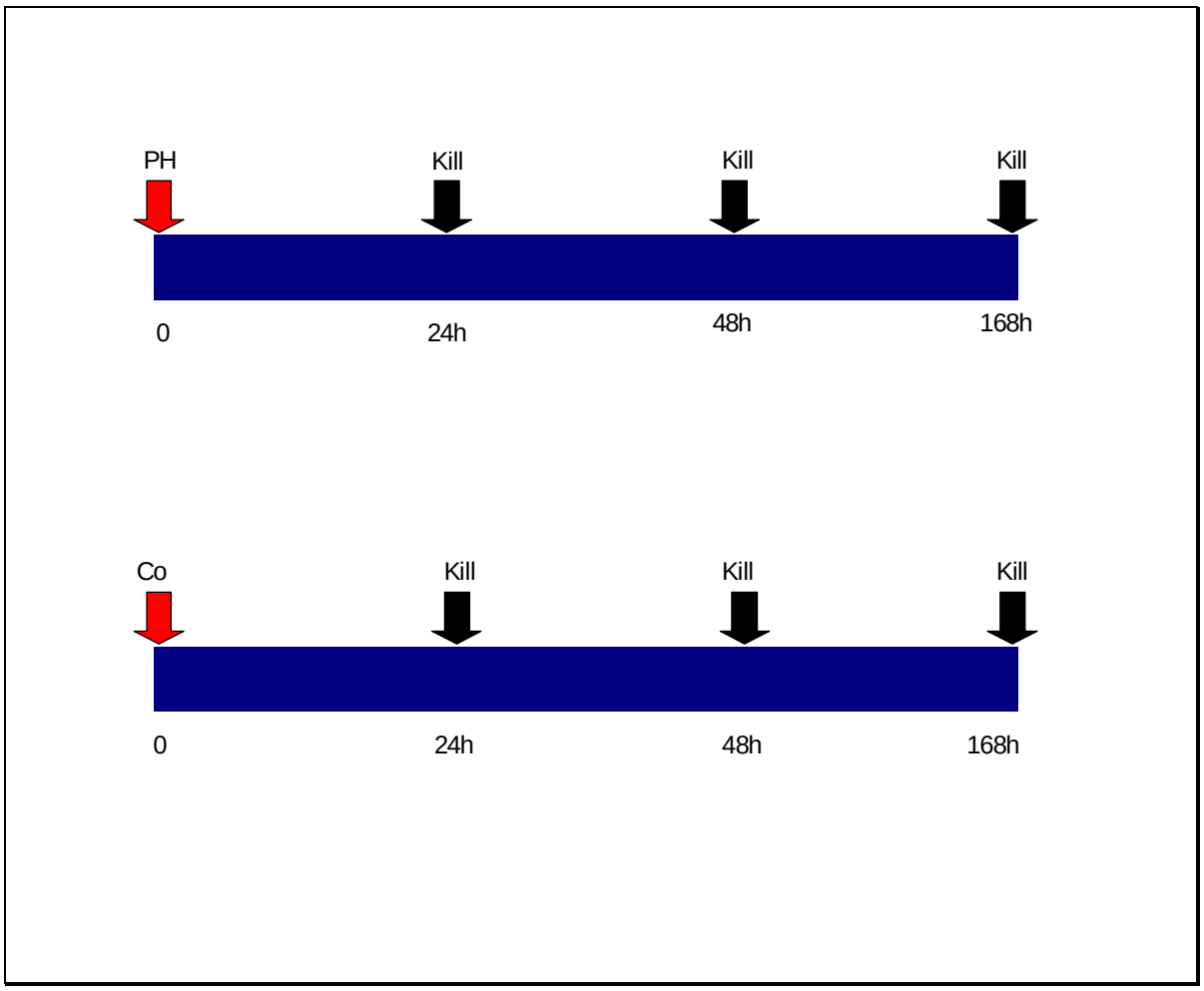
## Experimental Protocol 1




**Legend to experimental protocol 1:**

Fifteen 7 week-old male Fischer rats have been divided into 2 groups; animals of the first group were submitted to 2/3 PH while those of the second group served as control (CO) and were untreated. Four animals submitted to 2/3 PH and 1 control animal were sacrificed for each time point, according to figure. At time point zero all PH-animals (12) underwent surgery. Mice were sacrificed 24, 48 and 168 hours after time point zero. Two hours before sacrifice all animals received a single i.p. injection of BrdU at a dose of 100 mg/ Kg of body weight. Immediately after sacrifice, sections of the liver were fixed in 10% buffered formalin, processed and embedded in paraffin for staining with hematoxylin-eosin or immunohistochemistry. The remaining liver was snap-frozen in liquid nitrogen and kept at -80° C until use.

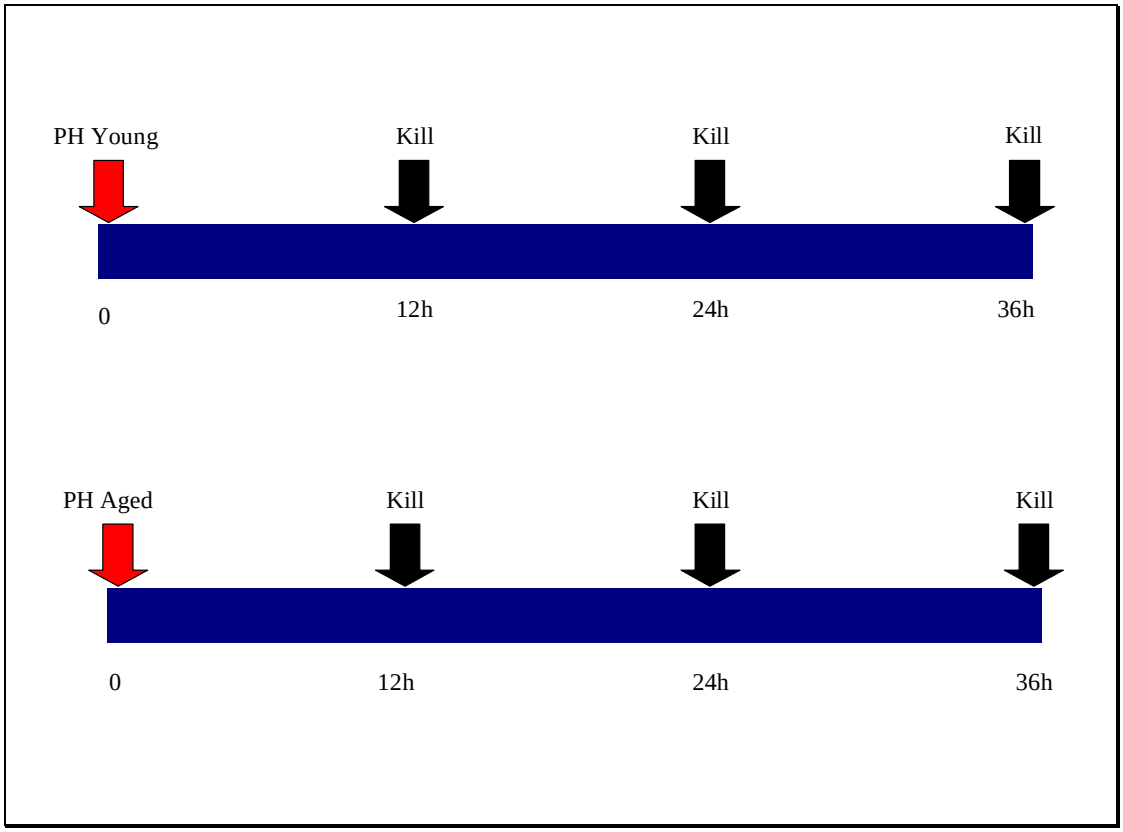
## Experimental Protocol 2




**Legend to experimental protocol 2:**

Fifteen 20 week-old female CD1-mice were divided in 2 groups; animals of the first group were submitted to 2/3 PH while those of the second group served as control (CO) and were untreated. Four animals submitted to 2/3 PH and 1 control animal were sacrificed for each time point, according to figure. At time point zero all PH-animals (12) underwent surgery. Sacrifices were done 24, 48 and 168 hours after time point zero. Immediately after sacrifice, sections of the liver were fixed in 10% buffered formalin, processed and embedded in paraffin for staining with the Ki67-Method. The remaining liver was snap-frozen in liquid nitrogen and kept at -80° C until use.

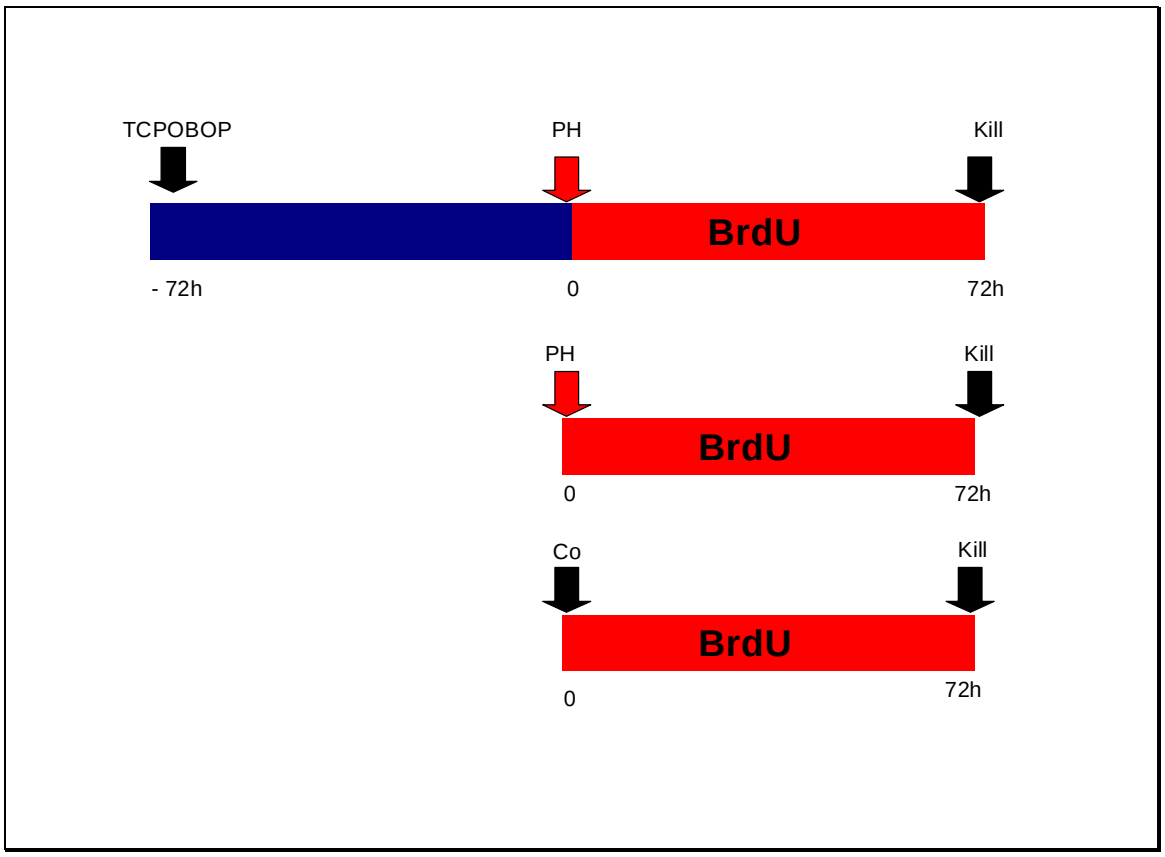
## Experimental Protocol 3




**Legend to experimental protocol 3:**

Twelve 8 week-old (young) and twelve 12 month-old (aged) female CD1-mice were divided both in 2 groups; animals of the first group were submitted to 2/3 PH while those of the second gr oup served as contr ol (CO) and were untreated. Three animals submitted to 2/3 PH and 1 control animal were sacrificed for each time point, according to figure. At time point zero all PH-animals (9 young mice and 9 aged mice) underwent surgery. Sacrificed were done from 12 to 36 hours, according to figure. Immediately after sacrifice, the livers were snap-frozen in liquid nitrogen and kept at -80° C until use.

## Experimental Protocol 4




**Legend to experimental protocol 4:**

Eleven 10 week-old female CD1-mice were divided in 3 groups; animals of the first group (6 animals) received a single i.p injection of the mitogen TCPOBOP (3 mg/kg of body weight) 72 hours prior to 2/3 partial hepatectomy; animals of the second group (6 animals) were submitted to 2/3 PH only, while those of the third group (3 animals) were untreated and served as controls (CO). All animals were sacrificed 72 hours after the time of 2/3 PH, considered as zero time point. In order to determine hepatocyte proliferation all mice received BrdU, dissolved in drinking water at a concentration of 1 mg/ml, starting from time point 0, as indicated in figure. Immediately after sacrifice, sections of the liver were fixed in 10% buffered formalin and processed and embedded in paraffin for staining with hematoxylin-eosin or immunohistochemistry. The remaining liver was snap-frozen in liquid nitrogen and kept at -80° C until use.

## 2.3 Preparation of cell lysates

Total cell extracts were prepared from frozen livers powdered in liquid nitrogen cold mortar. Equal amounts of tissue (~70 mg) per animal were re-suspended in 1 ml Triton lysis buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.4, 140 mM NaCL, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1mM PMSF, 5 mM iodoacetic acid, 10 mg/ml each of aprotinin, pepstatin, leupeptin). To minimize protein degradation during the isolation protocols several protease inhibitors were added to the isolation buffer. After vortexing, extracts were incubated 30 minutes on ice, centrifuged at 12000 rpm at 4°C and the supernatants were recovered. All inhibitors used were purchased from Boehringer Mannheim (Mannheim, Germany) with the following exception: PMSF, NaF and DTT, which were purchased from Sigma Chemical (St. Louis, MO, USA), and iodacetic acid, which was acquired from ICN Biomedicals (Irvine, CA, USA) Nuclear protein extracts were prepared according to Timchenko et al, 1996. The protein concentration of the extracts was determined according to the method of Bradford (1976) using bovine serum albumin as standard (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

## 2.4 Western blot analysis

For immunoblot analysis equal amounts (from 100 to 150 μg/lane) of proteins were electrophoresed on SDS-12% or -8% polyacrylamide gels. Polyacrylamide was purchased from Sigma Chemicals, St. Louis, CA. After gels electrotransfer onto nitrocellulose membranes (Osmonics, Westborough, MA, USA), to ensure equivalent protein loading and transfer in all lanes, the membranes and the gels were stained with 0,5% (wt/vol.) Ponceau S red (ICN Biomedicals, Irvine, CA) in 1% acetic acid for 5 min. Before staining, gels were fixed in 25% (v/v) isopropanol and 10% (v/v) acetic acid (Sigma Chemical, St. Louis, MO, USA). After blocking in TBS (Tris-buffered saline) containing 0,05% Tween 20 (Sigma Chemical, St. Louis, MO, USA) and 5% non-fat dry milk or BSA, membranes were washed in TBS-T and then incubated with appropriate primary antibodies diluted in blocking buffer. Whenever possible, the same membrane was used for detection of the expression of different proteins. Immunoreactive bands were identified with a chemiluminescence detection system,

as described by the manufacturer (Supersignal Substrate; Pierce, Rockford, IL, USA).

### 2.4.1 Antibodies used for immunoblotting

The following antibodies were used for immunoblotting: mouse monoclonal antibodies directed against Cyclin D1 (72-13 G) (Santa Cruz Biotechnology, CA), PCNA (PC-10) (Santa Cruz Biotechnology, CA, UAS), Actin (clone AC-40) (Sigma Chemical, St. Louis, MO, USA) and Albumin (Bethyl Laboratories, Montgomery, TX, USA); rabbit polyclonal antibodies directed against YAP, Phospho-YAP, purchased from Cell Signaling Technology (Danvers, MA, USA) and against Cyclin A (Santa Cruz Biotechnology, CA, USA). Anti-mouse and anti-rabbit horse radish peroxidase conjugated IgGs were acquired from Santa Cruz Biotechnology.

## 2.5 Immunohistochemistry

### 2.5.1 Preparation of liver tissue for immunohistochemistry

Immediately after sacrifice, the liver sections to be used for immunohistochemistry were fixed for 24 hours in a 10 % formalin solution. After fixation the processing of liver tissue was performed by an automatic processator (Citadel 200, Thermo Shandon). The processed liver samples were embedded in paraffin and later sliced via microtom (Microm HM 315) in 4 µm thick sections. Next, the slices were transferred on a glass slide with positive charge (SuperFrost-Plus).

### 2.5.2 BrdU Immunohistochemistry

Liver sections have been clearified by 2 hours of immersion in Bio-Clear, a solvent of vegetable origin in substitution of Xilene (Bio-Optica, Milano), then rehydrated by a descending alcohol series and immersion in distilled water. Then, the sections were incubated in a H2O2-solution (0,3 %) for 10 minutes to block the activity of endogenous peroxidases. To attain an optimal antigen exposition the slices were first

treated for 60 minutes in HCl 2N, which determines the opening of the DNA double helices, and then were transferred for 20 minutes in PBS at 0°C, to ensure the helices being separated. The treatment with formalin determines the cross-linking formation between the proteins which must be cut to avoid difficulties in antigene-antibody recognition, so the sections were incubated in 0,1 % tripsin (crude from porcine pancreas, Sigma Chem. Co.) for 30 minutes at a temperature of 37°C. The next step plans to block the natural epitopes which could lead to an unspecific binding of the secondary antibody. To ensure blocking, the slices were incubated in for 30 minutes in a 10 % solution of goat serum (Dako code n° X0907). Next, the slices were washed 3 times in PBS and then transferred for 2,45 hours in a humidity chamber in presence of the primary antibody (mouse monoclonal IgG, Necton Dickinson cat. N° 347580) anti-BrdU diluted 1:50. After this step, the secondary antibody (anti Fc) conjugated to a peroxidase (peroxidase goat anti ï mouse Sigma Aldrich A 2554), dilution 1:200, was added and incubation was done under the same conditions as the primary antibody for other 30 minutes. To confirm optically the binding site antigene-antibody, a solution of 3,3ôï diaminobenzidine (DAB) with a concentration of 0,5mg/ml in TRIS-HCl was used. The sections were incubated in this solution for 7 minutes in addition of 2µl of H2O2 (30%). Counter colouring was achieved by immersion in hematoxylin (Harris) for 4 seconds. At the end the slices have been dehydrated by an increasing alcohol series and the covered by a cover glass and fixed with a special synthetic glue called Bio-Mount (Bio-Optica). Labeling index (LI) was expressed as number of BrdU-positive hepatocyte nuclei/100 nuclei. Results were expressed as means ± S.E. of 3-4 animals per group. At least 3000 hepatocyte nuclei per liver were scored.

### 2.5.3 Ki-67 Immunohistochemistry

Liver sections have been clarified with Bio Clear (Bio-Optica, Milano), performing 2 steps of 15 minutes at room temperature. For hydration, the sections were transferred in a descendent alcohol series (100%, 90%, 80%, 70%), 5 minutes for every passage. To inhibit endogenous peroxidases the sections were incubated in a 3% H2O2 in Methanol solution for other 30 minutes followed by 5 minutes in distilled water. Unmasking of antigen sites was achieved by transferring the sections in

Sodium Citrate Buffer (10mM Sodium Citrate, 0,05% Tween 20, pH 6,6) and then for 2 cycles (12 minutes each) in micro wave (900 W). For cooling down, the sections then were incubated for 20 minutes at room temperature and, then, underwent two washing steps, 5 minutes each, first in distilled water, later in TBS (Tris Buffered Saline) 1x. Preincubation of sections was performed in a 5% saturated Goat serum (Dako code n° X0907) for 60 minutes at room temperature in humidity chamber. After incubation over night with the primary antibody anti-Ki-67 (1:25 in PBS monoclonal Rat anti-mouse, Dako code n° M7249), done in humidity chamber at 4°C, sections were incubated for 1 hour with the secondary antibody (DakoCytomation Polyclonal Rabbit anti-Rat Biotin; Diluted 1:200) at room temperature. Next they underwent two washings in TBS 1x ,5 minutes each, and after were incubated with the reagent ABC (Vector Laboratories, Vectastein Universal; Cat. No. PK-6100). The reagent was prepared by adding 2 drops of reagent A and B to 5 ml of TBS. To visualize the antigene-antibody binding sites Dako Liquid DAB Substrate Chromogen System 20 µl/ml was used (as described previously in chapter 5.2). Other 2 washing steps, 3 minutes each, were performed, first in TBS, then in distilled water, followed by counter colouring in hematoxylin (Harris) for 20 seconds at room temperature. Next the slices were washed briefly in water. For dehydration slices were transferred to an increasing alcohol series (70%, 80%, 90%, 100%) . To complete the procedure, slices were put in Bio-Clear for fixation and then covered with a cover-slide. Determination of Labeling Index was performed as described in chapter 5.2.

## 2.6 RNA Extraction

Total RNA of frozen liver was extracted after sacrifice and a fragment of liver tissue of about 50-100 mg per sample was used. The fragment was homogenized in 1 ml of TRIzol reagent (Invitrogen, No 15596-018). A centrifugation for 10 minutes at 12.000 x g at 4°C served to remove insoluble material from the homogenate and the supernatant was transferred into new tubes and incubated for 5 minutes at 21 °C to allow the complete dissociation of the nucleoprotein complexes. 0,2 ml of chloroform was added to the samples and then incubated for 3 minutes at room temperature and centrifuged for 15 minutes at 12.000 x g (4°C). The samples were so separated into 3 phases whereas the upper aqueous phase contained RNA and was transferred into

new tubes. To achieve a precipitation of the RNA 0,5 ml of isopropyl alcohol (Fischer Scientific, IL, USA; Cat No. A460-1GAL) was added to the samples followed by an incubation of 10 minutes at room temperature. After another centrifugation of 10 minutes (12.000 x g, 4 °C) the RNA formed a pellet on the bottom of the tube. The supernatant was removed and the pellet was washed by adding 1 ml of 75% Ethanol (Pharmacon). After vortexing the samples for 15 seconds they were centrifuged for 5 minutes at 7.500 x g ; 4 °C and the supernatant was removed again. At the end of the procedure the pellet was dried and 60 µl of Dnase/Rnase free distilled water (Invitrogen) was added. The tubes then were transferred into freezer for storage at ï 80 °C.

To determine the concentration and the purity of the extracted RNA the samples were analysed in a photospectrometer (Nano Drop) at wave lengths of 260 and 280 nm. The maximal absorbance of nucleic acids is at 260 nm and thus served to evaluate the concentration of the RNA. The ratio of the wave lengths 260/280 (maximal absorbance of proteins) nm permits to evaluate the purity of the sample. RNA quality was also confirmed by gel electrophoresis (1% agarose gel).

## 2.7 Real Time (quantitative) PCR and semiquantitative PCR

### 2.7.1 cDNA-Synthesis

To achieve retro transcription of RNA the kit *High Capacity cDNA Reverse Transcription* was used (Applied Biosystems). This kit contains reagents that when combined, form a 2X Reverse Transcription (RT) Master Mix. To prepare this mix the components of the kit have been unfrozen and transferred into ice. The quantities used for preparation of the kit are shown in the following table:

| Component | Volume(µl)/Reaction Kit |
|---|---|
| 10X RT Buffer | 2.0 |
| 25X dNTP Mix (100mM) | 0,8 |
| 10X RT Random Primers | 2,0 |
| MultiScribe Reverse Transcriptase | 1,0 |
| Rnase Inhibitor | 1,0 |
| Nuclease-free H20 | 3,2 |
| Total per reaction (Kit) | 10,0 |

After preparation the kit was incubated on ice. Per 10 µl of this mix 10 µl of sample were added containing 2000 ng of RNA.

The tubes then were sealed and centrifuged for a short period and transferred on ice till start of thermocycling. The thermocycler was programmed as indicated in the next table:

| | **Step 1** | **Step2** | **Step 3** | **Step 4** |
|---|---|---|---|---|
| Temperature | 25 °C | 37 °C | 85 °C | 4 °C |
| Time | 10 min | 120 min | 5 sec | |

## 7.2 Analysis of CTGF expression by semi-quantitative PCR

cDNA was amplified by using the semi-quantitative PCR method. A thermostable DNA-polimerase (Platinum Taq DNA Polmerase; Invitrogen) was added to the cDNA template in presence of a couple of primers specific for the gene sequence of CTGF. The following primers were used:

5ôï GCGCCAAGCAGCT GGGAGAA ï ó3 (forward)

5ôï GCAGCT AGGGCAGGGCCAAC ï ó3 (reverse)

Those primers bind to and amplify a fragment of the gene CTGF with a length of 364 base pairs, specific for mice and rats.

To perform PCR a mix of reagents was used (Platinum Taq DNA Polymerase kit); components are shown in the following table:

| **Components** | **Volume (µl)** |
|---|---|
| 10X PCR Buffer, Minus Mg | 5,0 |
| 10 mM dNTP mixture | 1,0 |
| 50 mM MgCl2 | 1,5 |
| Primer mix (10 µM each) | 3,0 |
| Platinum Taq DNA Polymerase | 0,2 |
| Water (Dnase/RNase Free) | 19,3 |
| ***Final Volume*** | 30,0 |

To the final volume 20 µl of sample at a concentration of 2,5 ng/µl were added.

Gene amplification was performed in a thermalcycler (9700 GeneAmp Thermalcycler; Applied Biosystems) using the following steps:

A step at 94°C for 5 minutes activated the polymerase, whereas the following 32 cycles of amplification were done at 94 °C for 30 sec, 58°C for 30 sec and 72°C for 1 minute. To confirm the ampflification of the target gene CTGF, the expression of the constitutively active gene 18 S was analyzed. To perform this analysis the ribosomal RNA of gene 18 S was examined to evaluate eventual variations of cDNA quantity. Conditions and parameters of semi quantitative PCR used for 18 S were the same as those used for CTGF with the exception of the primers which amplify for a fragment of 479 base pairs

5ôAAACGGCTACCACATCCAAG-3ô(forward)

5ôCCCTCTTAATCATGGCCTCA-3ô (reverse)

and the number of cycles used for amplification (20 cycles) due to the major expression of the gene 18 S compared to the expression of the gene encoding for CTGF.

### 2.7.3 Analysis of Survivin expression by Real time PCR

The reaction of amplification (in a volume of 10 µl) was performed while using 10 ng of cDNA, 5 µl dye TaqMan Gene Expression Master Mix (Applied Biosystems), 0,5 µl primer and probes which are specific for TaqMan (Mm00599749_m1) and amplify an amount of mRNA of the survivin gene in an thermalcycler (ABI PRISM 7300; Applied Biosystems). Parameters used consisted in an initial activation step at 50°C for 2 minutes and 95°C for 10 minutes, whereas 40 cycles of amplification were performed at 95°C for 15 sec and 60°C for 1 minute. The samples were analysed in series of three and beside the target gene, also the housekeeping gene beta-actin (beta-actin Endogenous Control, Applied Biosystems) was examined to evaluate eventual variations in the cDNA quantity. Relative differences between samples were evaluated with the æCt method using as reference the gene expression levels of the control group.

## 2.8 Densitometric Analysis

Densitometric analysis of western blot bands was done by using the program ImageJ ( Wayne Rasband, National Institute of Health).

## 2.9 Statistical Analysis:

All data are expressed as the mean ± Standard Error (SE) unless otherwise indicated. Differences between groups were compared by performing t-test while using the program BIOSTAT 1.0 (AnalystSoft, Vancouver, Canada).

# 3 Results

## 3.1 Aim 1: To investigate whether Hippo pathway is involved in control of compensatory regeneration after 2/3 PH in rats and mice.

*3.1.a Analysis of YAP expression during liver regeneration in F-344 rats*

As expected, peak of S-phase was evident 24 hours after 2/3 PH in rats (Fig.1 A , B and C), with return to control values 7 days after surgery. Indeed, the Labeling Index was found to be 18,2%, 11,6% and 0,69% in treated animals sacrificed at 24 h, 48 h and 7 days after 2/3 PH, respectively, vs a percentage of 0,52 in controls. The proliferative response seen in rats subjected to 2/3 PH in respect of untreated controls was confirmed by the analysis of hepatic protein levels of the cell cycle regulatory proteins PCNA and cyclin D1 (Fig. 2). In order to evaluate if during liver regeneration YAP expression correlates with liver enlargements, being activated while liver is reaching its original size and inhibited at seven days, when the process has been completed, we first used western blot analysis of total YAP (phospho-YAP and YAP) and phospho-YAP protein expression. As shown in Fig. 2, in rats subjected to 2/3 PH, levels of YAP protein correlate well with hepatocyte proliferation, being increased at 24 and 48 hours after PH, when hepatocytes were actively dividing, and decreased near to control values at seven days, when the liver has reached its original size. Due to the fact that the antibody which recognizes total YAP protein cannot discriminate between the active and the inactive (phosphorylated) form of YAP, in order to determine the contribution given by the two forms to total YAP expression, we also analyzed phospho-YAP protein expression. Data obtained (Fig.2) demonstrates that the inactive phosphorylated form of YAP was detectable at any tested time points, thus, also in those samples in which active YAP expression was low or absent, namely in controls and 7-days after PH. The active form of YAP was expressed at 24 and 48 hours after 2/3 PH, time points in which the liver was regenerating. Indeed, only at that time points total YAP expression was higher than that of phospho-YAP.

YAP protein acts as a co-activator, its induction leading to activation of YAP-dependent transcription factors. Among these factors, TEAD (Tea domain/transcription enhancer factor) seems to be a key mediator of YAP function

(Zhao et al 2008), with its transcriptional activation leading to induction of YAP target genes, such as Connective Tissue Growth Factor (CTGF) and survivin. Following, to determine if increased levels of YAP protein were associated to induction of YAP-mediated gene transcription, we analyzed, by semi-quantitative PCR, mRNA levels of the candidate YAP target gene CTGF. Data obtained (Fig.3) showed that the kinetic of CTGF mRNA expression levels essentially correlated with the kinetic of YAP protein expression, being increased compared to control values 24 and 48 hours after PH, with expression values at 7 days essentially similar to those observed in control animals.

*3.1.b Analysis of YAP-expression in CD-1 mice*

The peak of S-phase was evident 48 hours after 2/3 PH in mice (Fig. 4 A, B and C), with return to control values 7 days after surgery. Indeed, at 48 hours the L.I. was found to be of 28,8% in PH animals respect to a percentage of 13% and of 0,5% in animals sacrificed at 24 hours and 7 days after 2/3 PH, respectively (Fig. 4 C) vs a percentage of 0,1 of controls. In order to correlate L.I. with cell cycle protein expression, western blot analysis was performed pooling two liver protein samples for each time point. The higher DNA synthetic activity seen in PH animals was confirmed by the analysis of cell cycle proteins (Fig. 5) cyclin D1, a G1 phase marker, PCNA, a cell cycle regulatory protein expressed during G1 and S phase and of cyclin A, specifically expressed during S phase. As shown in Fig. 5, all these proteins were detected at significantly higher levels at 24 hours and 48 hours in treated animals in respect of controls, with cyclin A being exclusively expressed at 48h, due to its high specificity for S phase. As already observed in the rat model, in mice subjected to 2/3 PH, total Yap protein levels correlate with hepatocyte proliferation, being, increased at the time of maximal proliferative response, namely 24 and 48 hours, while phospho-YAP was expressed at similar levels at any tested time points (Fig. 6 A); densitometric analysis for total Yap and phospho-YAP, normalizing their expression in respect of actin, demonstrated, also in this case, that the active form of YAP was expressed at 24 and 48 hours after 2/3 PH, time points in which the liver was regenerating (Fig. 6 B ). Semi-quantitative PCR analysis revealed that the increased protein levels of Yap observed at 24 and 48 hours, were associated to higher CTGF mRNA levels (Fig. 7A) only at 24 hours with return to control values at 7 days (a time point characterized by a strong phospho-YAP protein expression). Next, we analyzed

the gene expression of another candidate YAP-target gene, namely survivin, by Real Time PCR. Data obtained (Fig. 7B) showed that survivin mRNA levels were strongly induced 48 hours after PH in mouse liver, being poorly expressed before and after that time point.

### 3.2 Aim 2: Analysis of YAP expression during compensatory regeneration in aged mice.

Western blot analysis of cell cycle proteins from 12 to 36 hours demonstrated that cell cycle entry was delayed and reduced in liver of aged animals (Fig. 8). Interestingly, as evaluated by densitometric analysis, comparing total and phospho-YAP protein expression normalized in respect of actin, active YAP levels were much higher in the liver of aged animals compared to young ones (Figures 9 A and B). The kinetic of increase of YAP protein levels were very different between the two groups, being in young animals essentially related to cell cycle progression, with a progressive increase from 12 to 36 hours, opposite to what observed in aged mice, in which total YAP expression levels were shown to be strongly increased as soon as 12 hours. Gene expression analyses of the YAP target genes CTGF and survivin were done by semi quantitative PCR and Real Time PCR on livers of young and aged mice collected from 12 to 36 hours after 2/3 PH. Data obtained (Fig. 10) demonstrated that, while CTGF mRNA levels increased in aged mice, starting from 12 hours after PH and maintaining a high expression thereafter, in young animals CTGF mRNA levels were high at 12 hours, with a decrease at 24 hours and returned to control values at 36 hours. Opposite, survivin mRNA levels were found in both groups to peak 36 hours after 2/3 PH, with decreased expression values from 12 to 24 hours compared to those found in control animals (Fig. 11 A). Itôs noteworthy, that in both young and aged mice survivin mRNA levels increased about 7 folds vs their respective control values at 36 hours after PH. By comparing the 36 hours survivin mRNA levels of both, the hepatectomized young and aged mice in respect of control aged animals, we found a 7 fold higher elevation in young animals compared to aged ones (Fig. 11 B).

### 3.3 Aim 3: Analysis of YAP expression after partial hepatectomy to be done in an enlarged liver.

The results showed that the liver weight/body weight ratio was much greater in mice which have received TCPOBOP in respect of those which were submitted to PH only (Fig 1 A left). That the increased liver mass observed in PH animals pretreated with TCPOBOP was a true regenerative response and not simply a hypertrophic response was shown by the analysis of the labeling index, that was similar in both the groups (76.3 and 74.1% in TCPOBOP plus PH and PH alone, respectively, vs 0,9% in controls, Figure 12 A right). Accordingly, western blot analysis of cell cycle proteins, namely cyclin D1 and PCNA, showed a similar expression pattern between PH and PH plus TCPOBOP animals (Fig. 13 A). In line with our hypothesis, when we analysed total YAP protein levels, we found that they were drastically decreased in livers of animals pretreated with the mitogen; this was associated with a strong increase of phospho-YAP protein levels in the same group (Fig. 13 A). In its active state YAP protein is located in the cell nucleus. To evaluate active YAP protein expression, we isolated nuclear protein extracts from livers of PH and TCPOBOP plus PH animals. Western blot analysis done on those nuclear extracts confirmed a decreased YAP activity in TCPOBOP plus PH livers (Fig. 13 B), with expression values near to be absent, as observed in control animals, and, additionally, showed that the co-activator was in an active state in PH animals.

# 4 Discussion

Accumulating evidence suggests the existence of a ósize checkpointô operating at the level of the organôs total mass, rather than regulating the size or the number of the constituent cells. Such a checkpoint could be responsible for blocking liver regeneration after partial hepatectomy, once the organ has regained its initial size. Recent genetic studies in Drosophila as well as in mammals delineated the importance of the Hippo (hpo) signalling pathway to play a key role in restricting organ size by controlling both cell proliferation and apoptosis (Edgar 2006; Harvey and Tapon 2007; Pan 2007). In mammals, dysregulation of the Hippo pathway results in activation of the potent growth promoter YAP, through loss of the kinase cascade (that normally inactivates YAP); upon dephosphorylation, YAP translocates into the nucleus where it binds to transcription factor(s) and regulates transcription of the Hippo pathway target genes. On this basis, the aim of this study was to analyse, the possible involvement of YAP in liver regeneration after partial hepatectomy in rodents. In particular, our interest was focused on determining:

- if during liver regeneration YAP expression correlates with liver enlargements (Aim 1);

- if impaired liver regeneration during ageing could be related to a sustained Yap activation (Aim 2);

- if YAP is a mediator of liver cell proliferation organ size-restricted (Aim 3).

### 4.1 Aim 1:

Data obtained clearly demonstrate that YAP protein expression during liver regeneration in rats and mice effectively correlates with liver enlargement, being activated when the liver is reaching its original size and inhibited once the process has been completed. Indeed, densitometric analisys of total (phospho-YAP + active YAP) and phospho-YAP, the inactive form of YAP, after normalization of their expression using actin protein as endogenous positive controls, allowed us to discriminate between the active and inactive form of the protein and to conclude that the active form of YAP was expressed when the liver was regenerating, namely 24

and 48 hours, while decreased near to control values when the regenerative process was completed. Analysis of the YAP target gene CTGF, have shown that in rat liver an increase in YAP protein levels correlate with an increase in YAP-dependent transcriptional activity, evaluated as CTGF mRNE expression levels. Opposite, such a strict correlation was not observed in mouse liver, where CTGF mRNA expression levels increased in respect of control values only 24 hours after PH, being at 48 hours and 7 days essentially similar to those observed in control animals. The difference between CTGF expression in mouse and rat liver observed in our study, seems to suggest that in mouse liver transcription of CTGF gene is a more regulated event, restricted to cell cycle phases earlier than S phase. Data obtained appear to be in contrast with those obtained by Ujike et al, 2000. In that study authors analyzed CTGF mRNA expression levels by Northern blot after 2/3 PH in rat liver starting from 2 hours to 7 days after surgery. They found that CTGF gene expression increased until 6 hours after partial hepatectomy and returned to control levels thereafter. These data suggest that induction of CTGF gene expression is an early event in liver regeneration and should be evaluated at earlier time points than those we have considered, from 24 hours to 7 days after 2/3 PH, chosen to observe a slight correlation between YAP expression and liver size. Nevertheless, our findings of increased CTGF mRNA expression also at 24 hours in mice, and from 24 to 48 hours in rats, could be related to the higher sensibility of the method we employed to evaluate gene expression (semi-quantitative PCR) compared to that used in the study of Uljike et al (Northern blot). Due to availability of a probe which only recognized mouse survivin gene, we also evaluated the expression of the candidate YAP- target gene survivin in mice after 2/3 PH. Data obtained showed a strong induction of survivin mRNA expression 48 hours after PH in mouse liver, being poorly expressed before and after that time point. The findings of increased survivin gene expression during liver regeneration after 2/3 PH starting from the time point corresponding to S phase peak, namely 48 hours in mouse liver, and with return to control values 7 days after 2/3 PH, are supported by the study of Baba et al, 2008. Indeed, when these authors analyzed protein and mRNA levels of survivin after 2/3 PH in rat liver, they found that they were significantly increased starting from 24 hours after PH, corresponding to S phase peak in rat liver, until 72 hours, with return to control values at 7 days. Summarizing, we can conclude from our data that YAP candidate target genes CTGF and survivin are regulated during liver regeneration

after 2/3 PH according to what reported in literature; however, based on the fact that their correlation with YAP has been essentially analyzed in tumors (Xie et al, 2001; Dornhofer et al. 2006; Zhao et al, 2008; Lehner et al, 2002; Dong, 2007), itôs still unclear if their regulation during compensatory regeneration is really YAP-dependent. It could be possible that CTGF and survivin only mediate the oncogenic potency of YAP, as already reported, while in more physiological conditions, their regulation could be mediated by factors, other than YAP, modulating inhibition of apoptosis, through survivin, and tissue repair, through CTGF, associated to liver regeneration. Data until reported didnôt allow us to distinguish between the role of YAP and that of the common cell cycle genes during liver regeneration, being their expression kinetic essentially similar.

### 4.2 Aim 2:

So we decided to evaluate YAP expression in condition of impaired liver regeneration, namely liver regeneration after 2/3 PH in aged mice. Several studies on the regenerative response that follows 2/3 PH have shown that the response to PH was both delayed and reduced with aging in rats and mice, being hepatocytes entering DNA synthesis in older animals (12 months of age) only 30% versus a percentage of 99% in young ones (Bucher et al, 1964; Fry et al, 1984). On this basis, we hypothesized that if YAP activation was really dependent upon liver size, results from aim 2 should have demonstrated a prolonged and robust YAP activation in liver of hepatectomized aged animals respect to young ones, despite of a reduced expression of cell cycle proteins in the first group. Indeed, the impairment of liver regeneration during aging, could lead to a sustained recruitment of proteins that ñsenseò the decreased liver mass and trigger an hepatocyte proliferation response, even if delayed and reduced, in an attempt to re-establish the original liver size related to organ homeostasis. Data obtained are in line with our hypothesis, showing a robust activation of YAP in aged animals as soon as 12 hours after PH, as evaluated by densitometric analysis comparing total and phospho-YAP protein expressions normalized with actin, despite of a detectable expression of the cell cycle proteins PCNA and cyclin D1 only at 36 hours after surgery. Opposite, in control hepatectomized young mice YAP expression essentially reflected progression into cell cycle, as already evaluated. Accordingly, analysis of CTGF gene expression in aged liver, evaluated by semi quantitative PCR from 3 to 36 hours after

2/3 PH, showed an increase in CTGF mRNA levels at any time points analyzed. Opposite, in young mice CTGF mRNA levels appeared to be increased only until 24 hours after PH, with return to control values thereafter; this confirmed what already observed in Aim 1, when we have analyzed CTGF mRNA expression in young animals starting from 24 hours to 7 days after 2/3 PH and detected an increase in CTGF gene expression only at 24 hours. Surprisingly, when we have analyzed survivin gene expression by Real time PCR at the same time points, from 3 to 36 hours after 2/3 PH, data obtained showed a similar expression pattern between young and aged animals. Indeed, in both groups survivin mRNA levels increased 36 hours after 2/3 PH, with values near to control animals before and after that time point, even if in young animals that increase was 5 fold more pronounced than in aged mice. As already discussed, in Aim 1 we observed survin gene expression increased 48 hours after 2/3 PH in young mice with return to control values 7 days after 2/3 PH, supporting a role of survivin in a cell cycle phase later than that in which CTGF gene is involved, namely S phase, according to what already observed by Baba et al during liver regeneration in rats. It is noteworthy that results obtained with Aim 2 demonstrated, when a survivin role in young mice during S phase or near to it was confirmed, that in aged animals survivin induction is anticipated, according to the early induction of YAP activity in this group. Indeed, it is to consider that, as liver regeneration after 2/3 PH is delayed during ageing, S phase occurs later than 36 hours, a time point at which cyclin D1, a G1 phase marker, became detectable in aged liver. Thus, results obtained in Aim 2 demonstrated an anticipated YAP activation after 2/3 PH during ageing despite of a delayed cell cycle entry, supporting a role of YAP as a cell cycle gene size regulated.

### 4.3 Aim 3:

In order to further exclude a YAP regulation merely dependent from signals related to cell cycle progression, and eventually to support the activation of YAP as a liver size-restricted process, we decided to analyze the role of YAP also in the opposite situation, that is during liver regeneration in an enlarged liver. It has been demonstrated that when 70% PH was performed in a liver made enlarged within 3 days by single injection of the mitogen TCPOBOP, a massive liver regeneration occurred, similar to that observed in mice subjected to PH alone (Columbano et al 2008). TCPOBOP is a mouse liver mitogen ligand of the nuclear receptor CAR, a

member of the steroid/thyroid hormone nuclear receptor superfamily. These ligand-activated nuclear receptors stimulate hepatocyte proliferation (Ledda-Columbano and Columbano 2003), acting as transcription factors. Notably, numerous early changes considered to be essential for liver regeneration after PH are not observed in nuclear receptor-mediated hepatocyte proliferation; the early signal transduction pathways involved in nuclear receptor-mediated hepatocyte proliferation are therefore quite different from those of liver regeneration (Ledda-Columbano et al 1998; Ledda-Columbano et al 2000a; Pibiri et al 2001). On this basis, the model of the mitogen-induced enlarged liver seemed to us to be particularly suitable to analyze the eventual dependence of YAP activation on liver size, unlike cell cycle common proteins. While data previously mentioned clearly suggest that the regenerative capacity after 70% PH is independent from the liver size at the time of surgery, the same should be not observed with YAP, if it is really a cell cycle regulator õsize restrictedò. Indeed, when 2/3 PH is performed on an enlarged liver, the original mass of the organ is recovered earlier in respect of what observed on a normal liver, so that if YAP expression is analyzed at 72 hours, a timepoint at which TCPOBOP+PH animals have been demonstrated to have the same liver weight of control animals, unlike PH animals (Columbano et al, 2008), we should be able to observe itôs up-regulation only in PH animals. Accordingly, we found that in an enlarged liver there was a notable difference between nuclear active YAP and cell cycle protein expressions 72 hours after 2/3 PH, being the former, in virtue of its organ size dependence, down regulated when the latter, merely related to cell cycle progression was up-regulated. Thus, phospho-YAP resulted greatly up-regulated in liver of TCPOBOP plus PH animals. Surprisingly, gene expression analyses of CTGF and survivin showed similar mRNA levels for the two genes in both PH and TCPOBOP plus PH groups, suggesting that YAP could not be the only determinant in survivin and CTGF regulation during compensatory regeneration. Indeed, factors other than YAP could exist that regulate expression of survivin and CTGF gene to inhibit the apoptotic process and to mediate the metabolic and adaptative responses associated to liver mass resection, in order to promote liver regeneration apart from liver size at time of 2/3 PH.

# 5 Conclusions

In conclusion, data obtained from this study clearly demonstrate, that during compensatory regeneration after 2/3 PH YAP acts as a key regulator strictly dependent from liver size, this aspect representing the discriminant that allows to distinguish it from classical cell cycle genes. In particular we have determined that:

- YAP is involved in liver regeneration after 2/3 PH, being increased when the organ is achieving its correct liver size and decreased when the regenerative process is terminated;

- YAP activation is sustained and anticipated during liver regeneration in aged mice, probably due to a decreased liver size present for a longer time, associated to impairment of the regenerative response after 2/3 PH during ageing, that determine an anticipated (survivin) and sustained (CTGF) activation of YAP-target genes;

- YAP activation is already abolished at 72 hours when 2/3 hepatectomy is done in an enlarged liver, due to excess of liver mass at surgery. When control liver size is attained further YAP recruitment is dispensable, supporting the dependence of YAP activation according to liver size.

# 6 References

Albrecht JH, Hansen LK; Cyclin D1 promotes Mitogen independent Cell Cycle Progression in Hepatocytes. Cell growth & differentiation (1999);10(6):397-404.

Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nature Medicine (1997); 3(8):917-21.

Apte U, Gkretsi V, Bowen WC, Mars WM, Luo JH, Shashikiran Donthamsetty S, Orr A, Monga SPS, Wu C, and Michalopulous GK. Enhanced Liver Regeneration Following Changes Induced by Hepatocyte-Specific Genetic Ablation of Integrin-Linked Kinase. Hepatology (2009); 50:844-851.

Bennett FC, Harvey KF; Fat cadherin modulates organ size in Drosophila via the Salvador/Warts/Hippo signalling pathway. Current Biology (2006);16(21):2101-10.

Bucher NLR, Swaffield MN, Di Troia JF; The influence of age upon the incorporation of thymidine-2-C14 into the DNA of regenerating rat liver. Cancer Research (1964); 24:509ï 512.

Buttitta LA, Edgar BA; How size is controlled: From Hippos to Yorkies. Nature Cell Biology (2007); 9: 1225-1227.

Callus BA, Verhagen AM, Vaux DL; Association of mammalian sterile twenty kinases, Mst1 and Mst2, with Salvador via C-terminal coiled-coil domains, leads to its stabilization and phosphorylation. The FEBS Journal (2006); 273:4264-4276.

Camargo FD, Gokhale S, Johnnidis JB, Fu D, Bell GW, Jaenisch R and Brummelkamp TR; YAP1 increases organ size and expands undifferentiated progenitor cells. Current Biology (2007); 17(23):2054-60.

Chan EH, Nousiainen M, Chalamalasetty RB, Schafer A, Nigg EA, Sillje HH; The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. Oncogene (2005); 24:2076-2086

Cho E, Feng Y, Rauskolb C, Maitra S, Fehon R, Irvine KD; Delineation of a Fat tumor suppressor pathway. Nature Genetics (2006); 38(10):1142-50.

Conlon I, Raff M: Size control in animal development. Cell (1999); 96:235-244.

Columbano A, Ledda-Columbano GM. Mitogenesis by ligands of nuclear receptors: an attractive model for the study of the molecular mechanisms implicated in liver growth. Cell Death & Differentiation ( 2003); 10:19-21.

Columbano A, Simbula M, Pibiri M, Perra A, Pisanu A, Uccheddu A and Ledda-Columbano GM; Potential utility of xenobiotic mitogens in the context of liver regeneration in the elderly and living-related transplantation. Laboratory Investigation (2008); 88:408ï 415.

Court FG, Wemyss-Holden SA, Dennison AR, Maddern GJ. The mystery of liver regeneration. British Journal of Surgery (2002); 89(9):1089-1095.

Dan I, Watanabe NM, Kusumi A; The Ste20 group kinases as regulators of MAP kinase cascades. Trends in Cell Biology (2001) 11, 220-230.

Deguchi M, Shiraki K, Inoue H, Okano H, Ito T, Yamanaka T, Sugimoto K, Sakai T, Ohmori S, Murata K, Furusaka A, Hisatomi H, Nakano T; Expression of survivin during liver regeneration. Biochemical and Biophysical Research Communications (2002); 297(1):59-64.

Denz U; Einfluss des IL-6/sIL-6R Fusionsproteins Hyper Interleukin-6 auf die Leber nach hydrodynamischer Transfektion in vivo. Freiburger Dokumentenserver (2004); URN: urn:nbn:de:bsz:25-opus-15274; URL: http://www.freidok.uni-freiburg.de/volltexte/1527/.

Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA, Gayyed MF, Anders RA, Maitra A, Pan D. Elucidation of a Universal Size-Control Mechanism in Drosophila and Mammals. Cell (2007); 130:1120ï 1133.

Dornhöfer N, Spong S, Bennewith K, Salim A, Klaus S, Kambham N, Wong C, Kaper F, Sutphin P, Nacalumi R, Hoeckel M, Le Q, Longaker M, Yang G, Koong A and Giaccia A; Connective Tissue Growth Factor-Specific Monoclonal Antibody; Therapy Inhibits Pancreatic Tumor Growth and Metastasis. Cancer Research (2006); 66(11):5816-27.

Edgar BA. From cell structure to transcription: Hippo forges a new path. Cell (2006); 124(2):267-73.

Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Journal of Hepatology (2006); 43: 45-53.

Fausto N. Liver regeneration. Journal of Hepatology (2000); 32: 19-31.

Fausto N; Hepatocyte differentiation and liver progenitor cells. Current Opinion in Cell Biology (1990); 2(6):1036-42.

Fry MF, Silber J, Loeb LA, et al. Delayed and reduced cell replication and diminishing levels of DNA polymerases-a in regenerating liver of aging mice. Journal of Cellular Physiology (1984);118:225ï 232.

Halbleib JM, Nelson WJ; Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. Genes & Development (2006); 20(23):3199-214.

Hamaratoglu F, Willecke M, Kango-Singh M, Nolo R, Hyun E, Tao C, Jafar-Nejad H, Halder G; The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. Nature Cell Biology (2006); 8:27ï 36.

Harvey K, Tapon N; The Salvador-Warts-Hippo pathway - an emerging tumour-suppressor network. Nat. Rev. Cancer (2007); 7(3):182-91.

Harvey KF, Pfleger CM, Hariharan IK; The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell (2003); 114:457-467.

Higgins GM, Anderson RM; Experimental pathology of the liver. Restoration of the liver of the white rat following partial surgical removal. Archives of Pathology & Laboratory Medicine (1931); 12:186-202.

Iakova P, Awad SS, Timchenko NA. Aging reduces proliferative capacities of liver by switching pathways of CEBP/al pha growth arrest. Cell (2003); 113:495ï 506.

Kishimoto T, Akira S, Taga T; Interleukin-6 and its receptor: a paradigm for cytokines. Science (1992); 258:593ï 597.

Koniaris LG, McKillop IH and Schwartz SI (2003). Liver regeneration. Journal of the American College of Surgeons (2003); 197:634ï 659.

Kountouras J, Boura P, Lygidakis NJ. Liver regeneration after hepatectomy. Hepato-Gastroenterology (2001); 48:556-562.

Ledda-Columbano GM, Curto M, Piga R, et al. In vivo hepatocyte proliferation is inducible through a TNF and IL-6-independent pathway. Oncogene 1998; 17:1039ï 1044.

Ledda-Columbano GM, Curto M, Piga R, Zedda AI, Menegazzi M, Sartori C, Shinozuka H, Bluethmann H, Poli V, Ciliberto G, Columbano A. In vivo hepatocyte proliferation is inducible through a TNF and IL-6-independent pathway. Oncogene (1998); 17(8):1039-44.

Ledda-Columbano GM, Pibiri M, Loi R, Perra A, Shinozuka H, Columbano A. Early increase in cyclin-D1 expression and accelerated entry of mouse hepatocytes into S phase after administration of the mitogen 1, 4-Bis[2-(3,5-Dichloropyridyloxy)] benzene. American Journal of Pathology (2000); 156(1):91-7.

Ledda-Columbano GM, Pibiri M, Cossu C, et al. Aging does not reduce the hepatocyte proliferative response of mice to the primary mitogen TCPOBOP. Hepatology (2004); 40:981ï 988.

Lehner R, Enomoto T, McGregor JA, Shroyer L, Haugen BR, Pugzhenthi U and Shroyer KR; Correlation of Survivin mRNA detection with histologic diagnosis in normal endometrium and endometrial carcinoma. Acta Obstetricia Gynecologica Scandinavica (2002); 81:162-167.

Marsman DS, Cattley RC, Conway JG, and Popp JA. Relationship of hepatic peroxisome proliferation and replicative DNA synthesis to the hepatocarcinogenicity of the peroxisome proliferators di(2-ethylhexyl)phthalate and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643) in rats. Cancer Research (1988); 48:6739-6744.

Matakatsu H, Blair SS; Separating the adhesive and signaling functions of the Fat and Dachsous protocadherins. Development (2006); 133(12):2315-24.

McClatchey AI, Giovannini M; Membrane organization and tumorigenesis ï the NF2 tumor suppressor, Merlin. Genes & Development (2005); 19:2265ï 2277.

Michalopoulos GK ; Liver Regeneration. Journal of Cellular Physiology (2007); 2:286-300.

Morello D, Lavenu A, Babinet C. Differential regulation and expression of jun, c-fos and c-myc proto-oncogenes during mouse liver regeneration and after inhibition of protein synthesis. Oncogene (1990); 5:1511-1519.

Okada T, You L, Giancotti FG; Shedding light on Merlinôs wizardry. Trends in Cell Biology (2007); 17:222ï 229.

Overholtzer M, Zhang J, Smolen GA, Muir B, Li W, Sgroi DC, Deng CX, Brugge JS, and Haber DA. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. Proceedings of the National Academy of Sciences (2006); 15:103(33):12405-10.

Pan D; Hippo signalling in organ size control. Genes & Development (2007); 21: 886-897.

Pibiri M, Ledda-Columbano GM, Cossu C, et al. Cyclin D1 is an early target in hepatocyte proliferation induced by thyroid hormone (T3). The FASEB Journal (2001); 15:1006ï 1013.

Pines J; Biochemical Journal (1995); 308:697-711.

Pines J; Cyclins and cyclin dependent kinases: a biochemical view. Biochemical Journal (1998); 308:696-711.

Praskova M, Xia F, Avruch J: MOBKL1A/MOBKL1B phosphorylation by MST1 and MST2 inhibits cell proliferation. Current Biology (2008), 18:311-321.

Sawada A, Kiyonari H, Ukita K, Nishioka N, Imuta Y and Sasaki H; Redundant roles of Tead1 and Tead2 in notochord development and the Regulation of Cell Proliferation and Survival. Molecular and Cellular Biology (2008); 28(10):3177-3189.

Sherr CJ, Roberts JM; Living with or without cyclins and cyclin-dependent kinases. Genes & Development (2004); 18(22):2699-711.

Starzl TE, Fung J, Tzakis A, Todo S, Demetris AJ, Marino IR, Doyle H, Zeevi A, Warty V, Michaels M. Baboon-to-human liver transplantation. Lancet (1993); 341:65-71.

Steinhardt AA, Gayyed MF, Klein AP, Dong J, Maitra A, Pan D, Montgomery EA, Anders RA ; Expression of Yes-associated protein in common solid tumors. Human Pathology (2008); 39:1582-1589.

Stocker E, Heine WD. Regeneration of liver parenchyma under normal and pathological conditions. Molecular and Cellular Biology (1971); 144:400-408.

Timchenko NA, Wilde M, Nakanishi M, Smith JR, Darlington GJ CCAAT/enhancer binding protein ŭ (C/EBPŭ) inhibits cell proliferation through the p21 (WAF-1/Cip-1/SD-1) protein. Genes & Development (1996); 10:804ï 815.

Udan RS, Kango-Singh M, Nolo R, Tao C Halder G; Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nature Cell Biology (2003); 5:914-920.

Ujike K, Shinji T, Hirasaki S, Hidenori Shiraha H, Nakamura M, Tsuji T, and Koide N; Kinetics of Expression of Connective Tissue Growth Factor Gene during Liver Regeneration after Partial Hepatectomy and D-Galactosamine-Induced Liver Injury in Rats. Biochemical and Biophysical Research Communications (2000); 277:448-454.

Wang X, Quail E, Hung N-J, et al. Increased levels of forkhead box M1B transcription factor in transgenic mouse hepatocytes prevent age-related proliferation defects in regenerating liver. Proceedings of the National Academy of Sciences USA (2001); 98:11468ï 11473.

Wu S, Huang J, Dong J, Pan D; Hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell (2003); 114:445-456.

Xie D, Nakachi K, Wang H, Elashoff R and Köffler PH; Elevated Levels of Connective Tissue Growth Factor, WISP-1, and CYR61 in Primary Breast Cancers Associated with More Advanced Features. Cancer Research (2001); 61:8917-8923.

Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, Xie J, Ikenoue T, Yu J, Li L, Zheng P, Ye K, Chinnaiyan A, Halder G, Lai ZC, Guan KL. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes & Development (2007); 21:2747-2761.

Zhao B, Lei QY, Guan KL; The Hippo-YAP pathway: new connections between regulation of organ size and cancer. Current Opinion in Cell Biology (2008a); 20:638-646.

Zhao B, Ye X, Yu J, Li L, Li W, Li S, Yu J, Lin JD, Wang CY, Chinnaiyan AM, Lai ZC, Guan KL. TEAD mediates YAP-dependent gene induction and growth control. Genes & Development (2008b), 22:1962-1971.

Zhou D, Medoff BD, Chen L, Li L, Zhang XF, Praskova M, Liu M, Landry A, Blumberg RS, Boussiotis VA et al; The nore1B/MST1 complex restrains antigen receptor-induced proliferation of naive T cells. Proceedings of the Nationall Academy of Sciences USA (2009) 105:20321-20326.

# 7 Appendix

## 7.1 Legends to figures

**Figure 1:**

Immunohistochemical analysis of BrdU-positive nuclei in Fischer rats; (A) control (B) animals submitted to 2/3 PH and sacrificed at 24 hours. All animals were given BrdU i. p. (100 mg/Kg b.w.) 2 hours before sacrifice.

(C) Labeling index of BrdU-positive nuclei in rats sacrificed 24, 48 and 168 hours after 2/3 PH. Each value represents the average of data obtained in three different samples.

**Figure 2:**

Western blot analysis of cell cycle proteins, cyclin D1 and PCNA, and of total and phospho-YAP in liver of Fischer rats submitted to 2/3 PH and sacrificed from 24 to 7 days. Each lanes represent an individual sample. Actin was used as loading control.

**Figure 3:**

Semi-quantitative PCR analysis of CTGF mRNA levels YAP in liver of Fischer rats submitted to 2/3 PH and sacrificed from 24 to 7 days. Each lane represents an individual sample. Data obtained were normalized using actin as endogenous control gene.

**Figure 4:**

Immunohistochemical analysis of Ki-67-positive nuclei in CD-1 mice;

(A) control (B) animals submitted to 2/3 PH and sacrificed at 48 hours.

(C): Labeling index of Ki-67-positive nuclei in mice sacrificed 24, 48 and 168 hours after 2/3 PH. Each value represents the average of data obtained in four different samples.

**Figure 5:**

Western blot analysis of cell cycle proteins, cyclin D1, PCNA and cyclin A in liver of CD-1 mice submitted to 2/3 PH and sacrificed from 24 hours to 7 days. Each lane represents a pool of two different samples. Actin was used as loading control.

**Figure 6:**

(A): Western blot analysis of total and phospho-YAP in liver of CD-1 mice submitted to 2/3 PH and sacrificed from 24 hours to 7 days. Each lane represents a pool of two different samples. Actin was used as loading control.

(B): Densitometric analysis of total and phospho-YAP expression normalized in respect of actin protein expression. Each value represents the average of data obtained in four different samples.

**Figure 7:**

(A): Semi-quantitative PCR analysis of CTGF mRNA levels in liver of CD-1 mice submitted to 2/3 PH and sacrificed from 24 hours to 7 days. Each lane represents a pool of two samples. Data obtained were normalized using actin as endogenous control gene.

(B): Real Time PCR analysis of survivin gene expression. Each value was determined using a pool of cDNAs from two different samples.

**Figure 8:**

Western blot analysis of cell cycle proteins cyclin D1 and PCNA in livers of young and aged CD-1 mice submitted to 2/3 PH and sacrificed from 12 to 36 hours. Each lane represents a pool of three samples. Actin was used as loading control.

**Figure 9:**

(A:) Western blot analysis of total and phospho-YAP in livers of young and aged CD-1 mice submitted to 2/3 PH and sacrificed from 12 to 36 hours after 2/3 PH. Each lane represents a pool of three samples. Actin was used as loading control.

(B): Densitometric analysis of total and phospho-YAP expression normalized in respect of actin protein expression. Aged animals on the left, young animals on the right. Each value represents the average of data obtained in three different samples.

**Figure 10:**

Semi-quantitative PCR analysis of CTGF mRNA levels in livers of young and aged CD-1 mice submitted to 2/3 PH and sacrificed from 12 to 36 hours. Each lane represents a pool of three samples. Data obtained were normalized using actin as endogenous control gene.

**Figure 11:**

Real Time PCR analysis of survivin gene expression, evaluated from 3 to 36 hours after 2/3 PH. Each value was determined using a pool of cDNAs from three different samples.

(A): Increases in surviving gene expression in hepatectomized young and aged animals vs their respective control values.

(B): Increases in surviving gene expression in hepatectomized young and aged animals vs aged mice control values.

**Figure 12:**

(A): Immunohistochemical analysis of BrdU-positive nuclei in control, PH and TCPOBOP plus PH CD-1 mice 72 hours after 2/3PH. All animals were given BrdU in drinking water at a concentration of 1 mg/ml, starting from time of surgery.

(B): Labeling index of BrdU-positive nuclei in CD-1 mice sacrificed 72 hours after 2/3 PH, with or without pre-treatment with TCPOBOP. Each values represent the average of data obtained in four different samples.

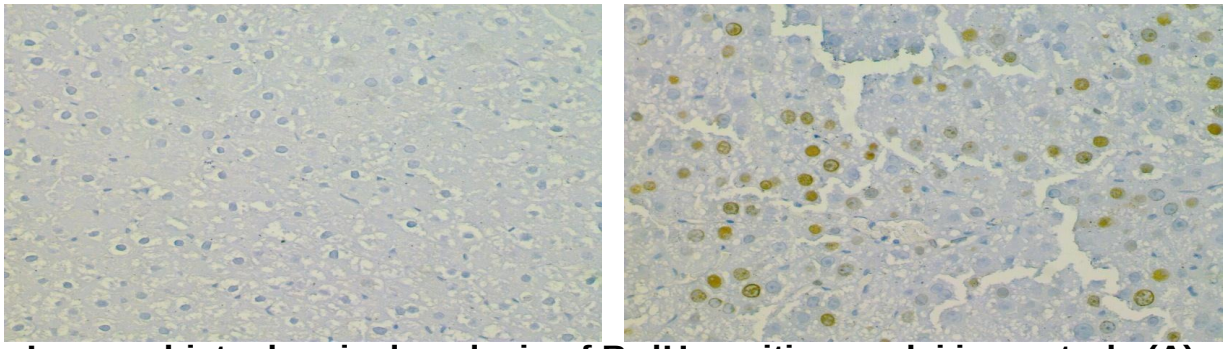
**Figure 13:**

(A): Western blot analysis of cell cycle proteins, cyclin D1 and PCNA, and of total and phospho-YAP in total liver protein extracts of CD-1 mice sacrificed 72 hours after 2/3 PH, with or without pre-treatment with TCPOBOP. Each lane represents an individual sample. Actin was used as loading control.

(B): Western blot analysis of total YAP on liver nuclear protein extracts of CD-1 mice sacrificed 72 hours after 2/3 PH, with or without pre-treatment with TCPOBOP. Each lane represents an individual sample. Albumin was used as loading control.

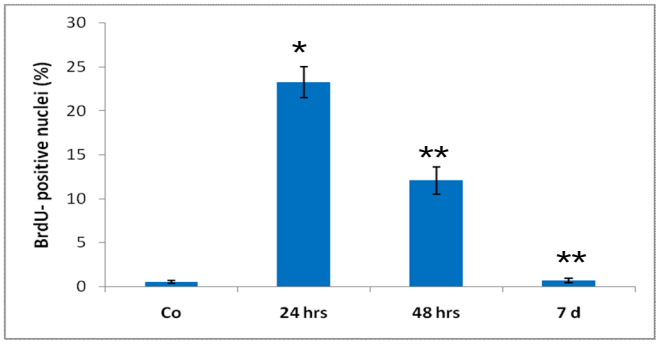
A

B



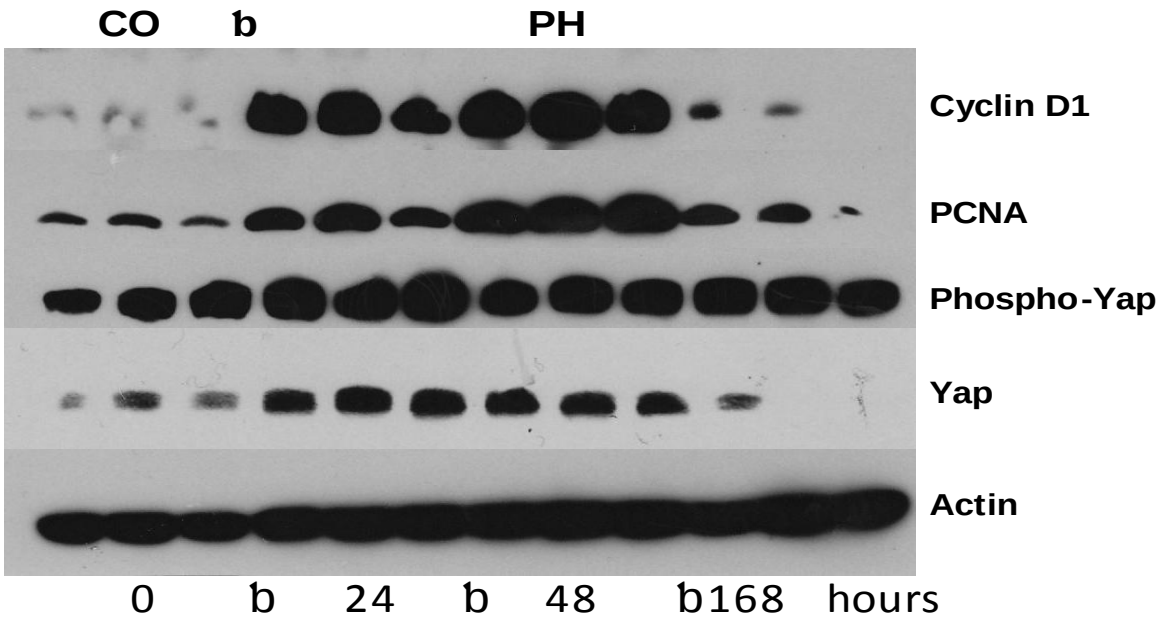
**Immunohistochemical analysis of BrdU-positive nuclei in controls (A) and 24 hours after 2/3 PH (B)**

C



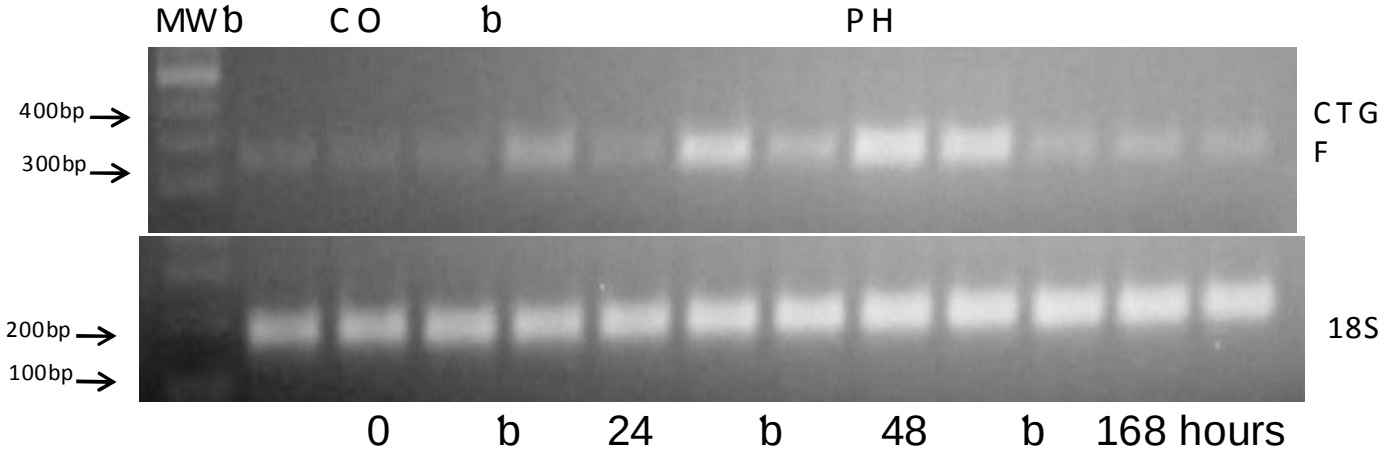

**Labeling Index**

**Fig. 1**




**Western blot analysis on total protein extracts**

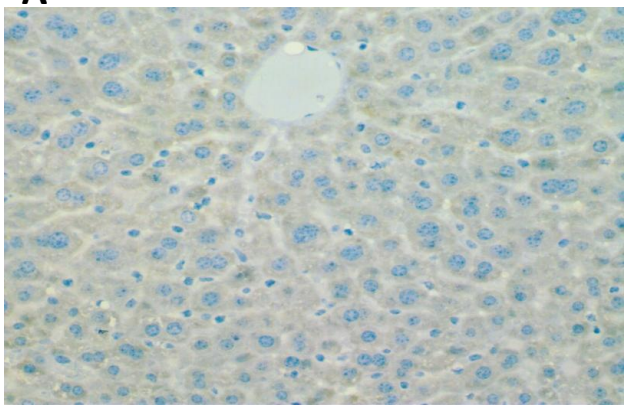
# Fig. 2



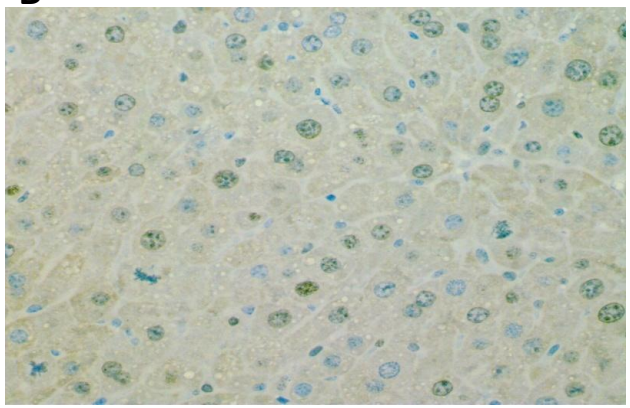

**Semiquantitative PCR analysis of CTGF gene expression**

# Fig. 3

A

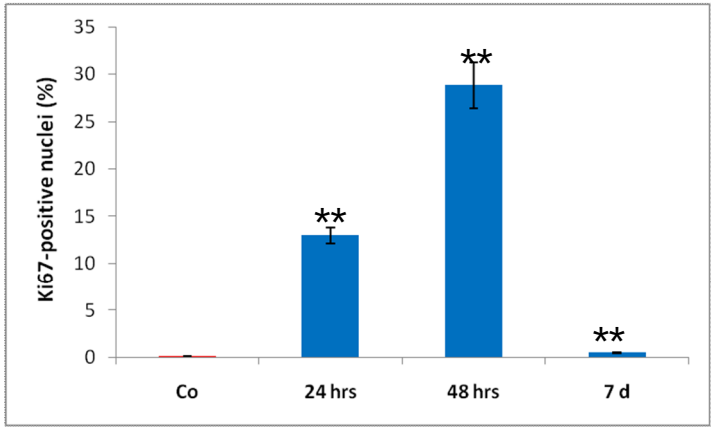


B



**Immunohistochemical analysis of Ki 67-postitive nuclei in controls (A) and 48 hours after PH (B)**

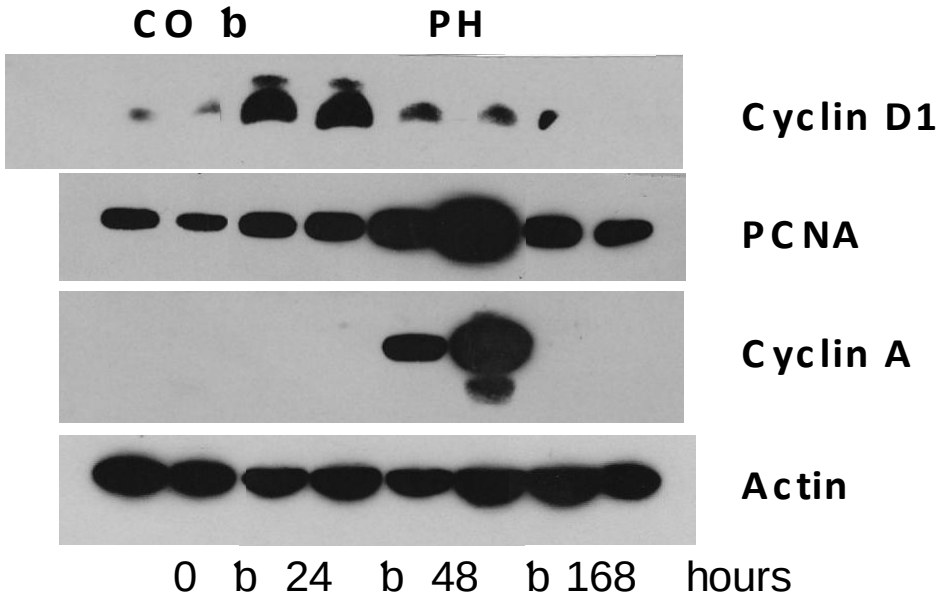
C




**Labeling Index**

*(*equal to p<0.005; ** equal to p<0,01; *** equal to p<0,001)*

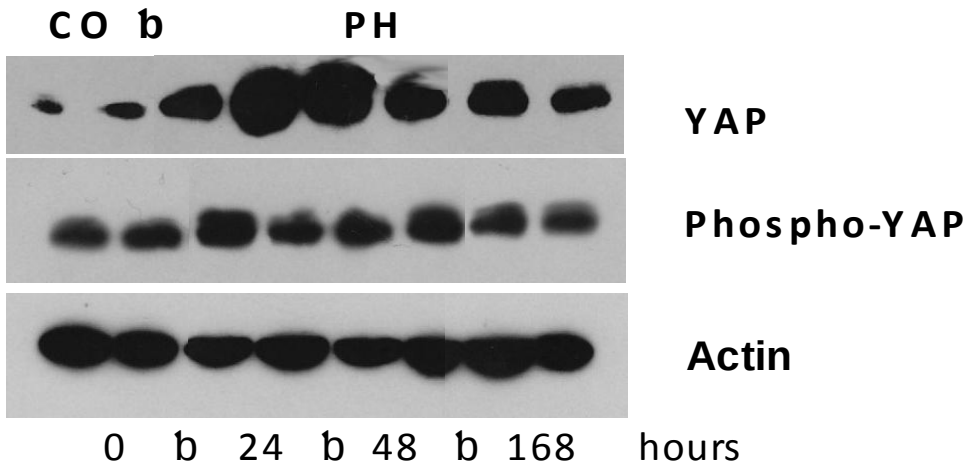
# Fig. 4




**Western blot analysis of cell cycle proteins**

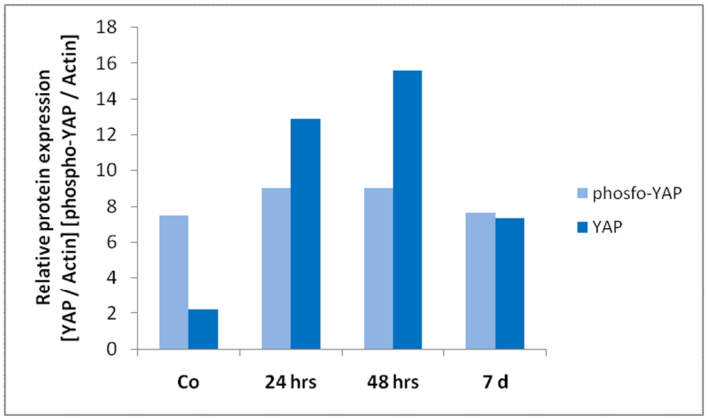
# Fig. 5

A




**Western blot analysis of total protein extracts**

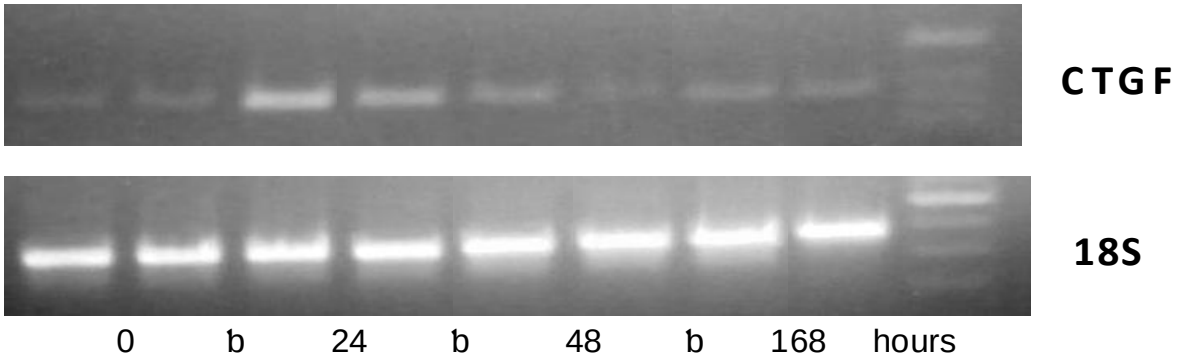
B




**Densitometric analysis of total- and Phospho-Yap proteins, normalized to Actin**

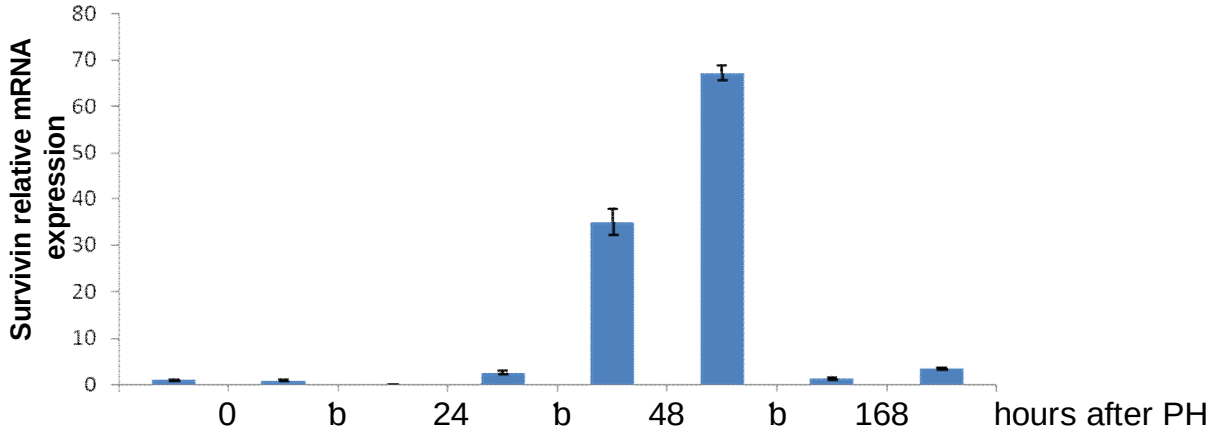
# Fig. 6

A



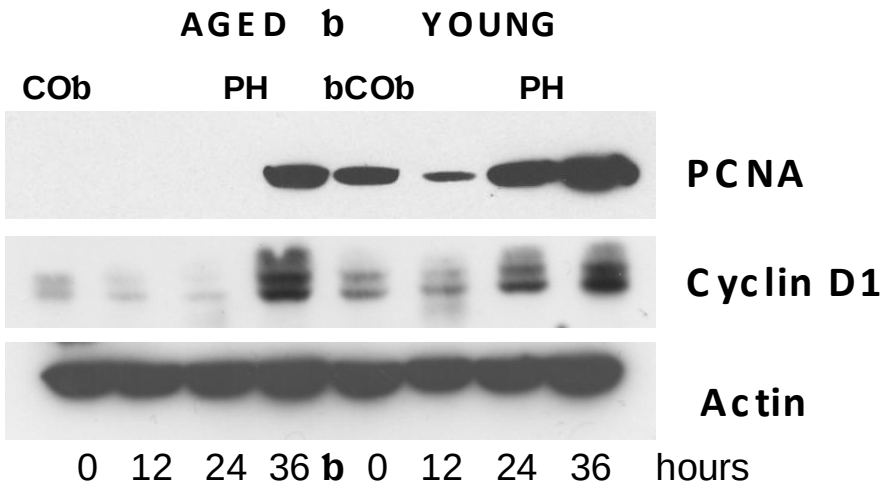

**Semiquantitative PCR analysis of CTGF gene expression**

B




**Real Time PCR analysis of survivin gene expression**

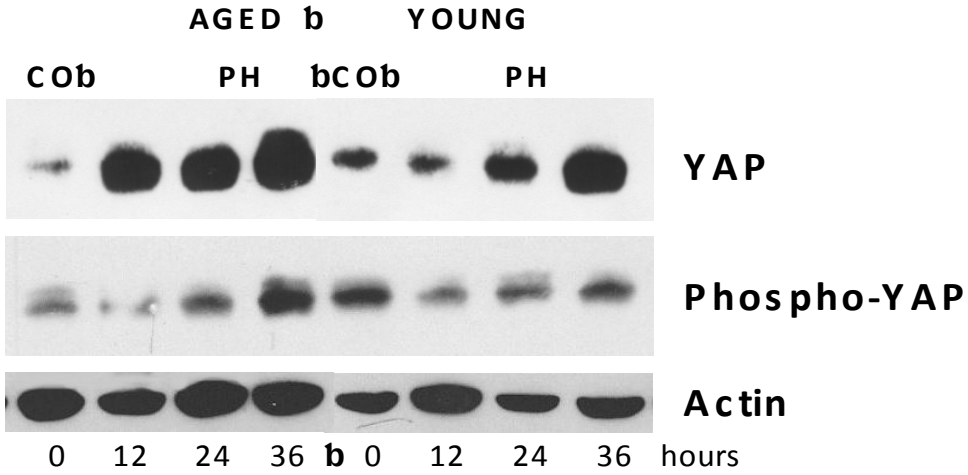
# Fig. 7




**Western blot analysis of cell cycle proteins**

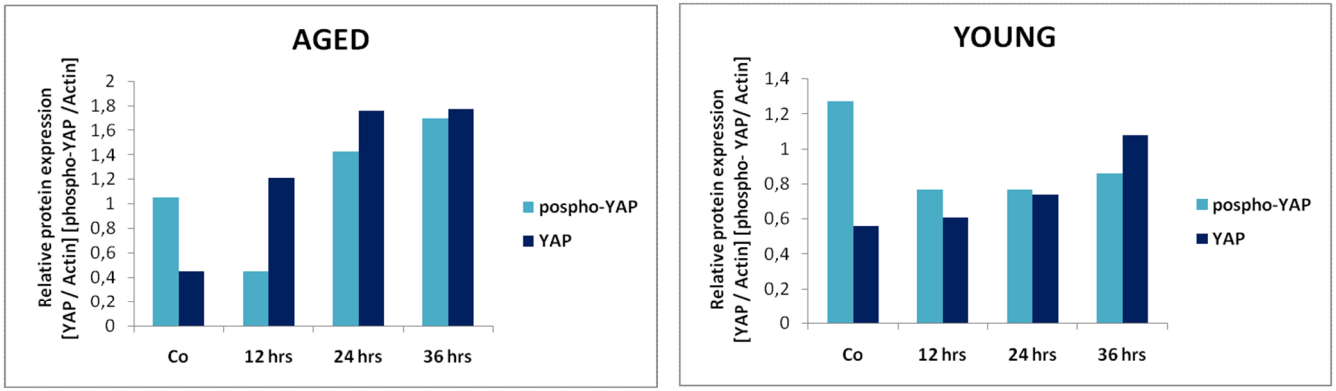
# Fig. 8

A



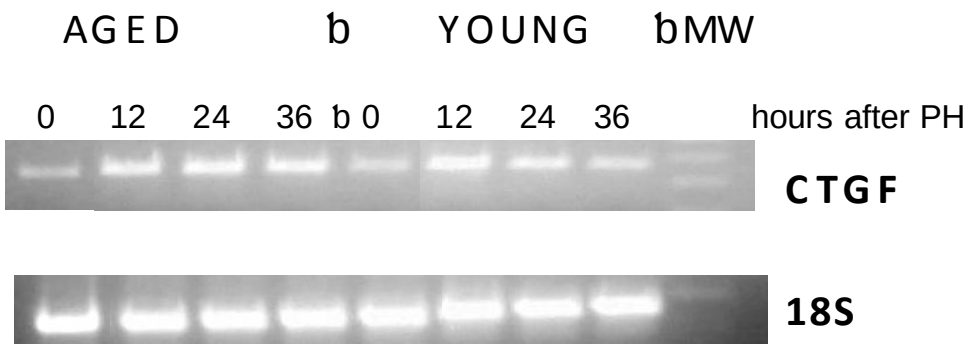

**Western blot analysis on total cell extracts**

B



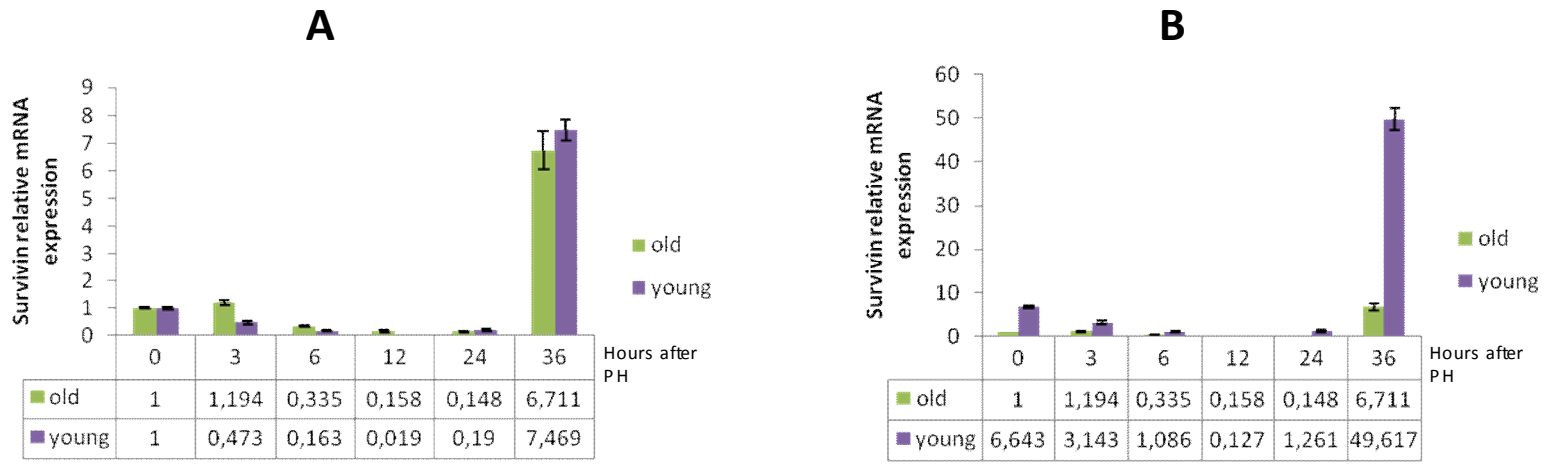

**Densitometric analysis of total- and Phospho-Yap proteins, normalized to Actin**

# Fig. 9




**Semiquantitative PCR analysis of CTGF gene expression**

**Fig. 10**



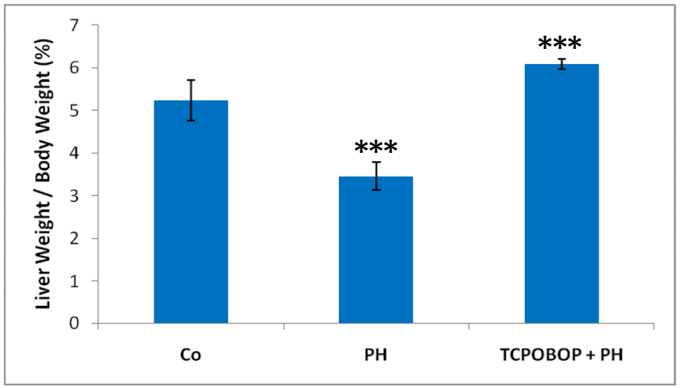

| | 0 | 3 | 6 | 12 | 24 | 36 |
|---|---|---|---|---|---|---|
| old | 1 | 1,194 | 0,335 | 0,158 | 0,148 | 6,711 |
| young | 1 | 0,473 | 0,163 | 0,019 | 0,19 | 7,469 |

| | 0 | 3 | 6 | 12 | 24 | 36 |
|---|---|---|---|---|---|---|
| old | 1 | 1,194 | 0,335 | 0,158 | 0,148 | 6,711 |
| young | 6,643 | 3,143 | 1,086 | 0,127 | 1,261 | 49,617 |

**Real Time PCR analysis of survivin gene expression in young and aged mice after PH: (A) fold of expression *vs* respective controls; (B) fold of expression *vs* aged controls**

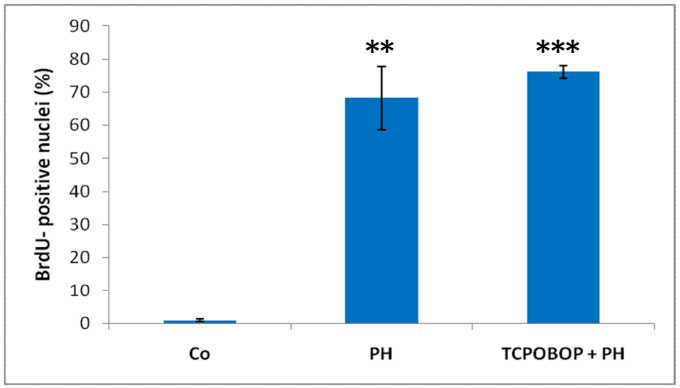
# Fig. 11

**A**




**Liver weight/Body weight ratio**

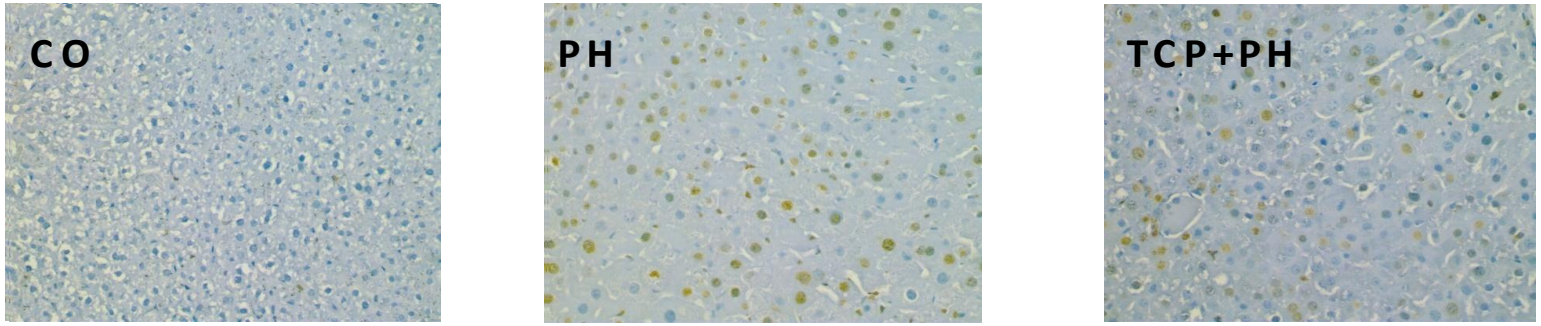
*(*equal to p<0.005; ** equal to p<0,01; *** equal to p<0,001)*




**Labeling Index**

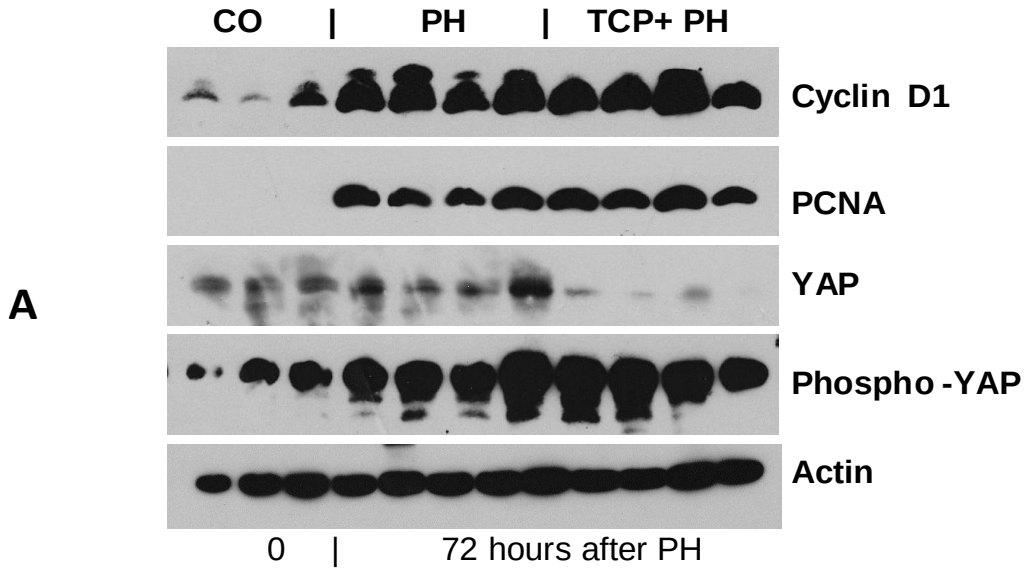
*(*equal to p<0.005; ** equal to p<0,01; *** equal to p<0,001)*

**B**



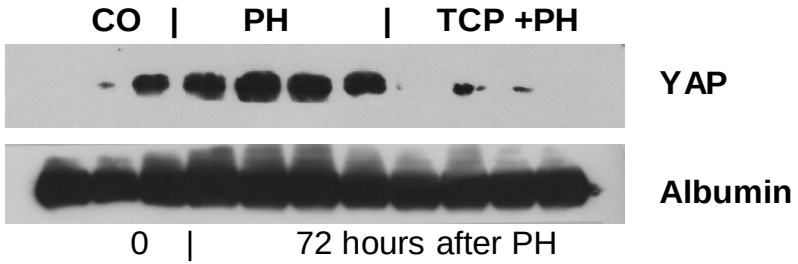

**Immunohistochemical analysis of BrdU-positive nuclei**

# Fig. 12




A

**Western blot analysis on total cell protein extracts**




B

**Western blot analysis on nuclear cell protein extracts**

**Fig. 13**

# Zusammenfassung

Das Gleichgewicht von Proliferation und Apoptose ist entscheidend für die Entwicklung und Beibehaltung der korrekten Größe eines Organs. Vorangegangene Studien weisen auf die Existenz eines ÄKontrollpunktesñhin, der über die Masse des Organs gesteuert wird. Dabei hat sich der Hippo-Signalweg mit seinem Effektor Yes-associated-protein (YAP), einem transkriptionellen co-Faktor, als besonders wichtig in der Steuerung dieses Prozesses entpuppt. Bei Verlust von Organmasse (Leber) kommt es zu einer verstärkten Expression von aktivem (dephosphoryliertem) YAP, was zu vermehrter Proliferation bei gleichzeitig verminderter Apoptose führt. Ziel dieser Arbeit war es insbesondere die Rolle von YAP bei der Wiederherstellung der ursprünglichen Organgröße nach 2/3 Teilresektion der Leber an Ratten- und Mausmodellen zu untersuchen. Die in dieser Arbeit gewonnen Daten demonstrieren, dass YAP bei der kompensatorischen Leberregeneration als Schlüsselregulator fungiert und abhängig von der Lebergröße ist. Dieser Fakt erlaubt es YAP von den klassischen Zellzyklusgenen abzugrenzen. Im speziellen wurde in dieser Arbeit gezeigt, dass:

- YAP an der Leberregeneration nach 2/3 Teilresektion beteiligt ist . Während der Leberregeneration wurde eine verstärkte YAP-Expression beobachtet, bei Terminierung des Prozesses eine verminderte;
- YAP-Aktivierung länger anhält und verfrüht vorkommt bei der Leberregeneration älterer Mäuse. Dies hängt vermutlich damit zusammen, dass es im höheren Alter zu einer Verlangsamung der regenerativen Antwort nach 2/3 Teilresektion der Leber kommt und die Lebergröße über längere Zeit vermindert ist. Das wiederum führt zu einer verfrühten (survivin) und länger anhaltenden (CTGF) Aktivierung von YAP-Zielgenen;
- 72 Stunden nach 2/3 Teilresektion einer vergrößerten Leber keine YAP-Aktivierung mehr feststellbar ist, was auf die schon größere Lebermasse zum Zeitpunkt der Operation zurückzuführen ist. Wenn zu diesem Zeitpunkt die Kontroll-Lebergröße erreicht wird, erfolgt keine weitere YAP-Aktivierung. Dies bestätigt die Abhängigkeit der YAP-Rekrutierung von der Lebergröße.