universität wien

DISSERTATION

Titel der Dissertation

Interplay between C-Raf kinase and Rassf1a putative tumour suppressor in liver cancer

Verfasserin ODER Verfasser

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angestrebter akademischer Grad

Doktor der Naturwissenschaften (Dr.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt:	A 091 490
Dissertationsgebiet lt. Studienblatt:	Dr.-Studium der Naturwissenschaften UniStG Doktoratsstudium
Betreuerin / Betreuer:	Univ.-Prof. Dr. Manuela Baccarini

Abstract

The Hippo/MST signalling cascade regulates cell survival and proliferation in metazoans and numerous reports indicate its further role in tumour suppression, especially in the liver. C-RAF, indirect activator of ERK in MAPK pathway, can interact with, and inhibit, MST2. In addition, C-Raf is implicated in the protection from apoptosis in mouse liver, and found overexpressed in human liver tumours. We investigate the possible interaction between the Hippo/Mst pathway and C-Raf in liver tumorigenesis.

Results from experiments on *c-raf* single knock-out animals revealed that C-Raf ablation in the hepatocyte leads to increased liver size, tumour mass and tumour number after chemical liver carcinogenesis. Similarly, ablation of C-Raf in hepatocyte and liver non-parenchymal cells before, but not after, tumour initiation leads to increased number of tumours, although not liver mass or tumour mass. The ablation of C-Raf in hepatocyte and liver non-parenchymal cells after development of macroscopic tumours did not affect their further growth or maintenance.

Further, we have crossed animals with hepatocyte-specific deletion of *c-raf* gene with animals harbouring a germline disruption of the gene encoding for Rassf1a, an upstream regulator of Hippo/Mst signalling with tumour suppressor functions lost in liver cancer through methylation of the isoform-specific promoter. The phenotype of hepatocyte-restricted *c-raf* knock-out after chemical carcinogenesis was absent on a *rassf1a* knock-out background, indicating genetic interaction between *c-raf* and *rassf1a*.

In most livers with *c-raf*-deficient hepatocytes we could detect progression to carcinoma stage, whereas in wild-type animals we detected almost exclusively benign nodules. On the other hand, double knock-out animals had similar numbers of carcinomas in comparison to those with sole *rassf1a* deletion. Similarly proliferation in tumour unaffected tissue was increased in livers with *c-raf*-deficient hepatocytes on the WT but not *rassf1a* knock-out background. Both *c-raf*-deficient and double KO livers exhibit increased numbers of Kupffer cells indicating increased inflammation. We correlate observed phenotypic changes in tumour-bearing livers with changes in activation of MAPK, Hippo and inflammatory signalling pathways. Finally, we use primary cell cultures to investigate acute effects of oxidative stress on Hippo pathway activation.

We propose a tumour suppressive function of C-Raf in the hepatocyte and tumour promoting role in liver non-parenchymal cells derived from hematopoetic line. Furthermore we unravel interaction between *c-raf* and *rassf1a* in chemically-induced mouse liver cancer model.

Zusammenfassung

Der Hippo/MST Signaltransduktionsweg reguliert das Zellwachstum in Metazoen und fungiert als potentieller Tumorsuppressor in Leberkarzinomen. C-RAF, der indirekte Aktivator von ERK im MAPK Signaltransduktionsweg, kann MST2 binden und inhibieren. Auβerdem schützt C-Raf Zellen vor Apoptose und zeigt eine deutliche Überexpression in humanen Leberkarzinomen. Diese Arbeit untersucht die potentielle Interaktion zwischen dem Hippo/Mst Signaltransduktionsweg und C-Raf in der Entstehung von Leberkrebs.

Chemische Tumorinduktion in *c-raf* Knock-out Mäusen zeigen, dass die Ablation von C-Raf in Hepatozyten zu einem gesteigerten Tumorwachstum und einer höheren Anzahl von Tumoren führt. Auf ähnliche Weise führt die Ablation von C-Raf in Hepatozyten und den nicht-perenchymalen Zellen vor der Tumorinitiation zu einer höheren Zahl von Tumoren, jedoch zu keinem gesteigerten Tumorwachstum. Die Ablation von C-Raf in Hepatozyten und den nicht-parenchymalen Zellen nach der Entstehung von makroskopische Tumoren zeigt keine Auswirkung auf deren Zahl und Wachstum.

Im nächsten Schritt wurde die chemische Tumorinduktion in einem *c-raf/rassf1a* doppel Knock-out Stamm durchgeführt. Rassf1a reguliert den Hippo/Mst Signaltransduktionsweg, und fungiert als Tumorsuppressor in der Leber, verliert diese Funktion jedoch im Zuge der Tumorprogression durch Promotor-Methylierung. Der Phänotyp Verlust des *c-raf* Knock-outs nach der chemischen Tumorinduktion im *rassf1a* Knock-out Hintergrund deutet auf die genetische Interaktion zwischen *c-raf* und *rassf1a* hin.

Die Mehrzahl der *c-raf*-Knock-out Lebern zeigt Tumore im fortgeschrittenen Karzinomstadium, während in Wildtyp-Mäusen nur Dysplasien detektiert werden konnten. Doppel Knockout Mäuse aber zeigen eine ähnliche Zahl an Karzinomen wie jene mit der alleinigen Deletion von *rassf1a*. Weiters war in tumorfreien Arealen in *c-raf* Knock-out Mäusen im Vergleich zum Wild-Typ eine erhöhte Proliferation zu beobachten, nicht jedoch im *rassf1a*-Knock-out Hintergrund. Sowohl C-*raf*- als auch *c-raf*/*rassf1a* doppel Knock-outs weisen eine erhöhte Anzahl von der Kupfferzellen auf, was auf eine Entzündungsreaktion hindeuten könnte. Die beobachteten Phänotypen der Lebertumore wurden mit veränderten Aktivitäten in MAPK-, Hippo- und Enzündungs Signaltransduktionswegen korreliert. Im letzten Schritt wurden die Akuteffekte von, oxidativem Stress auf den Hippo Signaltransduktionsweg analysiert.

Zusammenfassend konnten wir zeigen, dass C-Raf in Hepatozyten als potentieller Tumorsuppressor, in nicht-parenchymalen Leberzellen, die aus dem Knochenmark stammen, aber als Tumorpromoter fungiert. Weiters wurde die Interaktion zwischen *c-raf* und *rassf1a* im chemisch-induzierten Leberkrebsmodel analysiert.

Contents

Introduction

Liver physiology and architecture

Liver is the largest internal organ in human body divided into four lobes (right, left, caudate and quadrate). It is surrounded by external (Glisson) capsule of connective tissue and penetrated by portal vein that delivers nutrient-rich blood from intestines, hepatic artery that delivers oxygen-rich blood from the heart, hepatic vein that drains blood from the liver back to heart, bile-ducts that collect bile from the liver to the gall bladder, lymphatics that drain excess fluid and protein from the interstitial space of the liver and nerve fibres. Portal vein and hepatic artery branch in the liver into smaller portal veins followed by venules and hepatic arteries followed by arterioles, respectively, that finally open into sinusoids. Having perfused liver parenchyma via sinusoids blood enters terminal hepatic venules and is drained from the liver by the system of hepatic veins. Hepatocytes adjacent to each other form between their plasma membranes tight-junction-limited space that constitutes bile canaliculi, where the bile is secreted. Canaliculi drain into Canals of Hering that open to cholangioles (bile ductules), where the bile is collected from by the system of bile ducts and directed to the gall bladder (Figure 1) (Burt, Portmann et al. 2007).

Arrangement of vessels that penetrate the liver determines its microanatomy and functional unit. On the cross section portal triads (composed of artery, portal vein and bile duct) have hexagonal arrangement in the liver parenchyma with terminal hepatic vein placed in the middle - structure called **classical hepatic lobule**. Flow of oxygenated and nutrient-rich blood proceeds from the portal triads (periportal areas) in the direction of terminal hepatic veins (centrilobular areas) and determines microenvironment and metabolic function of hepatocytes depending on their localisation between portal triad and terminal hepatic vein. Functionally more relevant unit is **liver acinus** composed of 2 terminal hepatic veins from adjacent hepatic lobules with a portal triad in between. Space in the liver accinus that lies between portal triad and terminal hepatic vein is divided into 3 zones (1,2 and 3) with zone 1 being closest to the portal triad, zone 2 being intermediate, and zone 3, adjacent to terminal hepatic vein. Zonation along this porto-central axis is reflected by different ultrastructure, enzyme activities of hepatocytes and their metabolic function (Figure 2) (Burt, Portmann et al. 2007).

Hepatoctes constitute for 70% of liver cells and perform most of the functions of this organ: they secrete bile, release glucose and store it in the form of glycogen, biotransform and

detoxify drugs, metabolise heam, synthesise lipids and some amino acids, maintain nitrogen balance, produce plasma proteins and store iron and copper. They are large (30-40μm in diameter) epithelial cells facing sinusoidal space with their basolateral surface, bounding bile canaliculis with their canalicular surface and facing rest of extracellular space with their lateral surface. They maintain polarity by forming tight junctions in the lateral surface with neighbouring hepatocytes. They are often bi-nucleated, their nuclei are large and round encompassing 5-10% volume of the cell, and with age they become progressively polyploid. Endoplasmatic reticulum (ER) encompasses 15% of cytoplasm volume, with smooth ER twice as abundant in centrilobular as in periportal hepatocytes, what reflects their different metabolic functions. Hepatocyte has around 50 Golgi zones participating in bile secretion and 30 lysosomes used for autophagy and degradation of endocytosed extracellular proteins. It contains also around 300-600 peroxisomes that oxidise number of substrates including ethanol and as much as 1000 mitochondria that occupy around 20% of its cytoplasm and produce energy used in all metabolic processes. **Cholangiocytes** (bliary epithelial cells) are other type of epithelial cells of the liver that line bile ducts and modify composition of the secreted bile (Figure 1) (Burt, Portmann et al. 2007).

Normally quiescent hepatocytes maintain throughout the life of the organism the capability to re-enter cell cycle and repopulate liver in case of organ injury. Nevertheless, liver contains also population of stem cells named oval cells, that are reside in the vicinity of portal vein and are able to differentiate into hepatocytes as well as biliary epithelial cells (Figure 1). They are mobilized in case hepatocytes are not able to efficiently contribute to liver regeneration processes. For instance, in rodents in the protocol of choline deficient diet liver injury is accompanied by inhibition of hepatocyte proliferation. This leads to proliferation of oval cells, and in case of prolonged treatment, to HCC development. Oval cell activation is induced, among other stimuli, by growth factors like TGF-α and HGF secreted by stellate cells, and various inflammatory cytokines, like TNF superfamily members (TNF-α, TWEAK, lymphotoxins α and β, LIGHT) and activators of GP130 (Il-6, oncostatin M, leukaemia inhibitory factor) secreted by Kupffer cells and liver associated lymphocytes (Bird, Lorenzini et al. 2008). Research in the last years indicates that at least some human HCCs might result from abnormal oval cell proliferation as their number is elevated in various pathological liver conditions that precede HCC development (Knight, Matthews et al. 2005). Recently, HCC development associated with expansion of oval cell compartment in mice was found to be suppressed by Hippo/Mst signalling (Lee, Lee et al. 2010; Lu, Li et al. 2010).

Non-parenchymal cells of the liver localise to the sinusoids lined by **endothelial cells** that have numerous fenestrae which allows for the blood filtration and pass-through of solutes that contact hepatocytes. Endothelial cells produce some inflammatory cytokines and have endocytic activity that enables them removing protein aggregates and immune complexes from filtered blood. **Stellate cells** reside between endothelial cells and hepatocytes (in the space of Disse) and are of mesenchymal origin. They produce extracellular matrix that constitutes structural meshwork of the liver what is controlled by cytokines secreted by Kupffer cells and endothelial cells. They also store vitamin A, can stimulate hepatocyte proliferation during liver regeneration, and act as pericytes around the sinusoids by responding to vasoactive agents. **Kupffer cells** belong to mononuclear phagocytic system and play role of resident liver macrophages. They are able to proliferate in the liver, but in some part they are also derived from circulating monocytes. They phagocyte microorganisms, degenerated cells, tumour cells and various macromolecules, and by releasing cytokines they influence behaviour of hepatocytes, endothelial cells and stellate cells. **Liver-associated lymphocytes** are comprised in 65% of natural-killer (NK) cells, γδ T-cells and NK-molecules-expressing T- (NKT) cells. They are participating in response against various pathogens and tumour cells; especially hepatic NK cells, that comprise 50% of all liver-associated lymphocytes, have ability to lyse tumour cells and their number greatly increases in case of hepatic malignancy (Figure 1) (Burt, Portmann et al. 2007).

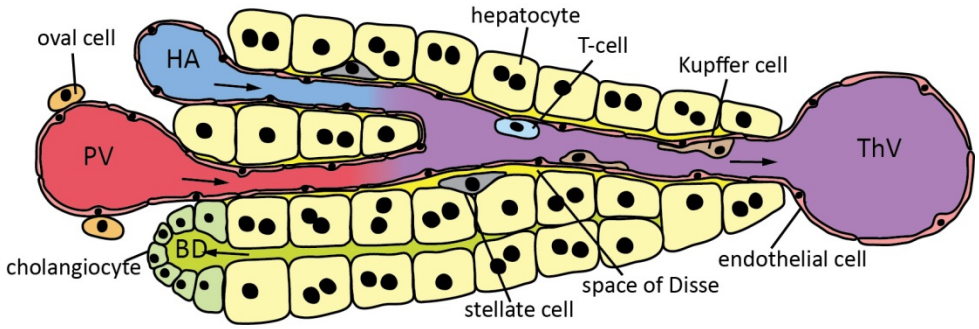

Figure 1. Architecture of the liver sinusoid with localisation of various cell types. For clarity only hepatocytes adjacent to the single sinusoid are depicted. Details in text.
HA - hepatic artery; PV - portal vein; BD - bile duct; ThV - terminal hepatic vein (also known as central vein); arrows indicate direction of blood and bile flow;

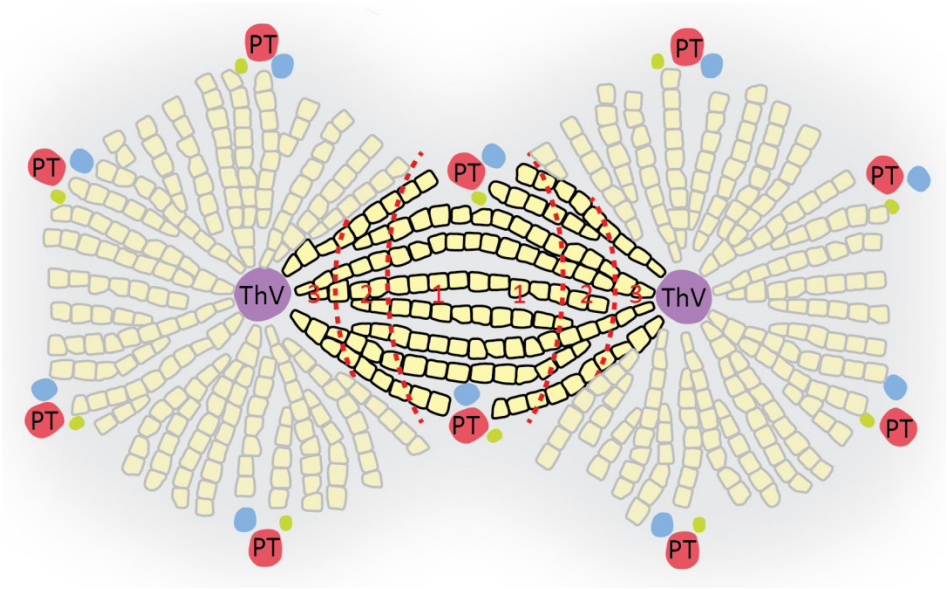

Figure 2. Architecture of hepatic lobule and liver accinus. Arrangement of portal triads (PT) consisting of portal vein (red), hepatic artery (blue) and bile duct (green) and two terminal hepatic veins of two adjacent hepatic lobules is depicted with cords of hepatocytes (light yellow) belonging to one liver accinus. Red dashed lines indicate division to different zones of the accinus. Details in text.

Hepatocellular carcinoma epidemiology, aetiology and treatment

Hepatocellular carcinoma (HCC) is the third cause of cancer-related death worldwide with more than 600000 patients succumbing to this disease each year. Its incidence is more frequent in men and geographically highly prevails in Asia and sub-Saharan Africa, though recently in Western countries number of diagnosed cases per year is increasing. HCC belongs to epithelial tumours and is the most common liver cancer comprising 83% of all primary tumours of this organ (Farazi and DePinho 2006; Ferenci, Fried et al. 2010).

HCC is usually preceded by liver cirrhosis, caused by excessive fibrosis resulting from continuous liver injury and inflammation. The main risk factors of HCC are infections with hepatitis B and C viruses (HBV and HCV). Virus-related factors that contribute to HCC development are complex and consist of both viral and host components. HBV integration into host's genome may cause microdeletions that include cancer-relevant genes. Some of HBV-encoded proteins, like HBx, function as activators of host's proto-oncogenic pathways. Host-viral interactions in case of HBV infection drive robust immune-response that triggers necrosis, inflammation and regeneration, leading to hepatocyte transformation. Moreover HBV, as well as HCV, is interacting with endoplasmic reticulum (ER), what induces oncogenic oxidative stress. As HCV is a RNA-virus, it cannot integrate into host's genome and has higher replication error rate that underlies its ability to evade immunological system. This results in higher rate of chronic infections, causing recurrent cycles of immune response and subsequent liver regeneration, what leads to liver cirrhosis with 10-20 times higher frequency than for HBV (Farazi and DePinho 2006). Another HCC risk factor is alcohol abuse that might contribute to HCC by activating monocytes and Kupffer cells to release proinflammatory cytokines. It also damages liver by oxidative stress, which in turn activates stellate cells, oncogenic pathways and causes mutations in DNA and telomere shortening (Farazi and DePinho 2006). Ingestion of aflatoxin B, a mycotoxin, can also be the cause of HCC, but unlike viruses or alcohol, it does not cause liver fibrosis - rather functions as a direct mutagen specific for gene encoding TP53. Other aetiological factors associated with HCC are genetic disorders like hemochromatosis, α_1-antitrypsin deficiency, tyrosinaemia and porphyria cutanea tarda; long-term contraceptive consumption in woman; diabetes and non-alcoholic fatty liver disease (Farazi and DePinho 2006).

Lack of early biomarkers makes it difficult to detect HCC at early stage. Current therapies are available almost exclusively for limited disease and include surgical tumour resection, local

ethanol injection, radiofrequency and transarterial chemoembolisation. Liver transplantation is also applied, although patients need to meet stringent criteria, and donor shortage also poses a great problem. In advanced disease only symptomatic treatment is applied, as there is no beneficial chemotherapy available. Just recently sorafenib, a multikinase inhibitor designed primarily against C-RAF, has been shown to increase survival of patients with unresectable disease by 3 months. In some countries it has been approved for clinical use, but its high cost (up to 7300$ dollars for one month of treatment in China) makes it impossible to use in developing countries, where HCC has especially high incidence (Ferenci, Fried et al. 2010).

Mouse models of HCC

Numerous mouse models are used to investigate molecular mechanism of HCC as well as responses to potential therapeutic interventions, as they allow achieving physiologically most relevant research conditions. Liver cancer in rodents may be induced by chemical carcinogens, modification of genes involved in tumorigenesis or transplantation of transformed cells.

Chemical liver carcinogenesis in mice

Chemicals that can induce liver cancer in mice are divided into genotoxic and non-genotoxic. The former ones are able to introduce mutations, usually by forming DNA-adducts, and in this way initiate cell transformation. The latter ones do not affect DNA structure directly, but rather create tumour-promoting conditions in the liver, i.e. by inducing uncontrolled hepatocyte proliferation or liver inflammation, that favour cell transformation and tumour growth (Leenders, Nijkamp et al. 2008).

Chemically-induced development of HCC in animal models is divided into initiation, progression and promotion. Initiation is an irreversible step induced by carcinogen in which hepatocytes undergo transformation. Promotion, a clonal expansion of these transformed cells to macroscopic-size foci, is reversible and may be enhanced by chemical compounds or regenerative stimuli that drive hepatocyte proliferation. In progression stage, growth of tumours from dysplastic lesions takes place what is accompanied by further genetic changes that eventually lead to malignancy of primary tumour (Durr and Caselmann 2000).

Numerous compounds have been shown to induce transformation of hepatocytes (Williams 1997), and some of them are used in animal liver cancer models. N-diethylnitrosoamine (DEN) is the most often used carcinogen to induce HCC in rodents. The efficiency of tumour induction by DEN is strain- (susceptible 129/Sv versus resistant C57BL/6), sex- (males more

prone than females) and age-dependent. Typically injection around 2 weeks of age with a low dose (5mg per g body weight) leads to 100% tumour penetrance by around 40 weeks of age. (Vesselinovitch and Mihailovich 1983). Injection may also be performed in older mice (4-6 weeks of age) but much higher dose is required (80-100mg per g body weight) and tumour latency is much longer. This is a result of lower proliferation in the livers of older animals that positively correlates with tumour initiation rate. In such case combination with partial hepatectomy or proliferation stimulation with other chemical, like phenobarbital (Pb), may be used to accelerate tumorigenesis.

Administration of DEN, apart from HCC might cause neoplasms of Kupffer cells, and tumours in other organs like skin, gastrointestinal tract, respiratory system and hematopoietic system. DEN is bio-activated in pericentral hepatocytes with the help of microsomal cytochromes P450, mainly CYP2E1 that is highly expressed in liver centrilobular area. Activated form of DEN is able to form DNA adducts on all 4 bases at positions with high electron density as well as phosphodiester bond, that may result in point mutations and single strand DNA breaks, respectively. Therefore DEN introduces mutations that, if affect sequence of cancer-relevant gene, may contribute to hepatocyte transformation. Apart from that DEN is cytotoxic and shortly after administration induces apoptosis of centrilobular hepatocytes that is compensated by proliferation in periportal areas (Verna, Whysner et al. 1996).

Phenobarbital function in liver tumorigenesis is not completely understood, i.e. Pb inhibits tumorigenesis if administered before, and promotes growth of DEN-initiated hepatocytes if administered after DEN-treatment. It induces cytochromes P450, promotes proliferation and inhibits apoptosis of initiated hepatocytes and selects for cells with activated β-catenin (Whysner, Ross et al. 1996).

Chemical models of liver carcinogenesis can help to establish relationship between carcinogen exposure and other environmental factors or specific genetic changes. But it usually takes months from the tumour initiation until macroscopic nodules appear and furthermore, nodules that arise carry heterogeneous, often unidentified mutations.

Transgenic mouse models

Transgenic mouse technology enabled generation of genetic HCC models based, first on transgenic expression of proto-oncogenes or viral proteins, and further on knock-outs of tumour suppressors. Induction of tumour growth in the liver is achieved by exploiting various hepatocyte-specific promoters (α-1-antitripsin, metallothionein, albumin) to express

oncogenes and growth factors like large T-antigen of SV40 (Sepulveda, Finegold et al. 1989), c-myc (Sandgren, Quaife et al. 1989), TGF-α (Jhappan, Stahle et al. 1990) E2F1 and c-myc/E2F1 (Conner, Lemmer et al. 2000) or c-myc/TGF-α combinations (Murakami, Sanderson et al. 1993). Investigation of hepatocarcinogenic viruses lead to development of mouse HCC models based on transgenic expression of HBV viral envelope protein HBsAg (Chisari, Klopchin et al. 1989) and gene expression transactivator protein HBx (Kim, Koike et al. 1991; Yu, Moon et al. 1999) or HCV core E1 and E2 proteins (Moriya, Fujie et al. 1998). Liver-restricted ablation of genes encoding components of NF-κB (Luedde, Beraza et al. 2007; Bettermann, Vucur et al. 2010; Inokuchi, Aoyama et al. 2010), Hippo (Zhou, Conrad et al. 2009; Lee, Lee et al. 2010; Lu, Li et al. 2010; Song, Mak et al. 2010) or PI3 kinase signalling (Horie, Suzuki et al. 2004) also lead to spontaneous liver tumour development in mice.

Great limitation of classical transgenic models is genetic modification of all cells in the target organ, including tumour microenvironment. Therefore conditional activation/ablation of HCC-regulating genes is applied to mimic somatic mutations that initiate single cells without initially affecting surrounding tissue. Polyoma virus middle T antigen (PyMT) viral delivery to hepatocytes expressing virus-receptor (Lewis, Klimstra et al. 2005), SV40 T-Ag transgene controlled with adeno-Cre-excisable "stop" cassete (Lou, Molina et al. 2005) or also adeno-Cre-mediated conditional ablation of Apc-encoding gene leading to β-catenin activation (Colnot, Decaens et al. 2004) are examples of such approach.

Numerous transgenic models of HCC induce hepatocyte transformation in cell non-autonomous manner. Mice knock-out for *mdr2*, gene encoding transporter of bile contents, develop cholangitis, inflammation and subsequent HCC (Mauad, van Nieuwkerk et al. 1994). Transgenic overexpression of urokinase plasminogen-activator (uPA) in hepatocytes induces their death, and repopulation of the liver with transgene-negative hepatocytes that later on form tumours (Sandgren, Palmiter et al. 1992). Similarly mouse livers that have genetically disrupted damaged DNA-binding protein 1 (DDB1) are repopulated by DDB1-positive hepatocytes and develop tumours after few months (Yamaji, Zhang et al. 2010).

Such transgenic models enable studying role of other genes or environmental factors in HCC, but often require long time until complete disease development and in case of multiple transgenics require time consuming mice crossing.

Xenograft mouse models

Immunologically compromised mouse strains allow xenotransplantation of HCC-derived cells or tumours from mice and men and study their growth under the skin of the animal. Such approach offers highly repeatable conditions of tumour growth that can be examined non-invasively and very rapidly. But models based on that technology are lacking *in vivo* relevance, as involve introducing often much modified cells into physiologically unusual locations. Othotopic transplantation models, though more laborious, can help to overcome these limitations (Leenders, Nijkamp et al. 2008).

Lately "mosaic" mouse HCC models have been introduced that bear some similarity with xenotransplantation models. They involve transformation of hepatic progenitors, their fluorescent labelling and manipulation of genetic element of interest. Such cells are then introduced to recipient liver and their orthotopic growth can be monitored by *in vivo* imaging. These models are rapid, offer defined genetic context and microenvironment of cancer cell that resembles physiologic conditions (Zender, Xue et al. 2005).

Conditional gene ablation in the liver

To study *in vivo* the role of genes in liver carcinogenesis that are also required during development, conditional gene ablation needs to be applied. Such approach has also advantage of affecting gene of interest only in certain cell types, what allows avoiding effects of gene deletion in other parts of the body.

Hepatocyte-specific gene ablation can be achieved with *alfp-cre* mouse line that bears a transgene encoding Cre-recombinase cDNA under the control of albumin promoter and α-fetoprotein and albumin enhancers. Expression from this transgene is activated during embryogenesis between day 9,5 and 10,5 *post coitus* in liver parenchyma, and leads to deletion of floxed alleles in hepatocytes, biliary epithelial cells and presumably, oval cells (Kellendonk, Opherk et al. 2000).

Other mouse line that can be applied to ablate gene expression in the liver is *mx-cre* line, in which Cre-recombinase cDNA is controlled by interferon-responsive promoter. It can be indirectly activated by inducing innate immune response and interferon-production with polyinosinic-polycytidilic acid (poly(I:C)). Cre recombinase is then expressed in any interferon-sensitive cell type, and so deletion is much less restricted then in *alfp-cre* line, encompassing liver, spleen, duodenum, and to some extent heart, lung, uterus, thymus and kidney (Kuhn, Schwenk et al. 1995). Specifically in the liver, Cre recombinase in *mx-cre*

mice is expressed not only in parenchyma but also in most non-parenchymal cells - Kupffer cells and liver associated lymphocytes (as coming from hematopoietic line) and partially in endothelial cells (unpublished data).

Molecular hallmarks of HCC

Main characteristics that cells need to acquire to become "successful" cancer cell have been summarised more than 10 years ago (Hanahan and Weinberg 2000) and recently revised (Hanahan and Weinberg 2011), and in an accompanying review we remind them in the context of C-RAF role in tumour biology (Maurer, Tarkowski et al. 2011).

Research on HCC done until now revealed numerous molecular pathways that need to be affected in hepatocytes to enable them acquiring each of six classical hallmarks of cancer. Moreover, recent studies indicate tumour-promoting inflammation as having a key role in HCC development. In the following sections for each hallmark I summarise briefly most significant pathways affected in HCC, elaborating in more detail on those important for MAPK signalling with emphasis on C-RAF, and MST/Hippo signalling, that are in the focus of this dissertation.

Sustaining proliferative signalling

C-MYC transcription factor is the central oncogene that sustains abnormal proliferative signalling in hepatocytes. Its overexpression in mouse liver drives spontaneous tumour development (Sandgren, Quaife et al. 1989; Shachaf, Kopelman et al. 2004), and deregulation of its activity in patients is associated with malignant tumour conversion (Kaposi-Novak, Libbrecht et al. 2009). Cyclin-dependent kinases (CDK) system that drives progression through cell cycle is also often deregulated in HCC. Cyclin D1 is overexpressed in 11% of clinical advanced HCC cases (Nishida, Fukuda et al. 1994), and in mouse liver can independently drive tumorigenesis. Moreover, CDK inhibitors are suppressed in HCC either by oncomiRs like in the case of p27 in HCC patients (Pineau, Volinia et al. 2010) or by JNK signalling like in the case of p21 in mouse chemically-induced HCC (Hui, Zatloukal et al. 2008) that also suppresses tumorigenesis in chronically inflamed liver in hereditary tyrosinemia I mouse genetic model (Willenbring, Sharma et al. 2008).

Cell proliferation is controlled from its outside by small peptides - growth factors (GFs), which upon binding to their respective receptors on the cell membrane activate intracellular signalling pathways. GFs relevant for HCC development are EGF receptor ligand TGF-α, which is upregulated in serum of patients suffering from HCC (Yeh, Tsai et al. 1987) and

enhances liver regeneration and tumorigenesis in various mouse models (Jhappan, Stahle et al. 1990; Lee, Merlino et al. 1992; Murakami, Sanderson et al. 1993; Sandgren, Luetteke et al. 1993), EGF, which accelerates tumorigenesis in *c-myc* transgenic mouse model (Tonjes, Lohler et al. 1995), and probably IGFII, whose scavenger receptor encoded by *IGF2R* gene is often lost in human HCC (De Souza, Hankins et al. 1995). Hepatocyte growth factor (HGF) and its receptor C-MET are another GF-receptor system relevant for hepatocyte homeostasis. C-MET is overexpressed in HCC patients (Ueki, Fujimoto et al. 1997) and mutated in the kinase domain in childhood HCC (Park, Dong et al. 1999). In mice c-Met drives spontaneous HCC development (Wang, Ferrell et al. 2001) and is required for liver regeneration by enabling Akt and Erk activation and hepatocyte cell-cycle re-entry (Borowiak, Garratt et al. 2004). Transgenic overexpression of HGF in the liver leads to spontaneous HCC (Sakata, Takayama et al. 1996) and accelerated DEN-induced hepatocarcinogenesis in mice, accompanied by c-Met activation (Horiguchi, Takayama et al. 2002). Still, function of HGF/C-MET system in HCC might also be tumour-suppressive, depending on the stage and aspect of liver tumorigenesis (Shiota, Rhoads et al. 1992; Santoni-Rugiu, Preisegger et al. 1996; Takami, Kaposi-Novak et al. 2007).

After ligand binding and transmitting the signal across cell membrane, cytoplasmic domains of the receptor assemble protein complexes that activate, among other signalling mediators, small GTPase proteins from RAS family. RAS-encoding genes are most frequently mutated genes in human cancers, mainly in codons 12, 13 and 61, what decreases their ability to hydrolyse bound GTP and causes their constitutive activation. Nevertheless such mutations are not found in human hepatocellular hepatomas or carcinomas, but rather cholangiocarcinomas (Tada, Omata et al. 1990). Still, in spontaneous and chemically induced rodent liver tumours mutations or elevated expression of genes encoding Ras proteins is found (Reynolds, Stowers et al. 1986; Wiseman, Stowers et al. 1986; Stowers, Wiseman et al. 1988; Buchmann, Bauer-Hofmann et al. 1991). Moreover, H-Ras expression is upregulated in rats during DNA synthesis phase after partial hepatectomy - strong proliferative stimulus to normally quiescent hepatocytes (Goyette, Petropoulos et al. 1983; Thompson, Mead et al. 1986), and recent studies indicate that HCC cell lines and tumours that do not have mutations in any of RAS-encoding genes, down-regulate at least one of their inactivating proteins, GTPase-activating proteins (GAPs) (Calvisi, Ladu et al. 2006; Jin, Wang et al. 2007; Calvisi, Ladu et al. 2011).

Activated RAS recruits one of three RAF proteins to the membrane compartments and by physical interaction increases their kinase activity, allowing further transmission of the signal by subsequent two core kinases of the pathway, MEK and ERK. B-Raf-encoding gene bears activatory mutations in chemically-induced mouse liver tumours (Jaworski, Buchmann et al. 2005) and C-RAF is overexpressed in human liver cancer (Hwang, Choi et al. 2004). Moreover, RKIP protein, whose function is to inhibit MEK activation by competing for binding to C-RAF, is found down-regulated in HCC cell lines and tumours (Schuierer, Bataille et al. 2006). MEK overexpression is found in human HCC specimens, and assays *in vitro* and mouse xenografts show sensitivity of HCC cell lines in terms of proliferation and survival towards numerous MEK inhibitors alone (Huynh, Nguyen et al. 2003; Klein, Schmidt et al. 2006; Huynh, Soo et al. 2007) or together with inhibitors of RTK and RAF (Huynh, Ngo et al. 2010). Finally, ERK activation is elevated in clinical HCC samples (Ito, Sasaki et al. 1998) similarly to other MAP kinase JNK1, which is also required for efficient chemical carcinogenesis and liver regeneration in mice. JNK1 performs this function by suppressing expression of cell cycle inhibitor p21 and inducing c-Myc oncogene, what drives proliferation of hepatocyte (Hui, Zatloukal et al. 2008).

Evading growth suppressors

TP53 is the most often lost tumour suppressor in all cancers, including human HCC where it is frequently deleted or mutated (Bressac, Galvin et al. 1990; Bressac, Kew et al. 1991; Hsu, Metcalf et al. 1991) and its even brief reactivation in mouse mosaic HCC model is sufficient to induce irreversible tumour regression (Xue, Zender et al. 2007). PTEN is another classical tumour suppressor that inhibits spontaneous HCC in mouse (Horie, Suzuki et al. 2004) and promoter of its gene is methylated in around half of HCC patients (Wang, Wang et al. 2007). Transforming growth factor β (TGF-β) and its receptor have tumour suppressive role in cancer, but in late carcinogenesis they drive metastatic behaviour of transformed cells (Massague 2008). TGF-β inhibits proliferation of hepatocytes *in vitro*, and together with its receptor during rodent liver regeneration (Russell, Coffey et al. 1988; Romero-Gallo, Sozmen et al. 2005). TGF-β receptor inhibits chemical liver carcinogenesis in mice (Tang, Bottinger et al. 1998; Kanzler, Meyer et al. 2001), in human HCC its expression is lost (Kiss, Wang et al. 1997), and its effector Smad3 transcription factor protects murine liver form chemical carcinogenesis by sensitising HCC cells to apoptosis in a p38 activation-dependent manner (Yang, Zhang et al. 2006). p38 is a MAP kinase activated by stress stimuli with tumour suppressive functions in various cancer types. In the liver it inhibits chemically-induced

carcinogenesis by suppressing activity of JNK-c-Jun pathway and in this way restricting proliferation of hepatocyte (Hui, Bakiri et al. 2007).

In recent years the Hippo/Mst signalling pathway that restricts cell proliferation and organ growth appeared to be crucial for suppressing development of liver cancer. Its main components and their mechanism of action are described in detail in a separate chapter, but in a nutshell, at least *in vitro,* the pathway inactivates YAP proto-oncogene by phosphorylation signal, transduced through MST-LATS kinase cascade. Mst kinases and WW45 protein required for their activation play tumour suppressive role in mouse liver, most likely by restricting oval cell proliferation, and MST activation is decreased in majority of human HCCs (Zhou, Conrad et al. 2009; Lee, Lee et al. 2010; Lu, Li et al. 2010; Song, Mak et al. 2010). LATS2-encoding gene is located in a region frequently lost in human HCC (Chen, Yeh et al. 2005) and YAP-encoding gene amplification leads to its overexpression in HCC samples what in mouse models drives growth of liver tumours (Zender, Spector et al. 2006; Camargo, Gokhale et al. 2007; Dong, Feldmann et al. 2007). Also upstream regulators of MST signalling seem to be affected in HCC. Nf2, which orthologue of activates drosophila Hippo, suppresses tumorigenesis in mouse liver, although whether this is because of Mst activation (Zhang, Bai et al. 2010) or suppression of Egfr activity (Benhamouche, Curto et al. 2010) is still debated. Expression of Ras-associated factor 1A (RASSF1A), that regulates Mst activity by direct association mammalian cells as well as in flies, is epigenetically silenced in majority of HCC cases (Schagdarsurengin, Wilkens et al. 2003).

Resisting cell death

In case of DNA damage or oncogenic stress TP53 stabilisation leads to programmed cell death through induction of proapoptotic genes, like some Bcl-2 protein family members (Vousden and Lane 2007). Therefore frequent *TP53* inactivation might desensitise HCC cells from intrinsic apoptotic cues (Bressac, Galvin et al. 1990; Bressac, Kew et al. 1991; Hsu, Metcalf et al. 1991). Moreover Tp53 function is found antagonised by c-Jun at early stages of DEN-induced liver carcinogenesis (Eferl, Ricci et al. 2003). BCL-X_L, anti-apoptotic member of Bcl-2 family, is overexpressed in human HCC and protects HCC cell lines from various apoptotic stimuli *in vitro* (Takehara, Liu et al. 2001). Other Bcl-2 family member with similar properties, Mcl-1, is a target of miR-101, down-regulated in HCC cell lines and clinical samples (Su, Yang et al. 2009). Target gene of MST/Hippo pathway encoding inhibitor of apoptosis protein (IAP) is amplified in human liver cancer and is crucial for unhalted growth

of tumours in mosaic mouse HCC model with 9qA1 amplifications that also contains gene encoding YAP (Zender, Spector et al. 2006).

Autophagy is in many aspects apoptosis-related process and recent data indicate its role in development of various cancers. Autophagy is a degradation of cellular organelles within autophagosomes - double-membrane compartments with lysosomal hydrolases. It provides cell with required nutrients during starvation or allows for degradation of damaged organelles. In cancer it plays pivotal role, where at early stages it suppresses tumorigenesis by limiting detrimental influence of aberrantly functioning organelles (like damaged ROS-overproducing mitochondria) on cellular homeostasis. At the late carcinogenesis stages it supports tumour progression by providing cell with additional nutrients for fast and uncontrolled proliferation (Kirkin and Dikic 2011). In HCC tumours, especially those that are Bcl-X_L positive, autophagy regulator Beclin-1 is down-regulated, and its decreased expression correlates with poor patient survival (Ding, Shi et al. 2008). In mice, Beclin 1 and other regulator of autophagy Atg7, suppress spontaneous liver carcinogenesis by degrading p62 protein. p62 accumulation may lead to ROS accumulation (Mathew, Karp et al. 2009) and/or stabilisation of oncogenic transcription factor Nrf2, found in 25% of human HCCs (Inami, Waguri et al. 2011). Therefore, similarly to apoptosis, autophagy in HCC may play tumour suppressive role by limiting amount of aberrantly functioning organelles in the tissue.

Activating invasion and metastasis

Unrestricted TGF-β signalling is required for efficient epithelial-to-mesenchymal transition (EMT) in HCC cell lines and patient tissues (Fransvea, Angelotti et al. 2008) and for vascular invasion of HCC cells (Fransvea, Mazzocca et al. 2009). TGF-β also regulates disintegrase and metalloproteinases (ADAMs) expression that allow efficient HCC cell line migration and invasion in the livers of nude mice and are targeted by miR-122, often down-regulated in human HCC (Tsai, Hsu et al. 2009). β-catenin activating mutations are associated with liver cancer progression in the clinic (Ogawa, Yamada et al. 1999; Zucman-Rossi, Jeannot et al. 2006; Rebouissou, Amessou et al. 2009) and in HCC mouse models, where β-catenin is present mostly in the cells at the invasive front of tumours (Calvisi, Ladu et al. 2004), induces spontaneous HCC (Colnot, Decaens et al. 2004) and accelerates progression of activated c-Met-driven HCC (Tward, Jones et al. 2007).

Rho is a family of small GTPases that plays substantial role in HCC progression (Grise, Bidaud et al. 2009). Its members are regulated similarly to RAS by GAPs and guanine-

nucleotide-exchange factors (GEFs) and influence actin dynamics, cell shape and migration. They achieve this by controlling cytoskeletal kinases ROCK1 and ROCK2, the latter one being inhibited in mouse by direct interaction with activated C-Raf (Ehrenreiter, Kern et al. 2009; Niault, Sobczak et al. 2009). RhoA GAP deleted in liver cancer 1 (DLC1) is encoded by the gene lying in the region on chromosome 8p frequently lost in human HCC through LOH or promoter methylation (Yuan, Miller et al. 1998; Wong, Lee et al. 2003), its knock-down accelerates c-Myc-driven tumorigenesis (Xue, Krasnitz et al. 2008), and similarly to its orthologue DLC2, it suppresses migration of HCC cell lines *in vitro* (Leung, Ching et al. 2005; Wong, Yam et al. 2005). RhoA and Rac1 activity is modulated by translation initiation factor eIF5A2 that induces EMT, cell motility and metastasis in mouse models (Tang, Dong et al. 2010), and whose localization is regulated by exportin 4 (XPO4) indicated in the screening for tumour suppressors of HCC in mouse mosaic HCC model (Zender, Xue et al. 2008). Transcription of GEF of another Rho GTPase Cdc42, ARHGEF9, is elevated in HCC as a result of overexpression of helicase domain-containing protein CHD1. This leads to uncontrolled Rho activation, EMT induction and increased migration on cellular level, and metastasis in mouse models and patients (Chen, Chan et al. 2010). RhoA direct effector Rho-associated kinase 2 (ROCK2) is overexpressed in human HCC and regulates cell motility *in vitro* and tumorigenicity of HCC cell line in orthotopic xenograft model (Wong, Wong et al. 2009). Activities of its close orthologue ROCK1 together with RhoA are required for migration of hepatoma cells in intraperitoneal-invasion model (Itoh, Yoshioka et al. 1999; Yoshioka, Nakamori et al. 1999). Moreover, apart from influence on cell motility, RhoA and RhoC might induce expression of matrix metalloproteinases (MMPs) to facilitate HCC progression (Xue, Takahara et al. 2008). MMPs secretion and cell migration in malignant HCC might also be regulated by GTPases form RAS family. Sprouty-related Spread 1 and 2 negative RAS regulators expression is found down-regulated in 70% of human HCCs, and in HCC cell lines Spreads inhibit ERK activation and tumour invasiveness by limiting secretion of MMPs and cell motility (Yoshida, Hisamoto et al. 2006).

Inducing angiogenesis

As in case of each solid tumour, growth of HCC is limited by diffusion of nutrients what is eventually overcome by induction of neovascularisation (Semela and Dufour 2004). Neovascularisation is induced by hypoxia in the tumour through up-regulation of transcription factor hypoxia-inducible factor 1α (HIF1α) that induces expression of vascular-endothelial growth factors (VEGFs). Secreted VEGFs bind to their receptors on endothelial cells and

pericytes and activate them to loosen their cell-cell contacts, proliferate and form new vessels within the tumour. In HCC cell lines HIF1α expression and transcriptional activity on VEGF promoter is regulated by inhibitor of differentiation 1 protein (Id-1), up-regulated in advanced HCC, and required in xenografted tumours for efficient neovascularisation (Lee, Poon et al. 2006). Moreover, subset of human HCCs has gains of chromosomal region 6p21 that contains gene encoding VEGFA, (Chiang, Villanueva et al. 2008) what might be one of the reasons for frequent VEGF overexpression in HCC (Mise, Arii et al. 1996; Suzuki, Hayashi et al. 1996; Yamaguchi, Yano et al. 1998). In mouse xenograft and orthotopic transplantation models HCC cell line growth is accelerated by VEGF overexpression in a VEGFR2-dependent manner (Yoshiji, Kuriyama et al. 1998; Yoshiji, Kuriyama et al. 1999). Here it is worth to mention that the first successful molecularly-targeted therapy against HCC applies sorafenib, the broad-specificity kinase inhibitor that most likely is imposing its positive effect on patient survival by inhibiting angiogenic signalling (Liu, Cao et al. 2006). The control of angiogenic signalling between transformed hepatocyte and endothelial cells is also performed by GFs like FGF and HGF. Mice with hepatocyte-restricted FGF receptor 1 activation or overexpression of HGF are more prone to DEN-induced liver carcinogenesis. In both cases it is associated with higher tumour vascularization and higher VEGF production (Horiguchi, Takayama et al. 2002; Huang, Yu et al. 2006). The stability of the vessels is provided by angiopoietin-1 (Ang-1) which binds to Tie-2 receptors on endothelial cells, inducing their maturation, process inhibited by Ang-2. Ang-2 levels in tumour tissue of HCC patients are increased and its overexpression in HCC cell lines injected to nude mice causes haemorrhage of developing tumours (Tanaka, Mori et al. 1999).

Enabling replicative immortality

Cancer cells are characterised by capability of unlimited divisions, and to achieve that they need to overcome “Hayflick limit”, resulting in normal cells from telomere shortening with each division. Replicative immortality is achieved by sustaining expression of telomerase, but in HCC development this enzyme seems to play a pivotal role. At early stages of liver carcinogenesis, telomere length is shortened and correlated with chromosomal aberrations (Plentz, Caselitz et al. 2004). Therefore telomerase might perform tumour suppressive functions by limiting genomic instability required for generation of dysplastic lesions. On the other side, telomerase activity is detected already in patient cirrhotic liver tissue on low levels and is further increased in HCC (Tahara, Nakanishi et al. 1995). Furthermore various chemical and genetic mouse HCC models indicate that eventually re-expression of telomerase

is required for sustaining proliferation and viability of transformed cells and progression to advanced HCC stage (Farazi, Glickman et al. 2003). Therefore at the advanced HCC stages telomerase performs oncogenic role by providing cancer cell limitless replicative potential.

Tumour-promoting inflammation

NF-κB pathway responds to wide variety of stimuli transduced i.e. by TNFα-receptor family, interleukin-receptors, or intracellular receptors RIG-I. Ligand binding to the receptor induces recruitment of TRAF and RIP proteins that, together with kinases NIK and TGFβ-activated MAP3K (TAK1), facilitate phosphorylation-mediated activation of IKK complex. IKK complex consists of two kinases, IKKα and β and regulatory protein NEMO. When activated it phosphorylates inhibitory proteins of the pathway from IκB family, what leads to their ubiquitinylation and degradation. This allows for import of NF-κB heterodimers consisting of p65 and p50 from cytoplasm to the nucleus, where they can activate transcription of their target genes that determine the survival of the cell (Hayden and Ghosh 2008). NF-κB pathway is activated by stimuli associated with inflammation which in turn lies at the very basis of liver carcinogenesis. Therefore it is maybe not surprising that NF-κB signalling plays role in liver cancer, although what is the exact influence of NF-κB on this process is a subject of vigorous debate.

In Mdr2-KO mice, where ablation of multi-drug resistance 2 membrane channel leads to chronic inflammation in the liver followed by hepatitis and cancer development, NF-κB pathway has an oncogenic function. It is activated in paracrine manner by TNF-α, and protects hepatocytes from apoptosis in condition of chronic liver inflammation, therefore sustaining carcinoma development (Pikarsky, Porat et al. 2004). Moreover in HCC from virus-infected livers higher expression of lymphotoxins α and β is present and in mice their overexpression leads to HCC development in NF-κB-dependent manner (Haybaeck, Zeller et al. 2009). But other reports indicate NF-κB pathway to have rather tumour-suppressive functions in the liver inflammation-associated tumorigenesis. Regulatory subunit of Ikk complex NEMO suppresses chronic liver inflammation and spontaneous liver cancer (Luedde, Beraza et al. 2007) and Ikkβ kinase suppresses liver chemical carcinogenesis (Maeda, Kamata et al. 2005). In both cases, NF-κB pathway activity is required for inhibition of ROS production that may induce cell death and lead to extensive compensatory hepatocyte proliferation. Similarly Tak1 protects mouse liver from fibrosis and hepatocyte compensatory proliferation resulting in spontaneous HCC, by restricting hepatocyte sensitivity to apoptosis induced by TNF-α-NF-κB pathway (Bettermann, Vucur et al. 2010; Inokuchi, Aoyama et al.

2010). Tumour suppressive role of NF-κB pathway in liver cancer likely depends on inhibition of JNK activity. JNK is required for efficient chemical carcinogenesis, acute apoptosis induction and following compensatory proliferation response after carcinogen administration (Sakurai, Maeda et al. 2006).

The confusion regarding NF-κB in liver cancer may partially arise from differences between models in terms of conditions of cancer development, timepoint of NF-κB inactivation or cell type-specific NF-κB function. In Mdr2-KO model tumour growth is driven by constant inflammation and NF-κB activity is required by tumours only at the late stages of their development for survival of transformed hepatocytes (Pikarsky, Porat et al. 2004). In the DEN-model accompanied by tissue-damage inflammation after carcinogen administration is only transient, and NF-κB activity also in this case protects hepatocytes from cell death. But this in turn restricts compensatory proliferation of remaining hepatocytes, and therefore in DEN-model NF-κB behaves as an "indirect tumour suppressor" (Maeda, Kamata et al. 2005). During progression of initiated hepatocytes in chronically damaged liver NF-κB pathway also may suppress tumour development. It limits ROS accumulation and maintains activity of Shp phosphatases that inhibits STAT3 activation (He, Yu et al. 2010). On the second hand in liver non-parenchymal cells NF-κB is sustaining tumour growth by mediating growth-promoting cytokine-release (Maeda, Kamata et al. 2005). For instance Kupffer cells can produce IL-6 in response to IL-1α released by dying hepatocytes, process inhibited by Ikkβ and p38α through suppression of ROS accumulation in the liver (Sakurai, He et al. 2008).

Interleukins are intercellular messenger molecules of immunological system that activate JAK-STAT signalling. Interleukin binding to their receptors causes receptor oligomerisation and recruitment of Janus kinases (JAK) that phosphorylate receptor cytoplasmic domains. This creates docking sites for STAT transcription factors, their phosphorylation while being bound to receptor cytoplasmic domains and subsequent translocation to the nucleus. There as dimers they activate transcription of target genes (Schindler, Levy et al. 2007). Lower susceptibility of females to HCC, at least in mouse models, is attributed to inhibition of Il-6 production by oestrogens (Naugler, Sakurai et al. 2007). Transgenic mice overexpressing Il-6 and soluble binding subunit of its receptor gp80 develop nodules of hepatocellular hyperplasia and adenomas (Maione, Di Carlo et al. 1998). Gene encoding GP130, co-receptor for IL-6, bears activatory mutations or is overexpressed in most benign hepatocellular tumours in human (Rebouissou, Amessou et al. 2009). Activation of STAT3 is observed in human HCC and growth of HCC-derived cell lines *in vitro* and in nude mice is dependent on STAT3

activity (Lin, Amin et al. 2009). Negative regulator of JAK-STAT signalling suppressor of cytokine signalling 3 (SOCS3) suppress liver regeneration, DNA-replicative capacity of hepatocytes and chemical liver carcinogenesis in mice (Riehle, Campbell et al. 2008). Other negative regulator of Stats, Shp2 phosphatase, protects mouse liver from inflammation, cirrhosis, spontaneous HCC late in life and, by suppressing STAT3 activation, from chemically-induced HCC (Bard-Chapeau, Li et al. 2011). In the liver Shp2 is inhibiting signalling through Stat1 and Stat3 transcription factors in response to pro-inflammatory stimuli like LPS or Il-6, but is required for Erk and Jnk activation.

RAF kinases in cancer-roles and therapeutic opportunities - review article

TITLE: Raf kinases in cancer–roles and therapeutic opportunities

PUBLISHED (online) in: Oncogene (2011) 1–12

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Oncogene (2011) 1–12

www.nature.com/onc

REVIEW

Raf kinases in cancer–roles and therapeutic opportunities

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Raf are conserved, ubiquitous serine/protein kinases discovered as the cellular elements hijacked by transforming retroviruses. The three mammalian RAF proteins (A, B and CRAF) can be activated by the human oncogene RAS, downstream from which they exert both kinase-dependent and kinase-independent, tumor-promoting functions. The kinase-dependent functions are mediated chiefly by the MEK/ERK pathway, whose activation is associated with proliferation in a broad range of human tumors. Almost 10 years ago, activating BRAF mutations were discovered in a subset of human tumors, and in the past year treatment with small-molecule RAF inhibitors has yielded unprecedented response rates in melanoma patients. Thus, Raf qualifies as an excellent molecular target for anticancer therapy. This review focuses on the role of BRAF and CRAF in different aspects of carcinogenesis, on the success of molecular therapies targeting Raf and the challenges they present.
Oncogene advance online publication, 16 May 2011; doi:10.1038/onc.2011.160

Keywords: Raf; Ras; ERK pathway; hallmarks of cancer; kinase inhibitors

Raf proteins and their effectors

The first member of the Raf family, C-Raf-1 (also known as Raf-1), was identified in a oncogene capture experiment in which its catalytic domain was found fused to the retroviral Gag protein, resulting in the constitutive activation of the serine/threonine kinase activity of C-Raf (Rapp *et al*., 1983; Moelling *et al*., 1984); 4 years later, B-Raf was discovered in a similar experiment (Marx *et al*., 1988). Within 10 years of its discovery, C-Raf was identified both as an interaction partner and activator of mitogen-activated protein kinase (MAPK)/ERK kinase (MEK), the dual-specificity kinase responsible for activation of extracellular signal-regulated kinase (ERK), and an effector of Ras, which was reported to recruit C-Raf to the membrane and stimulate its activation by mechanisms, which, roughly 18 years later, are still incompletely understood. Both the history and the regulation of Raf have been reviewed recently (Wellbrock *et al*., 2004; Niault and Baccarini, 2010). Suffice it to say here that a wealth of studies have led to a widely accepted model in which Raf activation primarily consists in the relief of the inhibition imposed on the Raf catalytic domain by an N-terminal regulatory domain, featuring both a Ras-binding domain and a cysteine-rich domain responsible for interaction with the kinase domain and for Raf autoinhibition (Figure 1a). This basic mechanism applies to all three Raf proteins (A-Raf, B-Raf and C-Raf), although both A-Raf and C-Raf need additional steps, such as phosphorylation of activating residues and dephosphorylation of negative regulatory residues, to reach maximal activation. Thus, B-Raf is the family member most easily activated by Ras (Wellbrock *et al*., 2004; Niault and Baccarini, 2010). In addition, the basal kinase activity of B-Raf is higher than that of C-Raf and, likely, A-Raf (Pritchard *et al*., 1995; Emuss *et al*., 2005). This provides a potential rationale for the frequent mutational activation of BRAF (for example by the prominent BRAFV600E mutation; (Davies *et al*., 2002)), but not CRAF or ARAF, observed in human tumors. A major advance of the past few years was the discovery that Raf kinases can homo- and heterodimerize (Garnett *et al*., 2005; Rushworth *et al*., 2006), and that, in fact, the structure of an active Raf kinase is that of a side-to-side dimer in which only one partner must have catalytic activity (Rajakulendran *et al*., 2009). Dimerization is enhanced by Ras (Weber *et al*., 2001) and is subject to negative feedback regulation by ERK (Rushworth *et al*., 2006; Ritt *et al*., 2010) (Figure 1b).

In the Raf/Mek/Erk pathway, dimerization can be used to exert tight temporal control of the signal, in cases in which one dimer subunit is more prone to negative feedback regulation than the other (C-Raf < B-Raf (Dougherty *et al*., 2005; Ritt *et al*., 2010) and Mek1 < Mek2 (Catalanotti *et al*., 2009); reviewed by Wimmer and Baccarini (2010)). A further level of control is exerted by the interaction with inhibitory proteins (Kolch, 2005). In the context of cancer, the most relevant of these is the Raf kinase-inhibitory protein, RKIP (Zeng *et al*., 2008) (Figure 1b). In addition, a high degree of spatial control is provided by the interaction of pathway components with scaffolds that direct them to distinct subcellular compartments (Kolch, 2005; McKay and Morrison, 2007).

Overexpression of full-length Raf or the truncated catalytic domain leads to the activation of the ERK pathway and increases proliferation in cultured cells and *in vivo*. Thus, MEK/ERK is undoubtedly a target of

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Received 15 February 2011; revised and accepted 3 April 2011

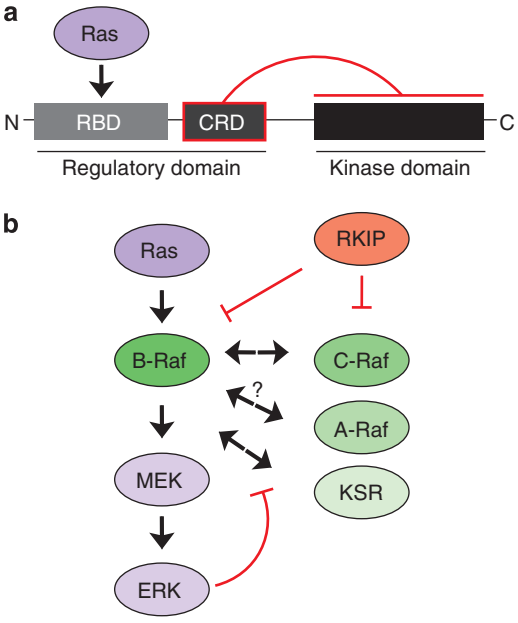

Figure 1 The structure of Raf and interactions within the Ras/Raf/MEK/ERK pathway. (**a**) A schematic view of Raf. All three Raf proteins consist of a regulatory and a kinase domain. In quiescent cells, the interaction between these two domains inhibits catalytic activity. The cysteine-rich domain (CRD) is necessary for this inhibition (indicated by the red blunt arrow), which is relieved by the binding of Ras to the Ras-binding domain (RBD). A-Raf and C-Raf need additional steps for full-fledged activation. (**b**) Regulation of the ERK pathway. Raf kinases can be activated by homo- and heterodimerization. Dimerization is induced by Ras and can occur in different combinations including not only the Raf kinases but also the pseudokinase KSR. Phosphorylation of Raf residues by activated ERK counteracts dimerization, allowing negative feedback control of the pathway (red blunt arrow). RKIP is an inhibitory protein whose expression is often lost in cancer and which can regulate pathway output at the level of Raf as well as MEK activation. ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; RKIP, Raf kinase-inhibitory protein.

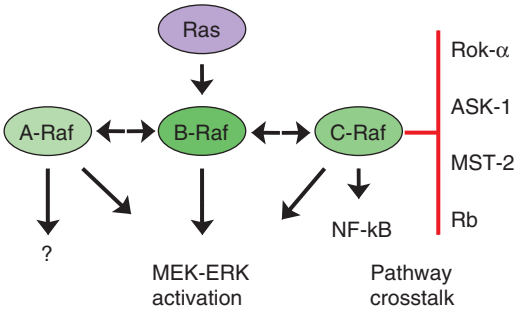

Figure 2 Functions of Raf. Gene ablation experiments have shown that B-Raf is essential for MEK–ERK activation in most systems. A-Raf and C-Raf heterodimerize with B-Raf and can participate in ERK activation (double-headed arrows). It is unclear whether A-Raf has functions outside the MEK/ERK pathway; C-Raf, however, can promote nuclear factor-κB activation and can inhibit (blunt-headed arrow) signal transducers involved in motility (Rok-α), apoptosis (ASK-1 and MST-2), proliferation and angiogenesis (Rb). ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase.

activated Raf in tumorigenesis. In the case of C-Raf, other targets potentially contributing to cell transformation have been proposed, such as the nuclear factor-κB pathway (Baumann *et al*., 2000), Rb (Kinkade *et al*., 2008) and BAD (Polzien *et al*., 2009), all reviewed by Niault and Baccarini (2010). In addition, C-Raf can inhibit apoptosis by binding to, and inhibiting, the stress-induced kinase ASK-1 (Chen *et al*., 2001) and the homolog of *Drosophila*'s Hippo, the MST-2 kinase (O'Neill *et al*., 2004; Matallanas *et al*., 2007); and finally, C-Raf interferes, by direct binding, with the activity of the cytoskeleton-based Rho effector Rok-α (also known as ROCK2), resulting in defects in cell migration, apoptosis and differentiation (Ehrenreiter *et al*., 2005, 2009; Piazzolla *et al*., 2005) (Figure 2).

Raf and the hallmarks of cancer

Six hallmarks of cancer, describing the acquired cell-autonomous capabilities of a cancer cell, were outlined in a legendary review by Hanahan and Weinberg (2000) more than 10 years ago. More recently, the list has been revised to include other features of cancer cells related to their interaction with the environment, such as avoidance of immunosurveillance (Dunn *et al*., 2004; Smyth *et al*., 2006; Zitvogel *et al*., 2006) and the stress phenotypes of cancer (Luo *et al*., 2009), as well as genomic instability (Negrini *et al*., 2010).

In the following section, we will highlight the contribution of Raf and of the Raf-dependent pathways to the hallmarks and states of cancer (Figure 3).

Genomic instability is a feature of almost all human cancers (Negrini *et al*., 2010). In hereditary cancers, germline mutations in caretaker genes (DNA-repair genes and mitotic checkpoint genes) promote tumor development by increasing the mutational rate and leading to chromosomal instability. In sporadic cancer, the caretaker genes are not mutational targets, and chromosomal instability is rather a consequence of the DNA replication stress induced by the activation of oncogenes, notably Ras.

Germline Raf mutations do not appear to contribute to cancer; instead, mutation in both BRAF and CRAF have been found in human genetic syndromes defined as 'Rasopathies' because they are caused by mutations in components of the Ras/ERK pathway (Tidyman and Rauen, 2009). The observed mutations cause activation of BRAF or CRAF, but the two kinases are not interchangeable in this context: mutations in the regulatory domain of CRAF are associated with the development of Noonan Syndrome, also caused by mutations in SOS1 and KRAS, and Leopard syndrome. By contrast, BRAF mutations are associated with Cardio-facio-cutaneous syndrome (CFC), also initiated by activating mutations of MEK (reviewed by Tidyman and Rauen, 2009).

In addition to the mutations identified in Noonan and Leopard syndrome, two weakly transforming germline mutations in the kinase domain of CRAF have been described in patients with therapy-related acute myeloid leukemia, which arises from concomitant

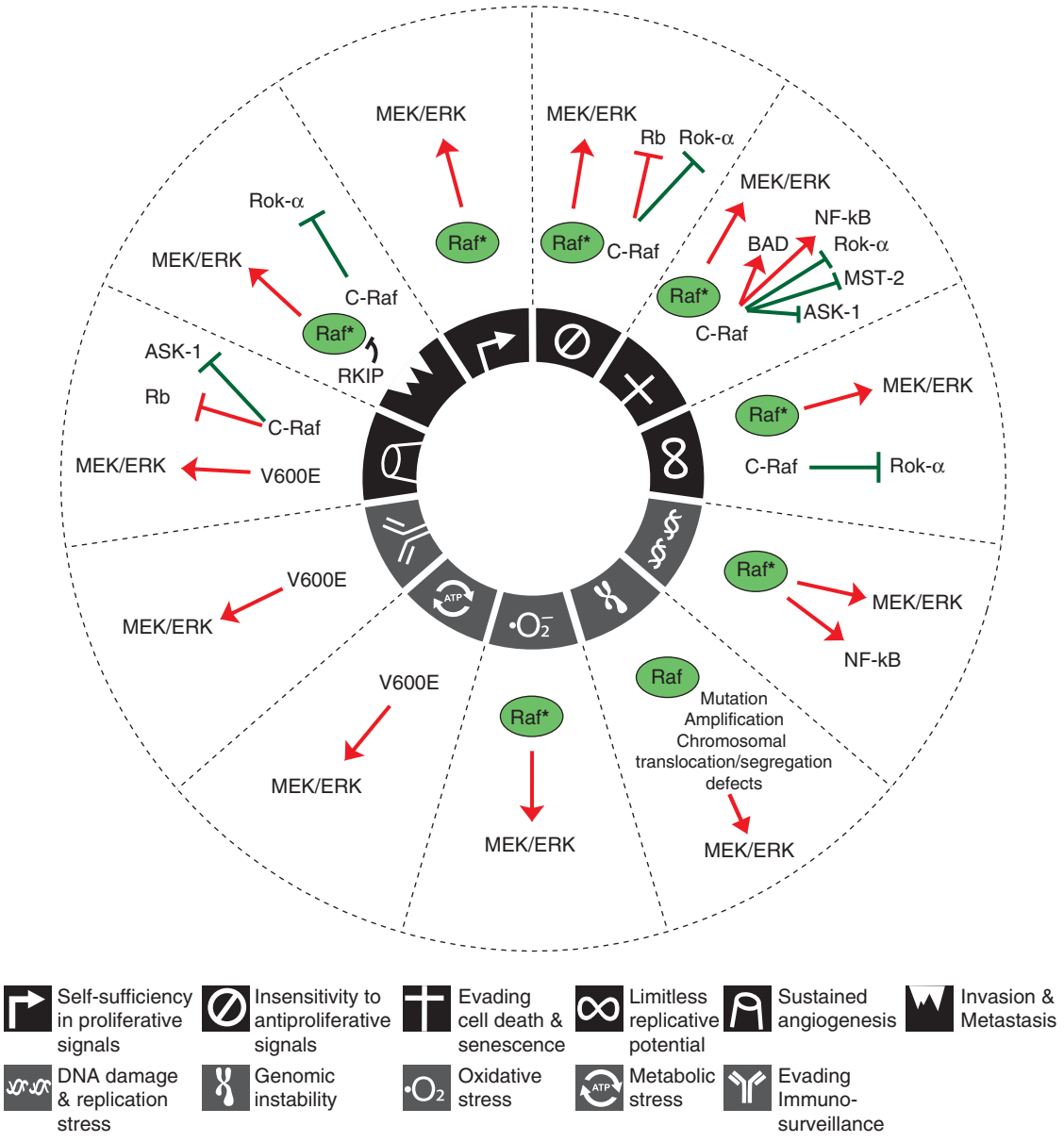

Figure 3 Contribution of Raf to the hallmarks and phenotypes of cancer. The hallmarks of cancer are depicted in black and the stress phenotypes associated with cancer in dark gray (adapted from Negrini *et al*. (2010)). The contributions of various Raf isoforms to each hallmark/phenotype and the downstream pathway mediating them are indicated. Raf* represents activated B-Raf or C-Raf; the red arrows indicate kinase-dependent functions of Raf; the red blunt arrows represent kinase-dependent inhibition of downstream pathways and the green blunt arrows represent kinase-independent inhibition processes. For clarity, only the hallmarks/phenotypes in which Raf has been implicated are depicted; mitotic stress and proteotoxic stress have been omitted.

loss of the Raf-inhibitory protein, RKIP (Zebisch *et al*., 2006, 2009). In general, the frequency of mutational changes of CRAF in human cancers is low (1%; http://www.sanger.ac.uk/genetics/CGP/cosmic). However, amplification of CRAF and other members of the ERK pathway have been observed during hormone escape in androgen-independent prostate cancer (Edwards *et al*., 2003), and both CRAF amplifications (4%) and deletions (2.2%) are strongly associated with tumor progression and an overall poorer survival in bladder cancer (Simon *et al*., 2001). Similarly, activation of the ERK pathway owing to BRAF gene duplication or mutation has emerged as a mechanism in the pathogenesis of low-grade astrocytomas (Pfister *et al*., 2008). Besides alterations in copy number, chromosomal translocations involving CRAF are found in certain human cancer sub-types such as stomach cancer (Shimizu *et al*., 1986) and pilocytic astrocytomas (Jones *et al*., 2009). The latter tumors also harbor chromosomal translocations involving BRAF activation (Jones *et al*., 2008); although seldom, such alterations have also been observed in nevi (Dessars *et al*., 2007) and radiation-induced thyroid cancer (Ciampi *et al*., 2005). In all cases, the alterations lead to constitutive RAF

activation through loss of the autoinhibitory N-terminal domain.

More recently, chromosomal translocations yielding gene fusion transcripts containing the C-terminal kinase domain of *CRAF* or *BRAF* have been identified at low frequency in prostate cancer, gastric cancer and melanoma. Both fusion proteins promoted MEK/ERK-dependent cell proliferation, migration and anchorage-independent growth in human prostate cells, but whereas expression of the BRAF fusion protein in NIH 3T3 cells induced tumor formation in nude mice, the CRAF fusion protein failed to do so (Palanisamy *et al*., 2010), implying crucial signaling differences between the BRAF and the CRAF fusion proteins. Interestingly, prostate cancer also harbored the reciprocal CRAF fusion, containing the CRAF-regulatory domain; this protein, however, has not been investigated in detail.

Besides being the target of chromosomal rearrangements, RAF has also been implicated in the induction of genomic instability. Two types of mutations have been associated with increased genomic instability thus far: BRAFV600E, the activating mutation observed with the highest frequency in melanoma and other cancers, induces genomic instability in a thyroid cell line (Mitsutake *et al*., 2005); in addition, expression of *B-RafD594A*, a transforming B-Raf mutant with impaired MEK kinase activity, can promote aneuploidy in a C-Raf-dependent, MEK-independent manner in mouse splenocytes and embryonic fibroblasts (Kamata *et al*., 2010).

C-Raf, too, has been implicated in promoting genomic instability, albeit indirectly. A balance between C-Raf and RKIP, the Raf inhibitor often lost in breast, prostate and melanoma tumors (Granovsky and Rosner, 2008), is necessary to guarantee fidelity of chromosome segregation. Loss of RKIP or C-Raf overexpression lowers the activity of the Aurora-B kinase, allowing cells to bypass the spindle assembly checkpoint and potentially resulting in genomic instability (Eves *et al*., 2006).

Self-sufficiency in proliferative signals is a crucial step on the road to transformation. In healthy tissues, soluble mitogenic growth factors are produced by one cell type and stimulate the proliferation of another. Many cancer cells are able to produce and respond to their own growth factors, resulting in a positive feedback signaling loop (autocrine stimulation), which makes them independent from their tissue environment. These proliferative signals include production of growth factors, overexpression/constitutive activation of growth factor receptors and alterations in downstream signaling cascades. The first two changes are likely to activate the Raf pathway, although the degree to which the resulting proliferation may depend on it may vary. In the context of alterations in signaling components, apart from mutational activation of RAF itself, the most direct connection is that between RAF and the members of the RAS gene family, which are activated in 33% of human cancers, particularly in those of epithelial origin (http://www.sanger.ac.uk/genetics/CGP/cosmic).

Several RAF mutations driving the proliferation of cancer cells have been described. The most frequent BRAF mutation, BRAFV600E, causes constitutive activation of the kinase as well as insensitivity to negative feedback mechanisms (Davies *et al*., 2002; Pratilas *et al*., 2009). In addition, less frequent BRAF mutations have been described that can stimulate the MEK/ERK pathway by activating wild-type CRAF in the context of a heterodimer (Davies *et al*., 2002; Garnett *et al*., 2005; Kamata *et al*., 2010). Mutations in CRAF itself are extremely rare, but overexpression has been reported at high frequency in subsets of human cancers, including hepatocellular carcinoma and squamous cell carcinoma of the head and neck (Riva *et al*., 1995; Hwang *et al*., 2004). CRAF overexpression is regarded as an early tumor marker for human lung adenocarcinoma (Cekanova *et al*., 2007); consistent with this, lung-restricted overexpression of full-length CRAF or of its truncated kinase domain causes the MEK-dependent formation of lung adenomas (Kerkhoff *et al*., 2000; Kramer *et al*., 2004). Similarly, elevated BRAF and CRAF expression and kinase activity have been observed in human glioblastomas, and a constitutive active CRAF mutant contributes to glioma formation in mice (Lyustikman *et al*., 2008). Cumulatively, these results imply that most alterations in Raf drive proliferation through stimulation of the MEK/ERK pathway. In addition to the dominant role played by BRAF oncogenic mutants, endogenous, wild-type BRAF mediates ERK activation and proliferation in uveal melanoma cells lacking RAS/RAF mutations (Calipel *et al*., 2006); conversely, CRAF, but not BRAF, is required for these process downstream from mutated NRAS in melanoma cell lines (Dumaz *et al*., 2006) or from mutated KRAS in non-small cell lung cancer cell lines (Takezawa *et al*., 2009). Studies in cultured cells and *in vivo* studies suggest that autocrine/paracrine factors resulting from ERK activation play a role in the self-sufficiency of cells harboring activating Raf mutations (Troppmair *et al*., 1998; Schulze *et al*., 2001, 2004; Vale *et al*., 2001), generating a feed-forward loop and promoting the concomitant activation of parallel proliferative pathways.

In addition to generating their own proliferative signals, either in a cell-autonomous or in a paracrine manner, cancer cells must develop *insensitivity to antiproliferative signals* that maintain tissue homeostasis. A crucial inducer of antiproliferative signals is transforming growth factor-β (TGFβ) (Seoane, 2008). Many tumors disable TGFβ signaling by downregulation or mutation of the TGFβ receptor, or through inactivation of its downstream targets SMAD4, $p15^{INK4B}$ and the retinoblastoma protein Rb (Hanahan and Weinberg, 2000). Activation of Raf and ERK induces TGFβ production but at the same time protects cells from differentiation and apoptosis (Lehmann *et al*., 2000; Park *et al*., 2000; Schulze *et al*., 2001, 2004; Wang *et al*., 2004; Riesco-Eizaguirre *et al*., 2009), enabling them to draw on the pro-tumorigenic effects of this cytokine such as promotion of proliferation, invasiveness, radioresistance and immunosuppression.

In addition, C-Raf interacts with, and phosphorylates, Rb. This interaction results in the recruitment of the Rb/C-Raf complex to proliferative promoters and increases E2F1-dependent transcriptional activity, counteracting the antiproliferative function of Rb (Wang *et al*., 1998; Dasgupta *et al*., 2006; Kinkade *et al*., 2008).

Induction of differentiation is a powerful obstacle to proliferative signals. As long as the initiated stem cells or early progenitor cells giving rise to a tumor have not lost sensitivity to differentiating signals, these can be exploited in therapy. A particularly good illustration of this is the introduction of a combination of chemotherapy and differentiation therapy, which has revolutionized the treatment of leukemia (Wang and Chen, 2008). Recently, we have shown that endogenous C-Raf is essential to maintain an undifferentiated status in Ras-driven epidermal tumors. Conditional ablation of C-Raf results in rapid regression of established tumors through MEK/ERK-independent activation of a differentiation program induced by hyper-activation of the cytoskeleton-based kinase Rok-α (Ehrenreiter *et al*., 2009). These data show that Ras-driven tumors are addicted to non-oncogenic C-Raf, and offer proof of principle that differentiation (co)therapy may be feasible in solid tumors.

Confirming the importance of Raf in the maintenance of an undifferentiated state, recent work has shown that amplification of CRAF leads to the ERK-dependent activation of β-catenin, and to the expansion of breast tumor-initiating cells in culture and cancer progression in xenografts (Chang *et al*., 2011). Thus, both MEK/ERK-dependent and -independent mechanisms can contribute to the maintenance of an undifferentiated state in tumor cells.

Evasion of senescence and apoptosis

From the above, it is clear that overexpression of full-length RAF or the truncated catalytic domain leads to the activation of the ERK pathway in cultured cells and *in vivo*. In both situations, strong activation of the pathway correlates with the induction of senescence, which has to be bypassed before hyper-proliferation ensues (Sewing *et al*., 1997; Woods *et al*., 1997; Ravi *et al*., 1998; Zhu *et al*., 1998; Roper *et al*., 2001). Thus, senescence is the Achilles' heel of the Ras/Raf/Erk pathway. Possible bypass mechanisms include direct regulation of the activity of the B-RafV600E mutant, to lower it to a level that would not induce senescence. In this context, candidates are Akt3, which can decrease the activity by phosphorylating negative-regulatory residues on BRAF (Cheung *et al*., 2008), and endogenous C-Raf, which has been shown to restrain B-RafV600E activity in the context of a heterodimer (Karreth *et al*., 2009).

Typically, however, senescence is disabled when tumor suppressors such as p16INK4a, p19ARF, p53 or PTEN are lost (Fedorov *et al*., 2003; Michaloglou *et al*., 2005; Goel *et al*., 2006, 2009; Gray-Schopfer *et al*., 2006; Dankort *et al*., 2007, 2009; Lyustikman *et al*., 2008; Dhomen *et al*., 2009; Yu *et al*., 2009; Carragher *et al*., 2010), or cooperating proto-oncogenes such as c-myc or Rac1b are expressed (Matos *et al*., 2008; Zhuang *et al*., 2008).

While RAF activation does not contribute to senescence evasion, it does have multiple, in part isoform-specific, roles in counteracting apoptosis. Downstream from activated Raf and Ras, but also from other oncogenes, the ERK pathway restrains apoptosis by regulating the expression and/or the activity of BCL-2 family members (Balmanno and Cook, 2009). In addition, MEK-independent pro-survival mechanisms, such as activation of MEKK1 and the nuclear factor-κB pathway (Baumann *et al*., 2000) and inactivation of the BH3-only BCL-2 family member BAD (Polzien *et al*., 2009), have been proposed for C-Raf (all reviewed by Niault and Baccarini, 2010). Reinforcing the connection between C-Raf and the Bcl2 family, Bcl2 deletion hinders the development of lung adenomas induced by the truncated, oncogenic form of C-Raf (Fedorov *et al*., 2002). In addition, endogenous C-Raf can restrain apoptosis in a kinase-independent manner by binding to, and inhibiting, the stress-induced, mitochondria-based kinase ASK-1 (Chen *et al*., 2001) as well as the homolog of *Drosophila*'s Hippo, the MST-2 kinase (O'Neill *et al*., 2004; Matallanas *et al*., 2007), and by regulating Fas trafficking through its interaction with the cytoskeleton-based kinase Rok-α (Piazzolla *et al*., 2005). By conferring a survival advantage, any of these events might potentially promote tumorigenesis, although their significance in this context has not yet been shown *in vivo*.

Limitless replicative potential can be achieved through avoidance of telomere shortening, which causes a DNA-damage response mediated by p53 and p21, and finally senescence. This senescent program, induced by telomere attrition, differs from the fast, oncogene-induced proliferation barrier observed, for instance, in BRAFV600E-expressing premalignant nevi (Michaloglou *et al*., 2005; Gray-Schopfer *et al*., 2006). An 85–90% portion of all cancer cells escape telomere attrition by upregulating telomerase, a reverse transcriptase that restores telomeric repeats after every cell division (Chan and Blackburn, 2004). Ets transcription factors, well-established targets of activated ERK, can stimulate the transcriptional activation of the telomerase catalytic subunit gene downstream from oncogenic growth factor receptor, Ras and Raf (Goueli and Janknecht, 2004; Dwyer *et al*., 2007), thereby potentially antagonizing telomere shortening and supporting the replicative potential of the mutated cells.

Sustained angiogenesis is absolutely required for growth of solid tumors beyond a size of about 3 mm^3. Tumor cells are able to initiate an angiogenic shift toward angiogenic initiating signals (for example, vascular endothelial growth factor (VEGF) and fibroblast growth factor-1 and 2 and suppress inhibitory signals (thrombospondin-1 and interferon-β). The impact of Raf on angiogenesis *in vivo* has been established by the delivery of a kinase-dead C-Raf construct to the tumor-associated vasculature in mice. The kinase-dead protein induced the apoptosis of both endothelial and tumor cells, leading to tumor regression

(Hood *et al*., 2002). C-Raf can promote endothelial cell survival by either MEK-dependent or -independent pathways, including ASK-1 inhibition (Alavi *et al*., 2003, 2007). In addition, selective disruption of the interaction between C-Raf and Rb inhibits the development of tumor-associated microvessels and suppresses the growth of tumor xenografts (Dasgupta *et al*., 2004; Kinkade *et al*., 2008). Thus, several C-Raf-dependent pathways can contribute to angiogenesis. By contrast, induction of angiogenesis by B-RAFV600E, involving expression of hypoxia-inducible factor-1α (Kumar *et al*., 2007) and VEGF (Sharma *et al*., 2005, 2006; Sumimoto *et al*., 2006), is entirely MEK-dependent. Conversely, conditional ablation of endogenous B-Raf prevents the angiogenic switch in a mouse model of pancreatic islet carcinoma driven by loss of function of the tumor suppressors p53 and Rb. B-Raf-deficient tumor cells proliferate normally despite decreased ERK activation, but produce insufficient amounts of the proangiogenic factors VEGF and TGFβ, resulting in reduced blood vessel density and tumor proliferation, and delayed tumor progression (Sobczak *et al*., 2008).

Tissue invasion and metastasis depends upon all the other hallmarks acquired during the process of tumor formation as well as on changes in proteins tethering cells to their surroundings. Changes in the expression of cell–cell adhesion molecules and/or in the binding specificities of integrins, as well as upregulation and activation of extracellular proteases, result in the ability of cancer cells to invade and colonize new terrain. Raf can influence invasion at several levels. First, activated Raf is involved in the production of TGFβ, which promotes invasion and metastasis (Lehmann *et al*., 2000; Sobczak *et al*., 2008; Riesco-Eizaguirre *et al*., 2009), as well as the epithelial–mesenchymal transition that precedes invasion in response to this factor (Janda *et al*., 2002). Second, both B-Raf and C-Raf have essential, if opposite, roles in cell contractility and migration: B-Raf increasing Rho-dependent contractility and opposing migration in an ERK-dependent manner (Pritchard *et al*., 2004), and C-Raf reducing contractility and increasing migration by inhibiting the Rho effector Rok-α (Ehrenreiter *et al*., 2005). In addition, B-RAFV600E/MEK/ERK are responsible for upregulation of several proteins involved in migration, and support integrin signaling, inducing melanoma cell invasion and metastases (Liang *et al*., 2007; Klein *et al*., 2008; Argast *et al*., 2009; Old *et al*., 2009). The B-RafV600E/MEK/ERK axis can also increase melanoma cell contractility and invasion by repressing the gene coding for a cGMP-specific phospshodiesterase, PDE5A (Arozarena *et al*., 2010). The resulting increase in the cGMP pool causes a raise in intracellular Ca^{++} and ultimately increased contractility, which boosts the rounded, bleb-associated mode of motility adopted during invasion (Sahai and Marshall, 2003).

PDE5A expression was found to be lower in metastasis-derived patient material than in primary tumors. As ERK is likely activated in both samples, it would have been interesting to know at which level is PDE5A expression further regulated, and what are the secondary events leading to full-fledged downregulation in metastasis. One possibility here are changes in tissue architecture, which in itself can exert strong antiproliferative effects and counteract invasion. Cadherin-based cell–cell adhesion, for instance, efficiently counteracts tumor proliferation, angiogenesis and metastasis in CRAF-driven lung adenomas (Ceteci *et al*., 2007). Additional regulators of tissue architecture are matrix metalloproteases, which are often overexpressed in cancer (Kessenbrock *et al*., 2010). Increased expression of one of these enzymes, matrix metalloprotease-9, by the Raf/MEK/ERK pathway in three-dimensional breast tissue cultures leads to a remodeling of the microenvironment, which induces loss of tissue polarity and re-initiation of proliferation (Beliveau *et al*., 2010).

In keeping with a role for the Raf/MEK/ERK pathway in invasion, the Raf-inhibitor protein RKIP has been identified as a suppressor of metastasis in many cancers (Granovsky and Rosner, 2008). Recently, a pathway has been discovered in which RKIP, through inhibition of the Raf/MEK/ERK module, increases the processing of the *let-7* miRNA. This, in turn, inhibits the chromatin-remodeling factor HMGA2, which contributes to the expression of several metastasis-promoting genes (Dangi-Garimella *et al*., 2009).

To colonize a new site, tumor cells must extravasate from the blood vessels. In melanoma cells, BRAFV600E promotes this process by causing the production of both tumor- and microenvironment-derived interleukin-8. This cytokine recruits polymorphonuclear leukocytes, which bind to melanoma cells ultimately facilitating their trans-endothelial passage (Liang *et al*., 2007).

Avoidance of immunosurveillance enables tumors to evade recognition and destruction by the immune system. Cancers escape surveillance by selecting for non-immunogenic tumor cells, such as those that have downregulated human leukocyte antigen class-I molecules and/or have become resistant to cytotoxic T-lymphocyte-induced killing (immunoselection). Alternatively, tumors can actively repress immune cells in various ways, creating an immune-privileged environment (immunosubversion) (reviewed by Zitvogel *et al*. (2006)). BRAFV600E, for instance, mediates immunosubversion by inducing the cytokines interleukin-10 and interleukin-6 (Sumimoto *et al*., 2006), and contributes to immune evasion by inducing an MEK/ERK-dependent decrease in the expression of melanoma differentiation antigens, which is recognized by antigen-specific T-lymphocytes (immunoselection). Unlike MEK inhibitors, treatment with a BRAF-specific inhibitor leaves the function of T-lymphocytes intact, raising hopes that such inhibitors might bypass immunoevasion (Boni *et al*., 2010).

Implementing the hallmark events described above comes at a high stress cost for tumor cells. Recently, five stress phenotypes of cancer have been defined (Luo *et al*., 2009): (1) DNA damage, resulting from telomere shortening, replication stress and oncogene activation, and from mutations of DNA-repair and DNA-damage

checkpoint genes; (2) mitotic stress, a consequence of chromosomal instability; (3) proteotoxic stress, caused by accumulation of misfolded proteins; (4) oxidative stress mediated by the generation of reactive oxygen species within a cancer cell and (5) metabolic stress (Kroemer and Pouyssegur, 2008), a consequence of enhanced aerobic glycolysis used by cancer cells for energy production.

How does RAF contribute to overcoming stress? In the case of genotoxic stress, activated Ras and Raf can induce the expression of the *mdm2* gene, leading to p53 degradation; at least in cells lacking the Mdm2 inhibitor p19ARF, this leads to reduced p53-dependent apoptosis following DNA damage (Ries *et al*., 2000). Downstream from p53, Ras or Raf activation by HB-EGF is responsible for the ERK-mediated induction of the cyclooxygenase-2 gene, which inhibits genotoxic stress-induced apoptosis (Han *et al*., 2002). Scatter Factor (hepatocyte growth factor), another growth factor that protects tumors from genotoxicity, uses Raf to signal survival through activation of nuclear factor-κB (Fan *et al*., 2007). In addition, an interplay between oncogenic Raf/ERK and ATM has been shown to promote homologous recombination repair in response to radiation (Golding *et al*., 2007), in line with previous reports showing a correlation between oncogenic Raf and radioresistance in tumors (Kasid *et al*., 1987, 1989, 1996). Finally, the RAF/MEK/ERK pathway protects multiple myeloma cells from DNA damage induced by treatment with a Chk1 inhibitor (Dai *et al*., 2008). These findings suggest that Raf and MEK inhibitors could be combined with cytostatic drugs or radiation in the therapy of cancer.

The relationships between Raf and oxidative stress are manifold: RAF/MEK/ERK activation can prevent the onset of oxidative stress in growth factor-deprived cells (Kuznetsov *et al*., 2008); on the other hand, generation of reactive oxygen species by derivatives of geldanamycin, a chemotherapeutic that inhibits the chaperone function of HSP90 and enforces the degradation of their client proteins, including Raf, is able to inhibit the activity of BRAFV600E (Fukuyo *et al*., 2008). Finally, a most interesting connection, relevant in terms of oncogene-selective therapy, has been reported recently between oncogenic activation of RAS and RAF, and the small-molecule drug erastin. Erastin causes mitochondrial dysfunction and oxidative cell death by activating voltage-dependent ion channels (voltage-dependent anion channels (VDACs)) on the mitochondria. In a panel of cancer cell lines of different origin, RAS and BRAF activation potentiated the lethality of the drug by inducing the expression of the VDACs (Yagoda *et al*., 2007). Thus, VDAC expression may represent a targetable weak spot in RAS- and RAF-driven tumors.

Metabolic stress, particularly lack of nutrients, imposes a number of metabolic checkpoints that cancer cells must bypass to continue proliferating under the dire conditions often found in the tumor microenvironment. BRAFV600E-expressing melanoma cells appear to solve this problem through ERK-mediated phosphorylation of the tumor suppressor and energy sensor LKB1. This phosphorylation, which occurs in the context of a physical complex including B-Raf V600E, prevents the LKB1-mediated activation of the AMP-activated protein kinase, which restricts protein synthesis (Zheng *et al*., 2009). The net result is normal operation despite nutrient shortage, and therefore a competitive advantage under conditions of metabolic stress.

RAF inhibitors–clinical success and challenges

From the above it is clear that Raf kinases are prime target for the design and application of molecule-target therapies of cancer, particularly melanoma. Several companies have generated Raf inhibitors currently in preclinical and clinical trials, and a drug specifically targeting BRAFV600E (PLX4032/RG7204; Plexxikon/Roche, Berkeley, CA, USA (Tsai *et al*., 2008; Joseph *et al*., 2010)) has recently produced dramatic results, with response rates of 70–80% as single agent in metastatic melanoma patients (Bollag *et al*., 2010; Flaherty *et al*., 2010). Similar results have been obtained with another ATP competitive BRAF inhibitor (GSK 2118436; GlaxoSmithKline, Brentford, UK (Kefford *et al*., 2010)). These drugs, which are currently being tested in clinical trials in patients affected by other solid tumors with BRAFV600E mutations, such as thyroid carcinomas or colon cancers (Arkenau *et al*., 2010; Puzanov *et al*., 2011), are reasonably well-tolerated. However, one intriguing and potentially worrying issue is the paradoxical increase in the proliferation and activation of the MEK–ERK pathway in cells not harboring the BRAFV600E mutation. The underlying mechanism is an allosteric effect of the drug, which enforces the dimerization of endogenous BRAF with CRAF or ARAF (Hatzivassiliou *et al*., 2010; Heidorn *et al*., 2010; Poulikakos *et al*., 2010). Within these dimers, only one active component is required for activation of the MEK–ERK pathway; therefore, at non-saturating concentrations, the inhibitors activate the pathway rather than disabling it, particularly in the presence of activated RAS, and it is possible that the rapid development of benign skin tumors in patients treated with RAF inhibitors might be fueled by such a mechanism (Degen *et al*., 2010; Robert *et al*., 2010) (Figure 4).

More troublesome is the fact that melanoma cells develop chemoresistance by a number of different molecular mechanisms (reviewed by Poulikakos and Rosen, 2011), leading to relapse of drug-responsive disease. Unlike the case of imatinib resistance, often caused by mutations in the kinase domain of the target BCR-ABL (Weisberg *et al*., 2007), *de novo* mutations in B-RAF have not been observed in relapsing tumors. Rather, acquired resistance involved reactivation of the ERK pathway by switching to other MEK kinases (other RAF isoforms (Villanueva *et al*., 2010) or COT/Tpl2 (Johannessen *et al*., 2010)), or by activating mutations in NRAS (Nazarian *et al*., 2010), but also upregulation of receptor tyrosine kinases driving other pathways (Nazarian *et al*., 2010; Villanueva *et al*., 2010)

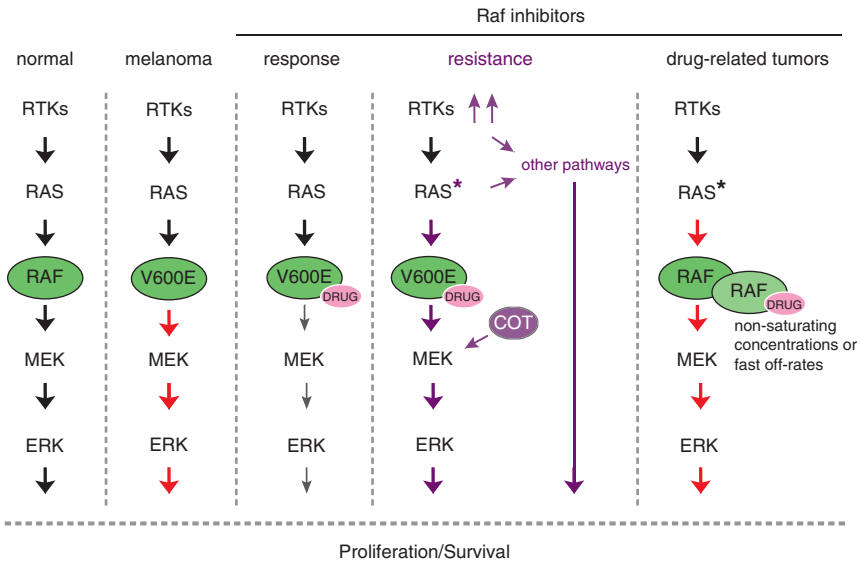

Figure 4 RAF inhibitors: response, resistance and drug-related tumors. In normal cells, RAF activation drives ERK activation downstream from RAS. In melanoma, BRAF mutants (V600E) with high kinase activity drive ERK activation (red arrows) independently of RAS. Cancer cells harboring these mutants are sensitive to BRAF inhibitors, which blunt kinase activity and reduce ERK activation as well as proliferation (thin arrows). Unfortunately, however, resistance arises, by mechanisms involving activation of other MEK kinases, such as COT, but also upregulation of receptor tyrosine kinases (RTKs) and of other pathways downstream from RAS (purple arrows). Finally, drug-related epidermal tumors have been observed in patients treated with RAF inhibitors. They correlate with ERK activation, which results from the ability of the drug to promote RAF dimerization. If only one subunit of the dimer is bound to the inhibitor (for instance at non-saturating inhibitor concentrations, or in the case of inhibitors with fast off-rates), the other subunit is activated and is capable of phosphorylating MEK with high efficiency, generating a tonic signal leading to increased proliferation. Thus, RAF inhibitors can paradoxically function as ERK activators, and potentially induce the development of drug-related tumors. ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase.

(Figure 4). Therefore, overcoming melanoma resistance might require modulation of multiple pathways.

Conclusions

The study of the Raf pathways has been extremely rewarding. The first serine/threonine kinase oncogene discovered has proven an excellent target in single-agent therapy of the disease it is most frequently associated with; in turn, investigation of the mode of action of RAF inhibitors has shed light on the mechanism of regulation of the cellular Raf enzyme. Animal models continue to delineate essential functions of the pathway components and the discovery of protein–protein interactions within the pathway and cross-pathways provides further potential leads for novel therapeutic strategies. Almost 30 years after its discovery, Raf is still a fascinating topic for basic and clinical researchers, and will remain so for many years to come.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank all the members of the Baccarini lab for helpful discussions and apologize to all the colleagues whose work could not be cited in this review for reasons of space. The Baccarini lab is supported by the Austrian Scientific Research Fund (Grants P19530 and SFB 021) and the European Community (Grants INFLA-CARE and GROWTHSTOP).

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Hippo signalling

Hippo signalling pathway was discovered in Drosophila in genetic mosaic screens of imaginal disc overgrowth. Genetic epistasis experiments quickly indicated interaction between its various components and established their order within the pathway. Later on it became obvious that all core components and the basic principles of their action in cell as well as their function on a tissue level are conserved throughout whole metazoan kingdom (Harvey and Tapon 2007). Maybe the strongest support for this comes from the experiments in which phenotypes of many mutants of the pathway core components in flies can be rescued by corresponding mammalian homologues (Saucedo and Edgar 2007; Zhao, Lei et al. 2008).

The purpose of the pathway is to maintain tissue homeostasis by restricting cell proliferation and survival. This is achieved by limiting activity of the pathway's ultimate effector Yorkie (or YAP in mammals) that is able to co-activate expression of proliferation and survival genes in the assist of variety of transcription factors. Yorkie/YAP inhibition is achieved by its phosphorylation by Warts kinase (LATS in mammals) that by phosphorylation is activated by Hippo kinase (MST in mammals). Phosphorylated Yorkie/YAP undergoes cytoplasmic retention by means of interaction with 14-3-3 protein.

Hippo, Warts and Yorkie together with adaptors Salvador and Mats are considered core components of the pathway, and their activity can be affected to various extents by many other Hippo pathway-associated proteins. In the following sections I summarize Hippo signalling principles that have been primarily deciphered in flies, similarities and differences that can be found in mammals Hippo/Mst pathway and its relevance for tumorigenesis.

Hippo signalling in flies

In flies signals from outside of the cell are reaching core components of the pathway through trans-membrane, atypical cadherin Fat (Ft) that interacts with other cadherin Dachsous (Ds) on another cell, as a receptor-ligand pair. Ft and Ds are phosphorylated on their cadherin domains by Golgi-resident kinase Four-jointed (Fj). Intracellular domain of Ft is also phosphorylated by casein kinase Discs overgrown (Dco). Ds stability is apart from that regulated by cytoplasmic protein Lowfat (Lft) that can bind cytoplasmic domains of both Ft and Ds (Pan 2010). Crumbs (Crb) is another trans-membrane protein with numerous EGF-like and laminin AG-like repeats, PDZ-binding motif and FERM-binding motif (FBM). With its FBM Crb may bind one of subsequent proteins in the pathway, Expanded (Pan 2010). Expanded (Ex) and Merlin (Mer) are both members of band 4.1 protein family and interact

with C2 and WW domain-containing protein Kibra. This triple-protein complex recruits pathways core components, Hippo, Warts, Salvador and Mats to the membrane for activation (Pan 2010). Core components activity is also regulated by Dco, but whether it is functioning in parallel or downstream of Ex and Mer also remains elusive. Dco and Warts are brought together by scaffolding protein Dachs, unconventional myosin which localisation of is regulated by palmitoyltransferase approximated (App) (Harvey and Tapon 2007).

Salvador (Sav) is another scaffold with WW domain binding to Warts (Wts) and SARAH domain binding to Hippo (Hpo), and brings together both kinases to facilitate signal transduction. dRassf protein also contains SARAH (Sav-Rassf-Hpo) domain and therefore is able to compete for binding to Hpo and inhibit pathway activation. Lately dRassf was shown to recruit PP2A dSTRIPAK that is able to inactivate Hpo by dephosphorylation. Hpo is serine/threonine kinase from sterile-20 kinase family that is able to phosphorylate and activate Wts, another serine/threonine kinase, member of NDR kinase family. Wts activation depends also on Mats and leads to phosphorylation of Yorkie (Yki) and its inactivation through cytoplasmic retention (Harvey and Tapon 2007).

When activation of upstream Hippo components is compromised Yorkie is imported to the nucleus by a co-transportation mechanism with associated transcription factor. As Yorkie is unable to bind DNA, in order to activate transcription of target genes, it needs to interact with transcription factors, like TEAD family member Scalloped, Homothorax or Smads. Yki is co-activating expression of genes including those encoding inhibitor of apoptosis DIAP, cyclin E, and oncogenic miRNA bantam (Harvey and Tapon 2007; Zhao, Lei et al. 2008). Yki also controls upstream activators of Hippo signalling, like Crb, Kibra, Ex and Fj, probably to achieve negative feedback regulation of its own activity. Furthermore, control of expression of E-cadherin, dMyc, Notch ligand Serrate, Wnt-pathway ligand Wingless, EGFR ligand Vein and proteoglycans Dally and Dally-like by Yki allows for extensive pathway cross talk (Pan 2010).

MST signalling in mammals

All components of the Hippo pathway are evolutionarily conserved in mammals, but functional connections of some of them with the pathway's core remains to be determined. Furthermore, in mammals most of these proteins are encoded by multiple genes, providing higher than in flies redundancy and probably number of roles that they may perform.

The role of homologue of drosophila Mer, Neurofibromin 2 (NF2) in activation of MST kinases *in vivo* is supported by one report (Zhang, Bai et al. 2010), while doubted by the other (Benhamouche, Curto et al. 2010). Further, there are two mammalian homologues of Hippo, kinases MST1 and 2 and two homologues of Warts, kinases LATS1 and 2. WW45 is a mammalian homologue of Salvador and MOBKL1A and B homologues of drosophila Mats that are very similar and often collectively referred to as MOB1. Yes-activated protein (YAP) and closely related transcriptional co-activator with PDZ binding motif (TAZ) are performing gene transcription co-activating function homologous to Yorkie. Apart from cytoplasmic retention as it is in flies, YAP is found to be regulated by degradation. Additional phosphorylation site on Serine 381 targeted by LATS kinases primes YAP for phosphorylation by CK1δ/ε followed by recruitment of ubiquitin ligase and targeting to proteasome (Pan 2010). Both YAP and TAZ, when not inhibited, activate transcription through binding to TEAD-family transcription factors, 4 mammalian homologues of drosophila Scalopped. So far they are also found to activate wider repertoire of additional transcription factors, including p73, PEBP2α, ERBB4 and ASPP2 (Saucedo and Edgar 2007; Zhao, Lei et al. 2008).

The exact external signals that send input to the Hippo pathway in mammalian cells are not well established. NF2 protein is known to be phosphorylated and activated in response to contact inhibition in cell culture (Morrison, Sherman et al. 2001). Moreover, similarly to LATS2 (McPherson, Tamblyn et al. 2004), it is required to inhibit cell growth upon high culture density (Lallemand, Curto et al. 2003). Further, NF2 is regulating transcriptional activity and localisation of YAP in confluent cell cultures (Zhao, Wei et al. 2007). In cell lines together with KIBRA and WW45 it stimulates LATS phosphorylation and in mouse livers is required for efficient Lats and Yap phosphorylation and cytoplasmic retention of the latter (Zhang, Bai et al. 2010). But in hepatoblasts Nf2 seems to be dispensable for regulation of Yap localisation in response to high cell density. Instead of that, it suppresses Egfr-mediated Akt and Stat3 activation (Benhamouche, Curto et al. 2010), probably by limiting Egfr availability on cell surface (Curto, Cole et al. 2007).

MST kinases can be activated during FAS receptor stimulation and are controlled by inhibitory interaction with MAPK pathway activator C-RAF (O'Neill, Rushworth et al. 2004). In response to FASL stimulation RASSF1A is displacing C-RAF from this complex that leads to MST2 activation (Matallanas, Romano et al. 2007). Activity of MST kinases is also associated with their proteolytic cleavage, possibly by caspases, and autophosphorylation

(Lee, Murakawa et al. 1998; Glantschnig, Rodan et al. 2002). In mouse liver Mst1 and 2 kinases are required for efficient Lats1/2 and Yap phosphorylation (Lu, Li et al. 2010) and in hepatocyte Yap is a target of Lats kinases (Benhamouche, Curto et al. 2010) but specifically Mst1/2-mediated Yap inactivation in the liver is likely achieved through intermediate kinase distinct from Lats1/2 (Zhou, Conrad et al. 2009).

YAP is also stabilised by c-Abl-mediated phosphorylation induced by DNA damage that confers p73 binding and apoptosis induction (Zhao, Lei et al. 2008). One of binding partners and activity modulators of TP53, ASPP1 is breaking interaction between LATS1 and YAP. Thereby ASPP1 inhibits cytoplasmic retention and degradation of the latter, leading to increased YAP-mediated cell survival (Vigneron, Ludwig et al. 2010). On the other hand, LATS2 in response to oncogenic stress phosphorylates ASPP1 what drives its nuclear translocation where it directs TP53 to promoters of pro-apoptotic genes, what in turn is inhibited by YAP (Aylon, Ofir-Rosenfeld et al. 2010).

Tumour suppressive functions of MST signalling

As Hippo signalling primary biochemical function is to inhibit the activity of proto-oncogene YAP, it is not surprising that many of its components perform tumour suppressive functions. Still, most of them are not often found mutated in human cancer, with few exceptions. NF2 is a tumour suppressor in hereditary cancer syndrome neurofibromatosis type 2 characterised by tumours of central nervous system. Apart from that, mutations in MOB1 are found in human melanoma and mouse mammary carcinoma.

On the other hand there is growing evidence that epigenetic silencing plays role in inactivation of many Hippo components in human cancers. LATS1 and 2 methylation-dependent silencing occurs in astrocytoma and correlates with aggressiveness of breast cancer. Furthermore mice deficient for Lats1 develop ovarian tumours and soft tissue sarcomas. LATS2 expression is also negatively regulated by oncomiRs in testicular germ cell tumours. MOB1 expression is lost in colorectal and lung cancers irrespective of its gene sequence and methylation status. MST kinases-encoding genes are found hypermethylated in soft tissue sarcoma. They also suppress liver growth and tumorigenesis in mice by restricting YAP activity, what is achieved most probably without direct involvement of LATS kinases. YAP, apart from being tumour proto-oncogene in the liver and causing hepatomegaly when over-expressed in this organ, is also amplified in mouse mammary tumour model. Moreover genomic locus that encodes YAP in humans is amplified in numerous cancers, including

ovarian, liver, lung, pancreatic, oesophegal and brain. Consequently, YAP overexpression is frequent in lung, ovarian, pancreatic, liver, colorectal and prostate carcinomas and gene encoding its partner, TEAD4, is also found amplified in various cancers. TAZ function in cancer was until now not investigated to such an extent as of YAP, but its overexpression is found at least in a subset of breast cancers. Finally, RASSF1 has originally been found as a tumour suppressor encoded within locus 3p21,3, which is frequently deleted in human tumours. Members of RASSF family are generally proposed to function as tumour suppressors, particularly isoform A of RASSF1, which is lost in numerous cancers due to gene promoter methylation (Harvey and Tapon 2007; Pan 2010).

Ras associated factors protein family

Ras-associated factors (RASSF) protein family in humans is encoded by 6 genes (RASSF1-6). They are putative RAS effectors, as in their sequence they contain RAS-association domain (RA). Nevertheless, apart from RASSF5, functionality and specificity of RA domain of RASSF proteins for RAS binding remains unproven. They all also contain SARAH (Salvador-Rassf-Hippo) domain responsible for the interaction with Hippo pathway components. Additional variety is provided by splicing and alternative promoter usage that leads to multiple isoforms in case of some of the members. Recently additional four members of the family have been identified that share lower homology with the previously known members and are lacking C-terminal SARAH domain (Avruch, Xavier et al. 2009).

Gene encoding RASSF1 undergoes differential splicing and has two transcription start sites what results in production of 7 different isoforms (A-G) that differ in protein domain composition. Until now biological relevance could be shown only for transcripts A and C, which are ubiquitously expressed in non-tumour tissues. Both isoforms share SARAH and RA domains and ATM-phosphorylation consensus sequence. Isoform A is transcribed from more upstream transcription start site, and results in the protein with additional C1 domain at the N-terminus. C1 domain is similar to diacylglycerol (DAG)-binding domain and contains a zinc-finger (Richter, Pfeifer et al. 2009).

Isoform A of *Rassf1* gene is subjected to epigenetic silencing in cancers as a result of hypermethylation of CpG islands in its promoter, whereas expression of isoform C is usually retained. Moreover, isoform A has tumour suppressive functions *in vitro* and *in vivo*, what is not the case for isoform C. Epigenetic silencing of RASSF1A expression occurs in various cancer types, correlates with cancer progression and poor survival (Richter, Pfeifer et al.

2009). Especially in HCC up to 90% of examined cases loose RASSF1A expression due to promoter hypermethylation (Schagdarsurengin, Wilkens et al. 2003).

Mice deficient for RASSF1A are tumour prone and late in life develop with varying frequencies spontaneous lung adenomas, lymphomas and breast adenocarcinomas (Tommasi, Dammann et al. 2005). They are also more susceptible to chemically induced skin carcinogenesis and have accelerated intestinal carcinogenesis in Apc^{Min} mouse model (van der Weyden, Arends et al. 2008).

Despite extensive research done in the last years, function of RASSF1A (and other RASSF proteins as well) is not ultimately determined. Apart from the role in influencing Hippo signalling, RASSF1A might perform its tumour suppressive function by controlling other apoptotic pathways, cell cycle, DNA-damage response and TP53 stability, or mictrotubule stability and mitotic progression. RASSF1A, by binding to MOAP-1, helps to activate BAX during TNF-α or TRAIL-induced apoptosis. It also inhibits accumulation of Cyclin D1 and AP-1 activity that positively influence cell cycle progression. In the interphase RASSF1A localises to microtubules (MTs) and during mitosis to centrosomes and mitotic spindle, most likely through the interaction with MT-associated proteins (MAPs). It also binds and is phosphorylated by serine/threonine mitotic kinase Aurora-A, and probably works as a scaffold for other Aurora-A targets. Moreover, region encompassing RA domain of RASSF1A confers binding of small GTPase RAN involved in mitosis. Therefore RASSF1A is able the influence microtubule stability and mitotic progression. Finally, because of its putative phosphorylation site for kinases ATM/ATR, it is proposed to sense DNA-damage.

Results

Rationale and aims I: C-Raf role in liver cancer

Previous research from the Baccarini lab shows crucial function of C-Raf in protecting mouse hepatocyte from apoptosis during development (Mikula, Schreiber et al. 2001) and in adult animal in response to FasL treatment (Matallanas, Romano et al. 2007). Apoptosis evasion is one of the classical hallmarks of cancer that needs to be achieved by tumour cells to resist anti-tumour defences of the organism (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). Furthermore, C-Raf is overexpressed in human HCC (Hwang, Choi et al. 2004) and sorafenib, primarily designed as C-Raf inhibitor is a first molecularly targeted drug approved for HCC treatment (Llovet, Ricci et al. 2008) (although recent research indicates that its use might be beneficial through inhibiting other kinases (Liu, Cao et al. 2006)). Therefore we set out to investigate role of C-Raf in hepatocarcinogenesis in the mouse model of HCC.

Mice with hepatocyte specific *c-raf* deletion are more prone to chemically-induced liver tumours.

We subjected 129/Sv mice with floxed exon 3 of *c-raf-1* gene and *alfp-cre* transgene to DEN/Pb chemical carcinogenesis protocol (Figure 3A). After 30 weeks carcinogenesis *alfp-cre;c-raf*$^{f/f}$ (*c-raf*$^{\Delta hep}$) mice have substantially more tumour-affected livers than *c-raf*$^{f/f}$ mice judging by their gross morphology (Figure 4A). They also have significantly increased liver/body weight ratio (Figure 4B), indicating increased liver mass, likely resulting from increased tumour load. Analysis of H&E-stained paraffin-embedded liver sections shows higher percentage of section affected by the tumours as well as higher number of tumours detected per designated area in *c-raf*$^{\Delta hep}$ mice (Figure 4C and D). This indicates that *c-raf*$^{\Delta hep}$ mice have more liver tissue affected by tumours and increased tumour number.

It is known that Cre-recombinase expression in cells may have genotoxic effect (Loonstra, Vooijs et al. 2001), what may influence liver carcinogenesis process (Takami, Kaposi-Novak et al. 2007). Apart from that location of *alfp-cre* transgene insertion within the mouse genome has not been determined so far. This leaves the possibility that *alfp-cre* transgene might affect some genomic element relevant to our studies. To determine whether phenotype in *c-raf*$^{\Delta hep}$ mice is a result of hepatocyte-restricted *c-raf* deletion, Cre-recombinase expression or other *alfp-cre*-transgene-specific effects we conducted liver carcinogenesis with the same protocol on WT and *alfp-cre* animals without floxed *c-raf* alleles. With similar numbers of animals we

could not detect significant differences between WT and *alfp-cre* mice in respect to their liver mass, tumour-affected area or nodule number estimated on H&E-stained liver sections (Figure 4E). Therefore we conclude that *alfp-cre* transgene does not influence liver carcinogenesis after 30-weeks in DEN/Pb model. It also ensures that observed phenotype is a result of disrupted *c-raf* expression.

C-Raf deletion prior to tumour initiation enhances liver carcinogenesis

Carcinogenesis is a multistep process where primarily initiated cells need to proliferate to form macroscopic nodules which then by acquiring genomic and epigenomic changes evolve to invasive and metastatic cancers. To determine the carcinogenesis stage in which *c-raf* deletion is required for enhanced liver tumour growth in *alfp-cre;c-raf*$^{f/f}$ mice we used same carcinogenesis protocol on *mx-cre*;*c-raf*$^{f/f}$ mice. In these mice deletion of *c-raf* floxed allele is mediated in the liver by poly(I:C)-inducible product of *mx-cre* transgene in hepatocytes and non-parenchymal cells. By injecting these mice with poly(I:C) we induced deletion of *c-raf* before, shortly after and late during carcinogenesis protocol to study its role in tumour initiation, promotion and regression, respectively (Figure 3B).

Deletion of *c-raf* before tumour initiation results in comparable liver gross morphology and liver mass of *mx-cre*;*c-raf*$^{f/f}$ (*c-raf*$^{\Delta liv}$) mice as *c-raf*$^{f/f}$ (Figure 5A and B). This is in contrast to the *alfp-cre*-mediated *c-raf* deletion, which leads to markedly more affected livers and increased liver mass (Figure 4A-B). Analysis of H&E-stained liver sections showed similar tumour-affected area in *c-raf*$^{\Delta liv}$ mice, but increased nodule number per defined area (Figure 5C and D). This indicates similar tumour mass but increased tumour number in livers of *c-raf*$^{\Delta liv}$ mice. These results show that *c-raf* deletion in the liver before tumour initiation enhances tumorigenesis in this organ. Some of the features of the *alfp-cre;c-raf*$^{f/f}$ phenotype, like increased liver mass and tumour-affected liver are not manifested in *mx-cre*;*c-raf*$^{f/f}$ mice. Therefore accompanying *c-raf* deletion in non-parenchymal cells in *mx-cre*;*c-raf*$^{f/f}$ mice is partially suppressing phenotype of sole *c-raf* deletion in the hepatocyte.

Similarly to deletion before, deletion of *c-raf* two weeks after tumour initiation also results in comparable liver gross morphology and liver mass of *c-raf*$^{\Delta liv}$ mice as *c-raf*$^{f/f}$ (Figure 5E and F). In contrast total tumour mass and tumour number as judged from H&E-stained liver sections remains unchanged (Figure 5G and H). Therefore, *c-raf* deletion after tumour initiation, unlike deletion before, is not affecting liver chemical carcinogenesis.

C-Raf plays role in protection of liver from apoptosis during development (Mikula, Schreiber et al. 2001) and in adult animal (Matallanas, Romano et al. 2007), and apoptosis evasion is one of classical hallmarks of cancer cells. Therefore, we checked cell death in developed tumours after 30 weeks carcinogenesis in *c-raf*$^{\Delta liv}$ mice in which *c-raf* deletion was induced after tumour initiation. We applied terminal deoxyunucleotydil transferase dUTP nick end labelling (TUNEL), which indicates DNA fragmentation in cells undergoing apoptosis or necrosis. Number of TUNEL-positive cells in tumours from livers of *c-raf*$^{\Delta liv}$ mice is similar to those from *c-raf*$^{f/f}$ (Figure 5I). Therefore, *c-raf* deletion in hepatocytes and non-parenchymal cells does not influence cell survival in DEN-induced liver tumours.

Sorafenib, small molecule inhibitor designed to block C-Raf activity, has recently been shown to prolong survival of patients with advanced, unresectable HCC (Llovet, Ricci et al. 2008). We decided to investigate whether in our model C-Raf ablation affects progression of developed tumours or their maintenance. We subjected to carcinogenesis protocol *c-raf*$^{f/f}$ and *mx-cre*;*c-raf*$^{f/f}$ mice for 28 weeks, induced *c-raf* deletion with poly(I:C) and kept them on Pb food for further 16 weeks. Isolated livers from animals of both genotypes have numerous large, vascularised tumours (Figure 5J), their mass is very high (12,4% ±8,7 for *c-raf*$^{f/f}$ and 15,9% ± 6,1 for *c-raf*$^{\Delta liv}$) (Figure 5K) and mice of both genotypes exhibit occasional lung metastasis (Gabriele Maurer, unpublished data). This indicates advanced stage of the disease and the capability of the protocol to induce fully progressed HCC able to form metastasis, resembling course of HCC in the clinic. Livers from *c-raf*$^{\Delta liv}$ and *c-raf*$^{f/f}$ have comparable gross morphology and liver mass (Figure 5J and K). This indicates that *c-raf* deletion is neither affecting tumour progression nor tumour maintenance in the liver chemical carcinogenesis model.

Deletion of floxed allele by inducible Cre recombinase might not be 100% efficient. In the case of negative selection against cells that have deleted targeted allele, tumours might preferentially arise from the clonal expansion of cells that retained it. Therefore, we checked efficiency of *c-raf* deletion by performing genotyping PCR on cell lysates from isolated tumours of *c-raf*$^{f/f}$ and *mx-cre*;*c-raf*$^{f/f}$ animals that were injected with poly(I:C) two weeks after DEN-injection. As a control, in parallel we also genotyped samples from *c-raf*$^{f/-}$ heterozygous mouse. All genotyped tumours from *mx-cre*;*c-raf*$^{f/f}$ animals showed efficient conversion of flox to Δ allele (Figure 5L). Similar genotyping PCRs were performed for selected tumours of each experimental *mx-cre*;*c-raf*$^{f/f}$ and *c-raf*$^{f/f}$ animal injected with poly(I:C) before, shortly after or late after tumour initiation, always confirming efficient *c-raf*

deletion (data not shown). Therefore *mx-cre*-mediated *c-raf* ablation in the liver is efficient and tumours that arise in these mice are not selected for *c-raf* expression.

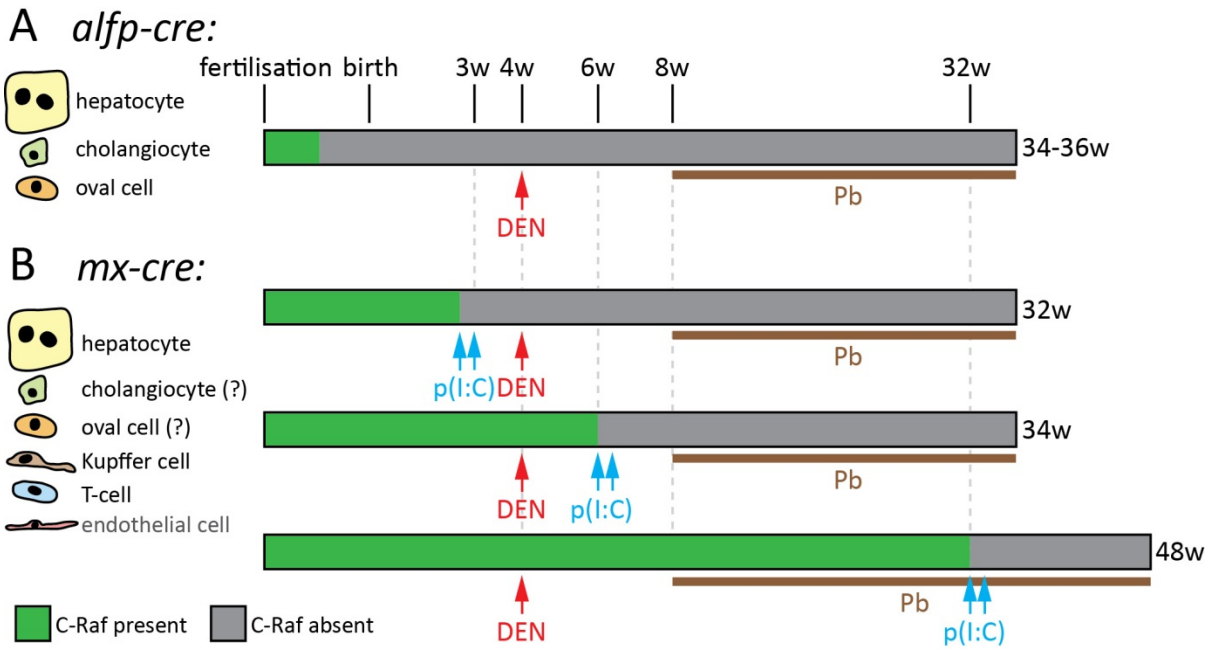

Figure 3. Chemical liver carcinogenesis and conditional C-Raf ablation in mice.
(**A**) In the *alfp-cre* line C-raf is deleted in parenchymal cells and most likely oval cells, as they also express α-fetoprotein. Expression of Cre recombinase begins between day 9,5 and 10,5 p.c. Tumours are initiated with single injection of DEN at 4 weeks of age and promoted by administration of phenobarbital-containing fodder (Pb) from 8th week on. Animals were euthanized at 34 weeks of age (single *c-raf*-knock out experiments) or 36 weeks of age (double knock-out experiments).
(**B**) In the *mx-cre* line *c-raf* is deleted in parenchymal cells (although efficiency of ablation in cholangiocytes and oval cells has not been investigated), cells derived from hematopoietic line (Kupffer cells, T-cells) and partially in endothelial cells (data not shown). Deletion is induced with 2 consecutive injections of poly(I:C) within 5 days, either one week before, or 2 weeks after, or 28 weeks after DEN injections. Animals were euthanized and their livers isolated at 32, 34 and 48 weeks of age, respectively. Details in text.

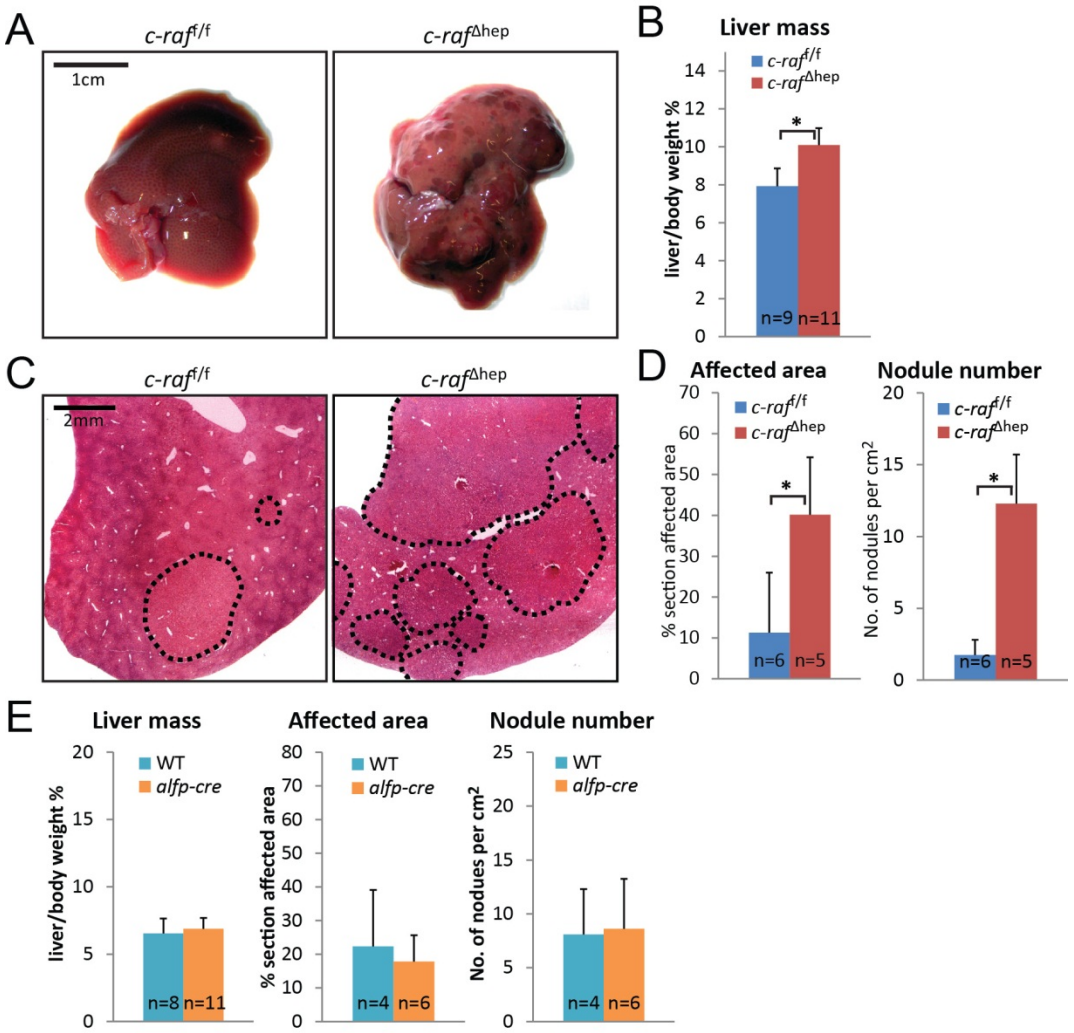

Figure 4. C-Raf is a tumour suppressor in a chemical model of HCC.
(**A**) Gross liver morphology, (**B**) quantification of liver mass as a percentage of body weight, (**C**) H&E staining of paraffin-embedded liver sections and (**D**) quantification of tumour-affected area and number of nodules per 1cm^2 of liver section from *c-raf*$^{f/f}$ and *alfp-cre*;*c-raf*$^{f/f}$ (*c-raf*$^{\Delta hep}$) male mice 30 weeks after DEN-treatment;
(**E**) Quantification of liver mass (as in B), tumour-affected area and nodule number (as in D) from WT and *alfp-cre* male mice after same treatment as in A-D;
Data in graphs is expressed as mean values; error bars - 95% confidence interval; * - p-value<0,05 as calculated from two-tailed student t-test for samples with equal variation; Figures A-D with contribution of Gabriele Maurer;

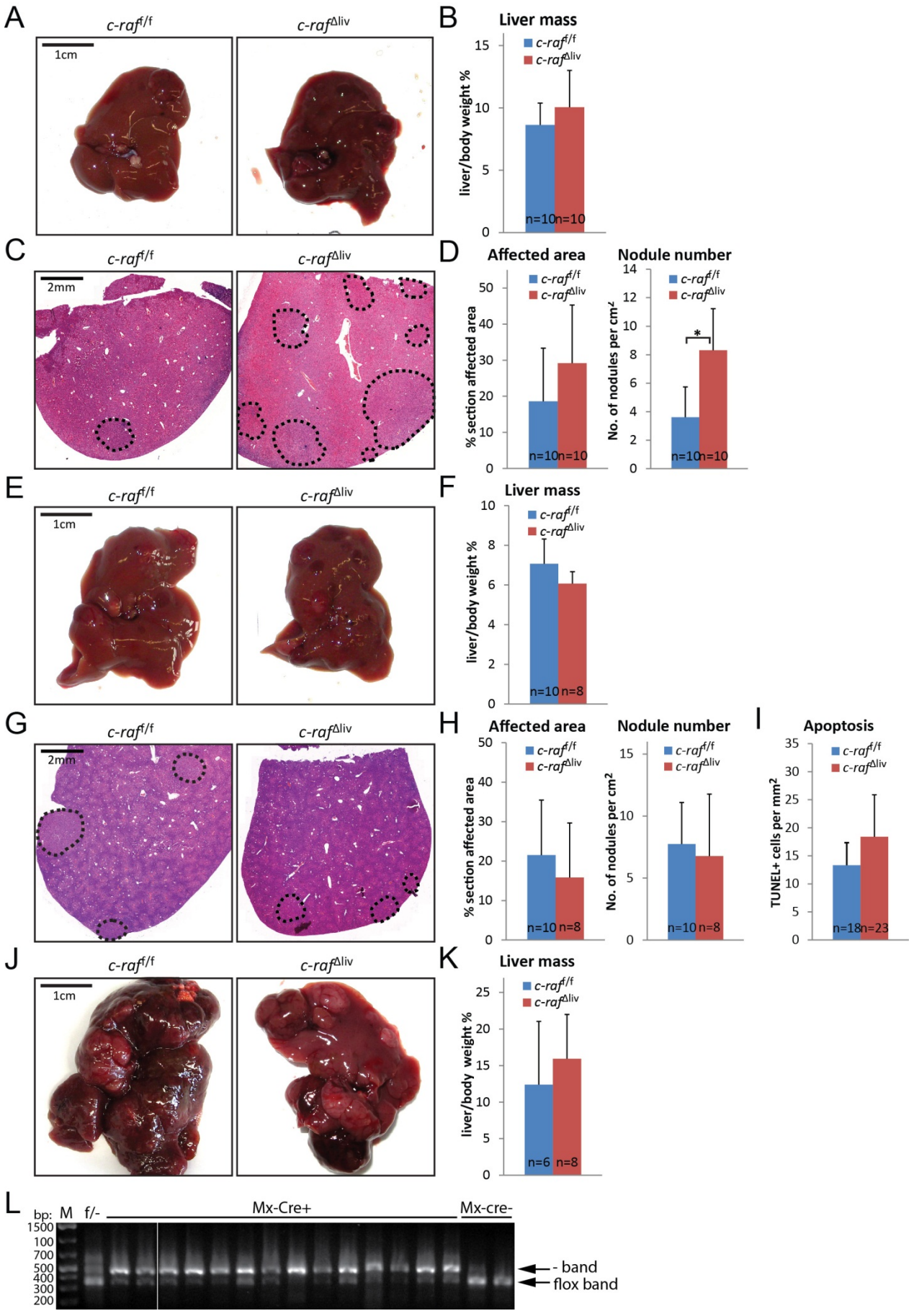
A
c-raf f/f
c-raf Δliv
1cm
B
Liver mass
c-raf f/f
c-raf Δliv
liver/body weight %
n=10 n=10
C
c-raf f/f
c-raf Δliv
2mm
D
Affected area
% section affected area
n=10 n=10
Nodule number
No. of nodules per cm²
*
n=10 n=10
E
c-raf f/f
c-raf Δliv
1cm
F
Liver mass
liver/body weight %
n=10 n=8
G
c-raf f/f
c-raf Δliv
2mm
H
Affected area
% section affected area
n=10 n=8
Nodule number
No. of nodules per cm²
n=10 n=8
I
Apoptosis
TUNEL+ cells per mm²
n=18 n=23
J
c-raf f/f
c-raf Δliv
1cm
K
Liver mass
liver/body weight %
n=6 n=8
L
bp: M f/-
Mx-Cre+
Mx-cre-
1500
100
700
500
400
300
200
- band
flox band

Figure 5. C-Raf suppresses tumour initiation and is dispensable for tumour maintenance.
(**A**) Gross liver morphology, (**B**) quantification of liver mass as a percentage of body weight, (**C**) H&E staining of paraffin-embed liver sections and (**D**) quantification of tumour-affected area and number of nodules per $1cm^2$ of liver-section from *c-raf*$^{f/f}$ and *mx-cre*;*c-raf*$^{f/f}$ (*c-raf*$^{\Delta liv}$) male mice 30 weeks after DEN-treatment that were poly(I:C)-treated two weeks before DEN;
(**E**) Gross liver morphology, (**F**) quantification of liver mass (as in B), (**G**) H&E staining (as in C), (**H**) quantification of tumour-affected area and number of nodules (as in D) and (**I**) quantification of apoptotic cells detected in tumours with terminal deoxyunucleotydil transferase dUTP nick end labelling (TUNEL) of liver paraffin-embed sections from *c-raf*$^{f/f}$ and *c-raf*$^{\Delta liv}$ male mice 30 weeks after DEN-treatment that were poly(I:C)-treated two weeks after DEN;
(**J**) Gross liver morphology and (**K**) quantification of liver mass (as in B) from *c-raf*$^{f/f}$ and *c-raf*$^{\Delta liv}$ male mice 44 weeks after DEN-treatment that were poly(I:C)-treated 28 weeks after DEN;
(**L**) Genotyping PCR for *c-raf* flox and Δ allele on DNA isolated from tumours of *c-raf*$^{f/f}$ and *c-raf*$^{\Delta liv}$ male mice 30 weeks after DEN-treatment that were poly(I:C)-treated 2 weeks after DEN;
Data in graphs is expressed as mean values; error bars - 95% confidence interval; * - p-value<0,05 as calculated from two-tailed student t-test for samples with equal variation; Figures A-K with contribution of Gabriele Maurer;

Rationale and aims II: Interplay between Rassf1a and C-Raf in liver cancer

Up to 90% of HCC clinical cases exhibit isoform-specific promoter methylation of *Rassf1a* that leads to loss of its expression (Dammann, Schagdarsurengin et al. 2003). Rassf1a was shown to have tumour suppressive functions *in vitro* (Dammann, Li et al. 2000) and *in vivo* (Tommasi, Dammann et al. 2005). Together with C-RAF, RASSF1A regulates activation of MST2 kinase (Matallanas, Romano et al. 2007), upstream regulator of Hippo signalling that in mammals controls liver homeostasis (Zhou, Conrad et al. 2009). Therefore we decided to investigate the role of interplay between C-Raf and Rassf1a in liver cancer in chemically induced mouse liver cancer.

Rassf1a is required for manifestation of *c-raf*$^{\Delta hep}$ phenotype in liver cancer

We have crossed 129/Sv *c-raf*$^{\Delta liv}$ mice with 129/B6 mice bearing conventional KO of *rassf1* isoform A and obtained at the mendelian ratio *c-raf*$^{\Delta liv}$;rassf1a$^{-/-}$ (double KO) animals that were viable and fertile as their *c-raf*$^{\Delta liv}$ littermates (data not shown). Untreated double KO animals do not exhibit any overt phenotype, have normal gross liver morphology and unaffected liver/body weight ratio at 36 weeks age (data not shown). Genotyping PCR on liver tissue lysate from *c-raf*$^{\Delta hep}$ and double KO mice amplified products both from flox and Δ allele, the former one presumably from non-parenchymal cells (Figure 6F). Indeed, western blot analysis confirmed efficient ablation of C-Raf on protein level in *c-raf*$^{\Delta hep}$ and double KO mice (Figure 8A).

We subjected *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *c-raf*$^{f/f}$;*rassf1a*$^{-/-}$ (referred to as *rassf1a*$^{-/-}$) and double KO animals to liver carcinogenesis protocol (Figure 3A). We could reproduce previously observed phenotype of *c-raf*$^{\Delta hep}$ mice in terms of increased liver mass, tumour-affected liver and number of tumours (Figure 6A-D). Strikingly, this phenotype is not exhibited on the background of *rassf1a* deletion. Gross morphology of livers from double KO mice resembles that of *rassf1a*$^{-/-}$ (Figure 6A) and liver mass of double KO mice is not increased in comparison to *rassf1a*$^{-/-}$ (Figure 6B). Furthermore, tumour-affected liver area and number of tumours is similar in H&E-stained liver sections from *rassf1a*$^{-/-}$ and double KO mice. Finally, nodule number in double KO mice is significantly decreased as compared to *c-raf*$^{\Delta liv}$ (Figure 6D). This indicates that Rassf1a deletion attenuates *c-raf*$^{\Delta hep}$ phenotype in chemical liver carcinogenesis mouse model.

Neoplastic nodules in the liver that result from hepatocyte transformation can be classified according to their morphological features like size, clear borders, congestion or invasion of surrounding tissue, hepatocyte size and morphology, growth pattern and eosinophylic or basophilic staining (Tamano, Merlino et al. 1994). They can be classified into several types of foci of cellular alteration, regenerative hyperplasia, adenomas and carcinomas with either normal or trabecular growth pattern. Different nodule classes are thought to reflect consecutive stages of tumour progression, with foci being the earliest, benign stage and carcinoma the final and most malignant one. Therefore relative amounts of each type of induced nodule in livers of DEN/Pb treated mice indicate rate of tumour progression.

To determine influence of *c-raf* and *rassf1a* gene deletions on tumour progression we classified nodules from H&E liver sections of *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *rassf1a*$^{-/-}$ and double KO mice according to their morphological features (in collaboration with Stratigoula Sakellariou and Vassilis Gorgoulis, Medical School, National Kapodistrian University of Athens, Greece). We detected mainly foci of cellular alteration (FCA) and hepatocellular adenomas (HCA) with few hepatocellular carcinomas (HCC). Percentage of nodules classified as HCC is significantly increased in *c-raf*$^{\Delta hep}$ but not in double KO mice, as compared to *c-raf*$^{f/f}$ mice, and it has tendency to increase, although without reaching statistical significance, in *rassf1a*$^{-/-}$ mice compared to *c-raf*$^{f/f}$ (p-value=0,067) (Figure6E). Moreover number of mice with detected nodules classified as HCC is significantly increased in *c-raf*$^{\Delta hep}$ but not in double KO mice. Apart from that, *rassf1a*$^{-/-}$ mice also have significantly higher incidence of HCC than *c-raf*$^{f/f}$ mice (Figure 6E). Therefore *c-raf* ablation in the hepatocyte enhances cancer progression what is attenuated by concomitant *rassf1a* deletion. Deletion of rassf1a alone on the other hand also enhances tumour progression.

Rassf1a deletion attenuates proliferation increase in *c-raf*$^{\Delta hep}$ liver

One of prime functions of MAPK signalling is to regulate proliferation of the cell. Uncontrolled proliferative signalling is a hallmark of cancer cells that allows them for uncontrolled growth. Therefore we checked the proliferation index in the *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *rassf1a*$^{-/-}$ and double KO mouse livers after carcinogenesis.

We performed immunohistochemistry for Ki67 on liver sections from experimental animals. In mice of all genotypes tumours exhibit ubiquitous staining for Ki67 whereas in tumour-unaffected tissue only rare positive cells were found (Figure 7A). This indicates high proliferation in the tumours and known quiescence of tumour-unaffected liver tissue. Mean

proliferation in tumour tissue in all genotypes is comparable, but in tumour-unaffected tissue of *c-raf*$^{\Delta hep}$ animals it is significantly increased compared to *c-raf*$^{f/f}$, what was recently observed in our lab (Gabriele Maurer, unpublished data). In contrast, proliferation is similar in tumour-unaffected liver tissue of double KO mice compared to that of *rassf1a*$^{-/-}$ (Figure 7B). Therefore, *c-raf* deletion in hepatocyte increases proliferative capacity in mouse tumour-unaffected liver tissue subjected to chemical carcinogenesis and *rassf1a* deletion attenuates this phenotype.

Rassf1a can influence apoptosis in the cell either by regulating activation of MST kinases that are activated in response to Fas stimulation (Matallanas, Romano et al. 2007) or by facilitating activation of Bcl-2 family protein Bax, that triggers efflux of cytochrome C from mitochondria (Baksh, Tommasi et al. 2005). Therefore we evaluated cell death in livers of the same mice as in case of Ki67 expression analysis with the help of TUNEL on liver sections. In general apoptotic indexes in both tumour and tumour unaffected liver tissue are very low. Neither in tumour, nor in tumour unaffected tissue we were able to detect any differences between mice of different genotypes (Figure 7C-D). This indicates that at least in the developed liver tumours neither C-Raf nor Rassf1a ablation affects hepatocyte survival.

Signalling pathway activation in livers after tumorigenesis

To assess state of signalling pathways activation that might be relevant for liver tumorigenesis and affected by *c-raf* and *rassf1a* ablation we isolated protein lysates form tumour-unaffected and tumour liver tissue from *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *rassf1a*$^{-/-}$ and double KO animals. These lysates were then analysed with western blotting for levels and modifications status of selected proteins from MAPK, Hippo, and stress and inflammatory signalling pathways.

The main indirect effector of RAF kinases is ERK which by phosphorylating numerous targets in the cytoplasm, nucleus and cellular membranes, influences cell proliferation and survival in response to extracellular signals. Erk activation results from threonine and tyrosine phosphorylation by MEK in TXY motifs. Western blot analysis of Erk activatory phosphorylation sites shows inconsistent Erk activation in different mice of the same genotype, especially in tumour tissue (Figure 8A). Still we observe lower Erk activation in tumour unaffected tissue of double KO animals compared to all other investigated genotypes.

In drosophila, dRassf protein is a Hippo inhibitor (Polesello, Huelsmann et al. 2006), but mammalian RASSF1A activates MST by displacing C-RAF from its inhibitory complex with MST2 (Matallanas, Romano et al. 2007). Loss of Mst activity in mouse liver leads to

spontaneous liver cancer development (Zhou, Conrad et al. 2009), and Yap overexpression in the liver leads to hepatomegaly followed by HCC (Dong, Feldmann et al. 2007). Hippo pathway activation is associated with proteolytic cleavage of MST kinases (Lee, Murakawa et al. 1998), phosphorylation of LATS kinases on specific serines and threonine residues (Chan, Nousiainen et al. 2005), and inhibitory YAP phosphorylation on S127 (Oka, Mazack et al. 2008). Western blot analysis of Mst2 levels shows decreased Mst2 levels in both tumour-unaffected and tumour tissue of *rassf1a*$^{-/-}$ and double KO animals compared to *c-raf*$^{f/f}$ and *c-raf*$^{\Delta hep}$ animals (Figure 8B). This might indicate either lower expression or enhanced proteolytic Mst2 cleavage in cells lacking Rassf1a. Analysis of Lats1 phosphorylation on S908 (homologous to human S909) shows increased Lats1 activation in tumour unaffected and tumour tissue of double KO animals compared to 3 other investigated genotypes (Figure 8B). This indicates that deletion of both C-Raf and Rassf1a in the hepatocyte leads to enhanced Lats1 activatory phosphorylation. Surprisingly, western blot analysis of total Yap and its inhibitory phosphorylation site shows no differences depending on the genotype. But expression of total Yap is increased in *c-raf*$^{\Delta hep}$ mice compared to *c-raf*$^{f/f}$ and double KO animals compared to rassf1a$^{-/-}$ in both tumour unaffected and tumour tissue (Figure 8B). This indicates either higher expression or increased Yap stability in C-Raf-deficient hepatocytes. Finally, in tumour tissue Lats1 phosphorylation inversely correlates with Yap levels, apart from tissue of double KO animals, where both Lats1 phosphorylation and Yap levels are relatively high (Figure 8B). This indicates that concomitant *c-raf* and *rassf1a* deletion leads to uncoupling of Mst signalling and Yap levels in liver tumours.

Chronic inflammation is associated with HCC development and signalling pathways like NF-κB or p38 that respond to inflammatory or stress stimuli influence liver homeostasis. NF-κB transcription factor can be activated downstream of TNF-α-receptors, it regulates cell survival and its activation is either suppressing or enhancing liver cancer development depending on experimental model (Pikarsky, Porat et al. 2004; Luedde, Beraza et al. 2007). NF-κB is normally inhibited by IκB which degradation of occurs upon phosphorylation by IKK kinases. We investigated IκB status and observed its similar levels in animals of all genotypes. This indicates that neither Rassf1a nor C-Raf ablation influences NF-κB pathway activation in the liver.

p38 is a MAP kinase implicated in stress signalling with tumour suppressive function in various settings, including liver (Wagner and Nebreda 2009). p38 suppresses liver tumour development by antagonising c-Jun (Hui, Bakiri et al. 2007) and is itself suppressed by c-Jun

during liver regeneration (Stepniak, Ricci et al. 2006). Its activation, similarly to ERK, is a consequence of dual phosphorylation on TXY motif. We evaluated p38 activation by using phospho-specific antibodies and found decreased p38 phosphorylation in tumour unaffected tissue only from double KO animals. This indicates that genetic inactivation of both C-Raf and Rassf1a leads to lower activation of p38.

Macrophage number is increased in *c-raf*$^{\Delta hep}$ irrespectively of Rassf1a

Although HCC is a primary liver tumour that arises as a result of hepatocyte transformation, non-parenchymal cells of the liver also influence hepatocyte transformation, progression of neoplastic nodules and growth of tumours. For instance, inflammatory cells can secrete cytokines and produce ROS that might enhance cell growth and transformation. Kupffer cells, resident liver macrophages, seem to play crucial role in mediating tumour-promoting inflammation in this organ. They are for instance activated by factors released from dying hepatocytes to release Il-6 and TNF-α that in return can stimulate proliferation of surviving hepatocytes (Sakurai, He et al. 2008).

To assess numbers of macrophages in livers after tumorigenesis we performed immunohistochemistry for macrophage marker F4/80 on liver sections from *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *rassf1a*$^{-/-}$ and double KO animals. Livers from all genotypes showed substantial numbers of mostly elongated cells with abundant filopodia prevailing around portal areas, mostly with sinusoidal localisation. Numbers of detected macrophages were higher in *c-raf*$^{\Delta hep}$ compared to *c-raf*$^{f/f}$ but also in double KO compared to *rassf1a*$^{-/-}$. Therefore deletion of *c-raf* in hepatocytes leads to increased numbers of macrophages in the liver and concomitant Rassf1a deletion does not affect this phenotype.

It is known that macrophages can be activated by various stimuli and the type of stimulus can determine subsequent macrophage behaviour. Macrophage stimulation with interferons, TNF-α or LPS leads to classical activation of macrophage, or M1 phenotype. Such macrophages are considered to have higher microbicidal and tumoricidal activity and release high amounts of pro-inflammatory cytokines. Macrophage stimulation with IL-4 or Il-13 leads to alternative activation, or M2 phenotype. They express distinct set of cytokines than M1 macrophages and participate in parasite clearance, tissue remodelling and angiogenesis and in this way are able to facilitate tumour progression (Biswas and Mantovani 2010).

To determine whether macrophages in tumour-bearing livers are activated we performed immunohistochemistry for INOS macrophage activation marker which shows low numbers of

positive cells in tumour bearing livers from mice of all genotypes. For comparison, in *c-raf*$^{f/f}$ we detect only 19±8 INOS-positive cells per mm^2 of tissue section, whereas in the same mice we detect 582±86 F4/80-positive cells per mm^2 tissue section. Moreover INOS-positive cells have rounded morphology (Figure 9C) and most of F4/80-positive cells have elongated morphology and abundant filopodia (Figure 9A, arrows) although some of them also have morphology similar to that of INOS-positive cells (Figure 9A, arrowheads). Therefore most of F4/80-positive cells in the liver do not express classical macrophage activation markers. Quantification of INOS-positive cells shows no differences between *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *rassf1a*$^{-/-}$ and double KO animals. This indicates that hepatocyte-restricted *c-raf* and *rassf1a* deletion do not influence macrophage classical activation in the liver during chemical carcinogenesis.

Oval cells are not mobilised during early stages of liver carcinogenesis in *c-raf*$^{\Delta hep}$ mice

Oval cells, liver progenitor cells able to differentiate into hepatocyte and cholangiocytes, might play role in development of HCC. At least in some carcinogenesis protocols, especially those accompanied by hepatocyte proliferation inhibition, oval cell expansion might be induced and lead to tumour development. Moreover, recently Hippo signalling pathway was implicated in maintaining quiescence of oval cells (Zheng, Wang et al. 2011), as mice with liver epithelial cell-restricted Nf2 or Mst kinases deficiency show expansion of liver progenitor cell compartment (Benhamouche, Curto et al. 2010; Lee, Lee et al. 2010; Lu, Li et al. 2010). Therefore, we decided to investigate whether increased number of tumours in *c-raf*$^{\Delta hep}$ mice results from oval cell induction.

Oval cells in normal liver reside in the vicinity of portal vein and similarly to bile duct epithelial cells express large amounts cytokeratins. We used immunohistochemistry for pan-cytokeratins to visualise oval cells in *c-raf*$^{f/f}$ and *c-raf*$^{\Delta hep}$ mice at 4 weeks of age, 5 days and 2 weeks after DEN-treatment. In both genotypes we detect strong staining of bile ducts and some single positive cells around portal veins that presumably are oval cells (Figure 10A). We do not detect any change in abundance of cytokeratin-positive cells 5 days or 2 weeks after DEN-treatment. The amounts of cytokeratin-positive cells are similar in *c-raf*$^{\Delta hep}$ and *c-raf*$^{f/f}$ mice.

Recently novel antibodies with high specificity towards oval cells were generated using immunisation of animals with sera from rodents subjected to oval cell-chemical induction protocols (Dorrell, Erker et al. 2008). We applied one of these monoclonal antibodies, MIC1,

for immunohistochemistry on liver sections from *c-raf*$^{f/f}$ and *c-raf*$^{\Delta hep}$ mice 8 weeks after DEN-injection. Again, we observed staining of bile ducts and single cells around portal veins, with no difference between *c-raf*$^{\Delta hep}$ and *c-raf*$^{f/f}$ mice (Figure 10B). This indicates that during early stages of DEN-induced liver carcinogenesis proliferation of oval cells is generally not induced and *c-raf* deletion does not influence oval cell response in this system.

alfp-cre transgene-specific effects on hepatocyte acute response to DEN

c-raf$^{\Delta hep}$ mice have increased number of tumours that indicates more efficient cell transformation or increased survival of initially transformed cells. Apart from that, *c-raf*$^{\Delta liv}$ mice exhibit either increased or unchanged tumour number, depending whether *c-raf* deletion is induced before or after DEN-injection, respectively. Considering this, *c-raf* seems to play role during the tumour initiation. Therefore, we decided to investigate early metabolic response in the livers to DEN-treatment.

DEN is a cytotoxic agent and causes extensive cell death of hepatocytes shortly after administration. This is followed by compensatory proliferation of surviving hepatocytes. Hepatocyte-metabolised form of DEN is able to form DNA-adducts and therefore is causing mutations in surviving hepatocytes that may lead to cellular transformation.

We treated *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *rassf1a*$^{-/-}$, double KO as well as WT and *alfp-cre* animals without floxed *c-raf* alleles with DEN and isolated livers after 48 hours. Extensive hepatocyte death was observed on similar level in *c-raf*$^{\Delta hep}$ and *c-raf*$^{f/f}$ animals around central veins - site of metabolic DEN activation (Gabriele Maurer, unpublished data). Histochemical staining showed increased lipid accumulation around central veins in *c-raf*$^{\Delta hep}$ mice compared to *c-raf*$^{f/f}$, as well as in double KO compared to *rassf1a*$^{-/-}$, but also in *alfp-cre* animals compared to WT (Figure 11A). This indicates transgene-specific effect on metabolic response of hepatocyte to the DEN-treatment.

We also observed compensatory proliferation of hepatocytes in response to DEN (Gabriele Maurer, unpublished data). This response is compromised in *c-raf*$^{\Delta hep}$ mice compared to *c-raf*$^{f/f}$, as well as double KO compared to *rassf1a*$^{-/-}$, but also *alfp-cre* animals compared to WT (Figure 11B-C). This indicates that not only lipid accumulation but also inhibition of compensatory proliferation after DEN-treatment can be attributed to *alfp-cre* transgene.

Acute effects of DEN-treatment on Hippo pathway activation

Hippo signalling cascade regulates activity of liver proto-oncogene Yap by inhibiting its nuclear accumulation. Nuclear Yap can induce cell proliferation and inhibit apoptosis by modulating gene expression together with certain transcription factors in the nucleus.

To investigate activity of Hippo signalling during acute response to DEN-treatment we performed immunohistochemistry for Yap on liver sections from *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *rassf1a*$^{-/-}$ and double KO animals without and 48 hours after DEN treatment. In untreated livers from mice of all investigated genotypes Yap is expressed mostly in the cytoplasm in centrilobular liver areas and on a lower level in the nuclei in periportal areas (Figure 12A). 48 hours after DEN-treatment we observe strong accumulation of Yap in nuclei around central veins to similar extent in mice of all investigated genotypes. Western blot analysis indicates that 48 hours after DEN treatment there is no genotype-depending differences in total Yap amounts (Figure 12D). This indicates carcinogen-induced translocation of Yap from the cytoplasm to the cell nucleus of hepatocytes around central veins, probably as a result of Hippo pathway inhibition. This process as well as Yap expression is not affected by hepatocyte-restricted *c-raf* or *rassf1a* ablation.

To investigate even more acute effects of carcinogen-treatment on Hippo signalling in the hepatocyte we employed hepatocyte primary cultures. Hepatocytes from 3-4 months old male *alfp-cre*, *c-raf*$^{\Delta hep}$ and double KO animals were isolated with a 2-step perfusion method, plated on collagen-coated dishes and cultures in medium with serum and growth factors. Isolated cells compose homogenous population with morphological features resembling hepatocytes - they are bi-nucleated and relatively large (Figure 13A).

Currently the exact ligands that specifically stimulate Hippo signalling in mammalian cells are not well established. In cells isolated from mouse HCCs activation of Mst kinases can be stimulated with H_2O_2 (Zhou, Conrad et al. 2009). H_2O_2 induces oxidative stress in cell culture, and in the liver ROS enhances tumorigenesis (Maeda, Kamata et al. 2005; Luedde, Beraza et al. 2007). Therefore we investigated the role of *c-raf* and *rassf1a* in Hippo pathway activation in response to oxidative stress in primary hepatocytes. After few days in culture we stimulated them with H_2O_2 for 20 minutes and harvested with Laemmli buffer to obtain protein lysate. Western blot analysis shows elevated YAP inhibitory phosphorylation on serine 112 (homologous site to human S127) after H_2O_2-treatment in cells of all investigated genotypes. Moreover, double KO hepatocytes show stronger Yap phosphorylation than *alfp-*

cre or *c-raf*$^{\Delta hep}$ cells. Therefore H_2O_2-mediated oxidative stress induces Yap inhibitory phosphorylation in primary hepatocytes and this induction is increased in hepatocytes lacking both *c-raf* and *rassf1a*.

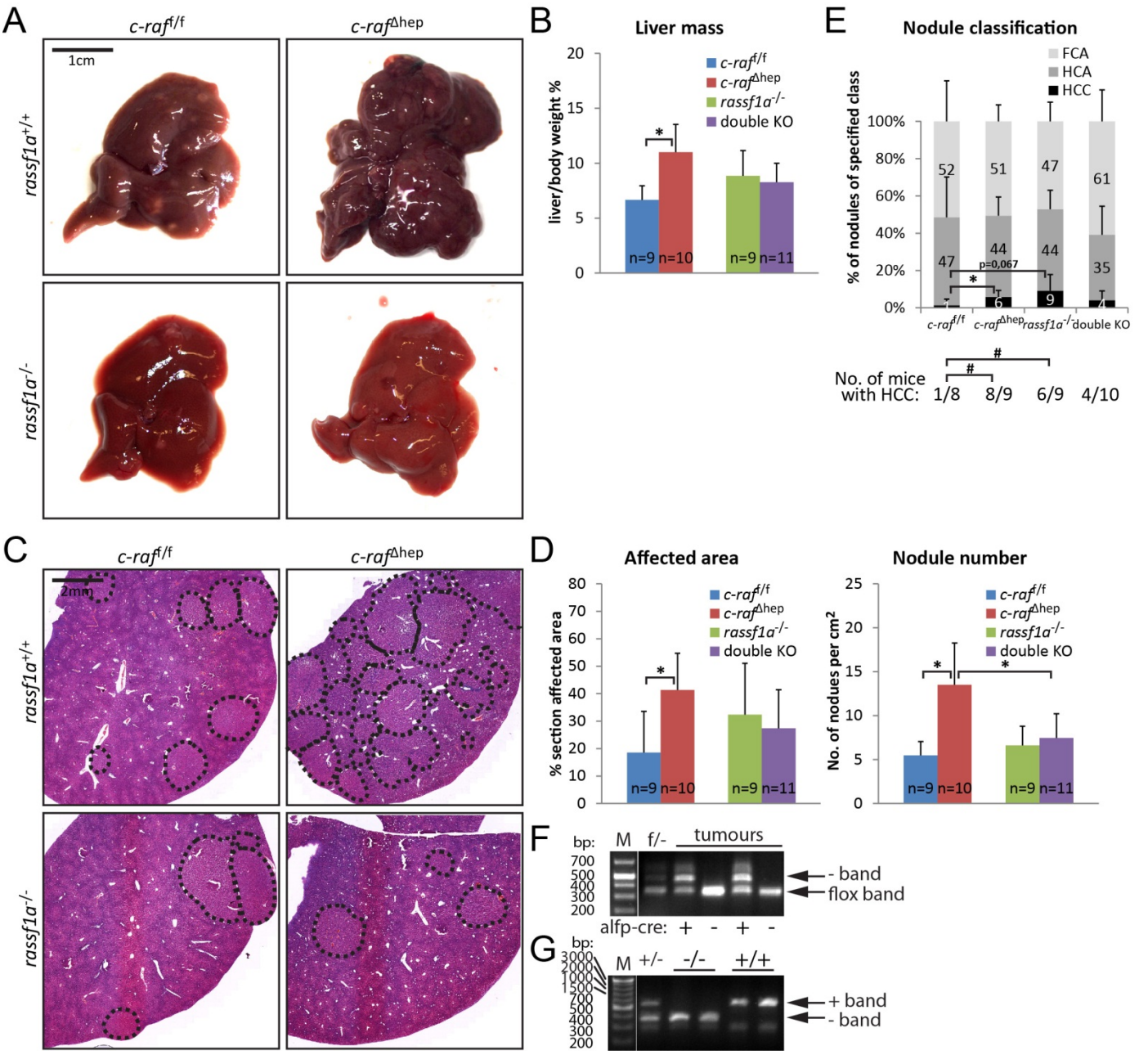

Figure 6. Rassf1a interacts genetically with C-Raf in chemical HCC model.
(**A**) Gross liver morphology, (**B**) quantification of liver mass as a percentage of body weight, (**C**) H&E staining of paraffin-embedded liver sections and (**D**) quantification of tumour-affected area and number of nodules per 1cm^2 of liver section from *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *rassf1a*$^{-/-}$ and *c-raf*$^{\Delta hep}$;*rassf1a*$^{-/-}$ (double KO) male mice 30 weeks after DEN-treatment;
(**E**) Quantification of percentage of nodules classified on H&E-stained liver sections as foci of cellular alteration (FCA), adenoma (HCA) or carcinoma (HCC) from same animals as in A-D. Below numbers of mice with detected HCC per total numbers of mice analysed for each genotype;
(**F**) Genotyping PCR for *c-raf* flox and Δ allele on DNA isolated from tumours of same animals as in A-E; (**G**) Genotyping PCR for *rassf1a* + and - allele on DNA isolated from tails from same animals as in A-E;
Data in graphs is expressed as mean values; * - p-value<0,05 as calculated from two-tailed student t-test for samples of equal variation; # - p-value<0,05 as calculated from Fisher's exact test; error bars - 95% confidence interval; Figures A-D with contribution of Zeynep Erdem and Martin Künzl; Figure E with contribution of Stratigoula Sakellariou;

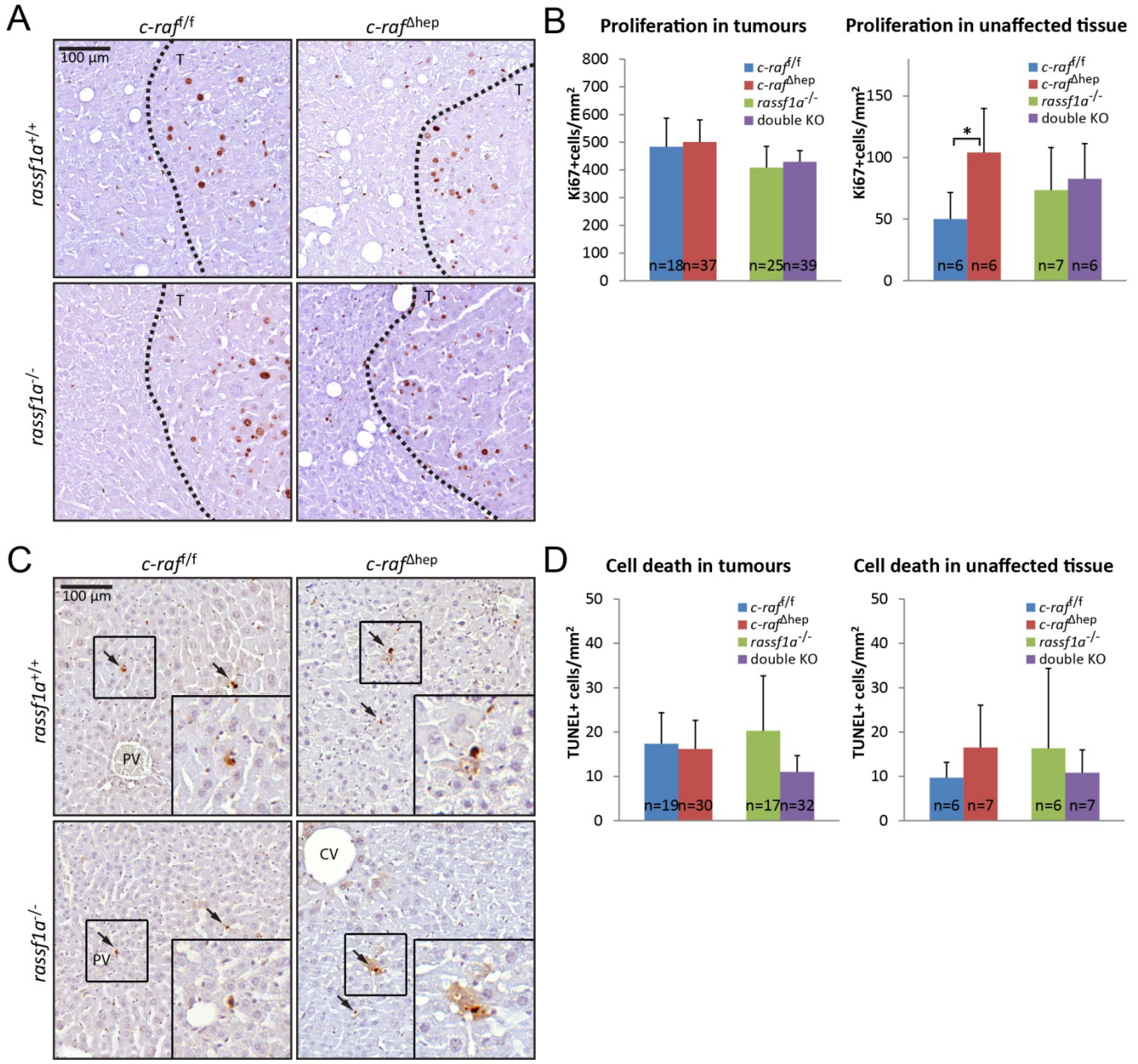

Figure 7. In double KO livers proliferation is not increased during chemical carcinogenesis.
(**A**) Immunohistochemical staining of Ki67 on paraffin-embedded liver sections - dashed line indicates border between unaffected and tumour (T) tissue - and (**B**) quantification of Ki67-positive cells per 1mm^2 of tissue section in tumours and unaffected tissue from *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *rassf1a*$^{-/-}$ and double KO male mice 30 weeks after DEN-treatment;
(**C**) TUNEL on paraffin-embedded liver sections (only unaffected tissue shown) and (**D**) quantification of TUNEL-positive cells same as in A-B;
Data in graphs is expressed as mean values; error bars - 95% confidence interval; * - p-value<0,05 as calculated from two-tailed student t-test for samples of equal variation; insets on images are 2x magnified; PV - portal vein; CV - central vein;

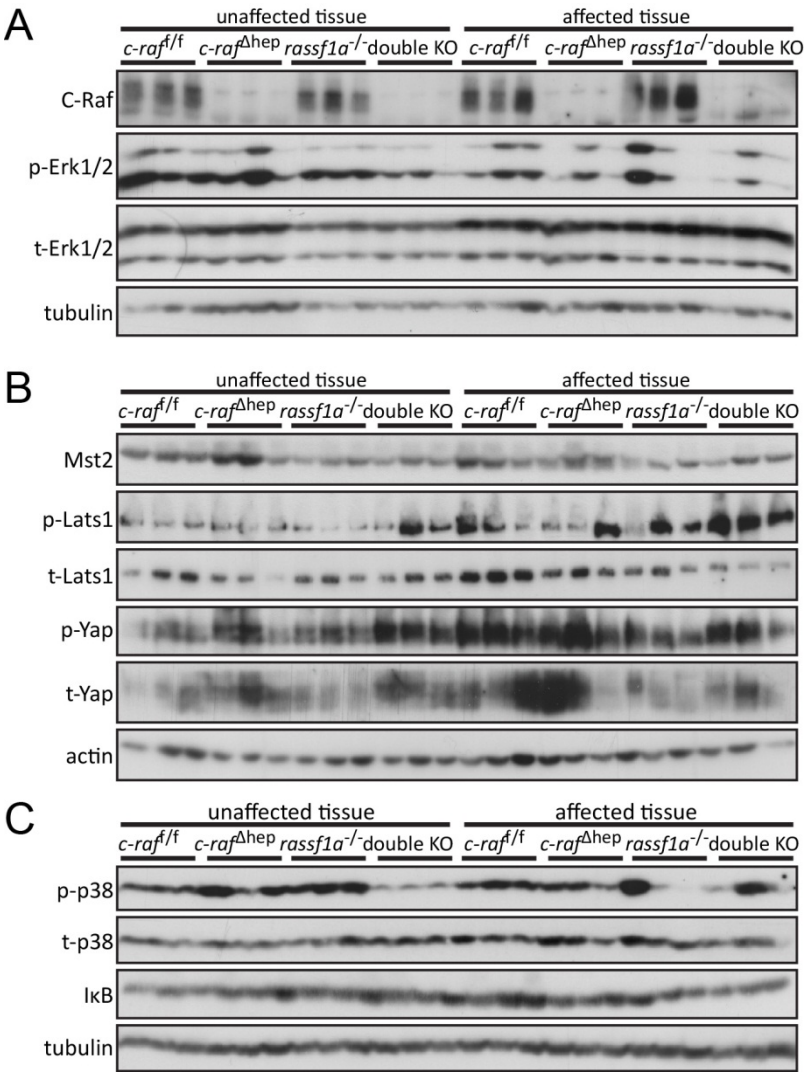

Figure 8. Signalling pathway activation in tumour-affected livers.
Western blot analysis of protein lysates from liver tumour-unaffected and tumour tissue isolated from *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *rassf1a*$^{-/-}$ and double KO male mice 30 weeks after DEN-treatment. For MAPK signalling (**A**) total C-Raf, activatory Erk1 and 2 phosphorylation (T203/Y205 and T183/Y185), total Erk1 and 2 and tubulin as loading control were detected; For Hippo signalling (**B**) total Mst2, activatory Lats1 phosphorylation (S908), total Lats1, inhibitory Yap phosphorylation (S112), total Yap and actin as loading control were detected; For inflammatory signalling (**C**) activatory p38 phosphorylation (T180/Y182), total p38, total IκB and tubulin as loading control were detected;
Figure A-C with contribution of Ines Jeric.

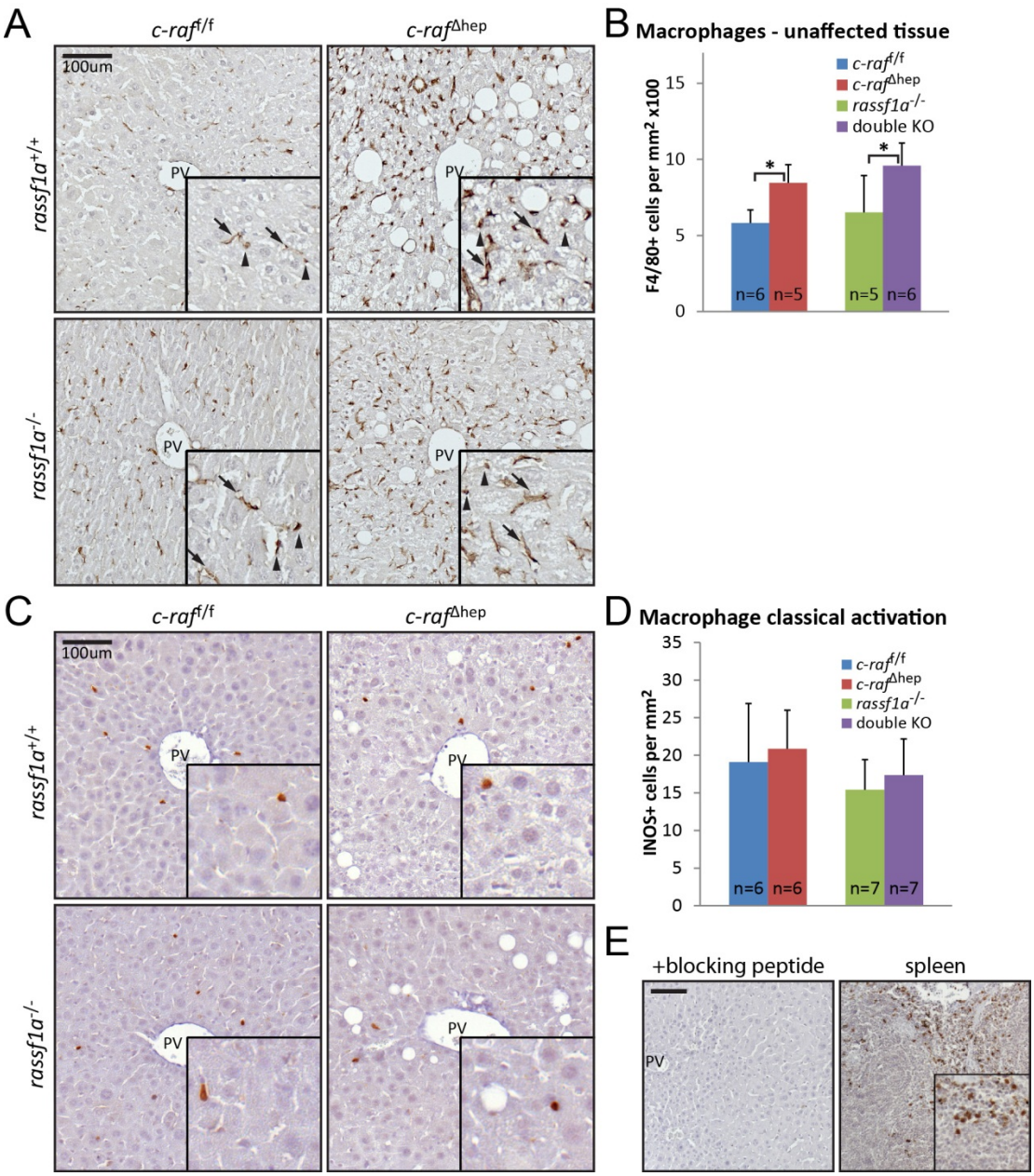

Figure 9. Macrophage number is increased in *c-raf*$^{\Delta hep}$ livers irrespectively of Rassf1a.
(**A**) Immunohistochemical staining of F4/80 on paraffin-embedded liver sections, (**B**) quantification of F4/80-positive cells per 1mm^2 of tissue section in tumour-unaffected tissue section from *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *rassf1a*$^{-/-}$ and double KO male mice 30 weeks after DEN-treatment;
(**C**) Immunohistochemical staining of INOS and (**D**) quantification of F4/80-positive cells same as in A-B; (**E**) Immunohistochemical staining of INOS on liver section using antibody pre-incubated with neutralising peptide as negative control (left panel) and spleen section using standard procedure as positive control (right panel);
Data in graphs is expressed as mean values; error bars - 95% confidence interval; * - p-value<0,05 as calculated from two-tailed student t-test for samples of equal variation; insets on images are 2x magnified; PV - portal vein;

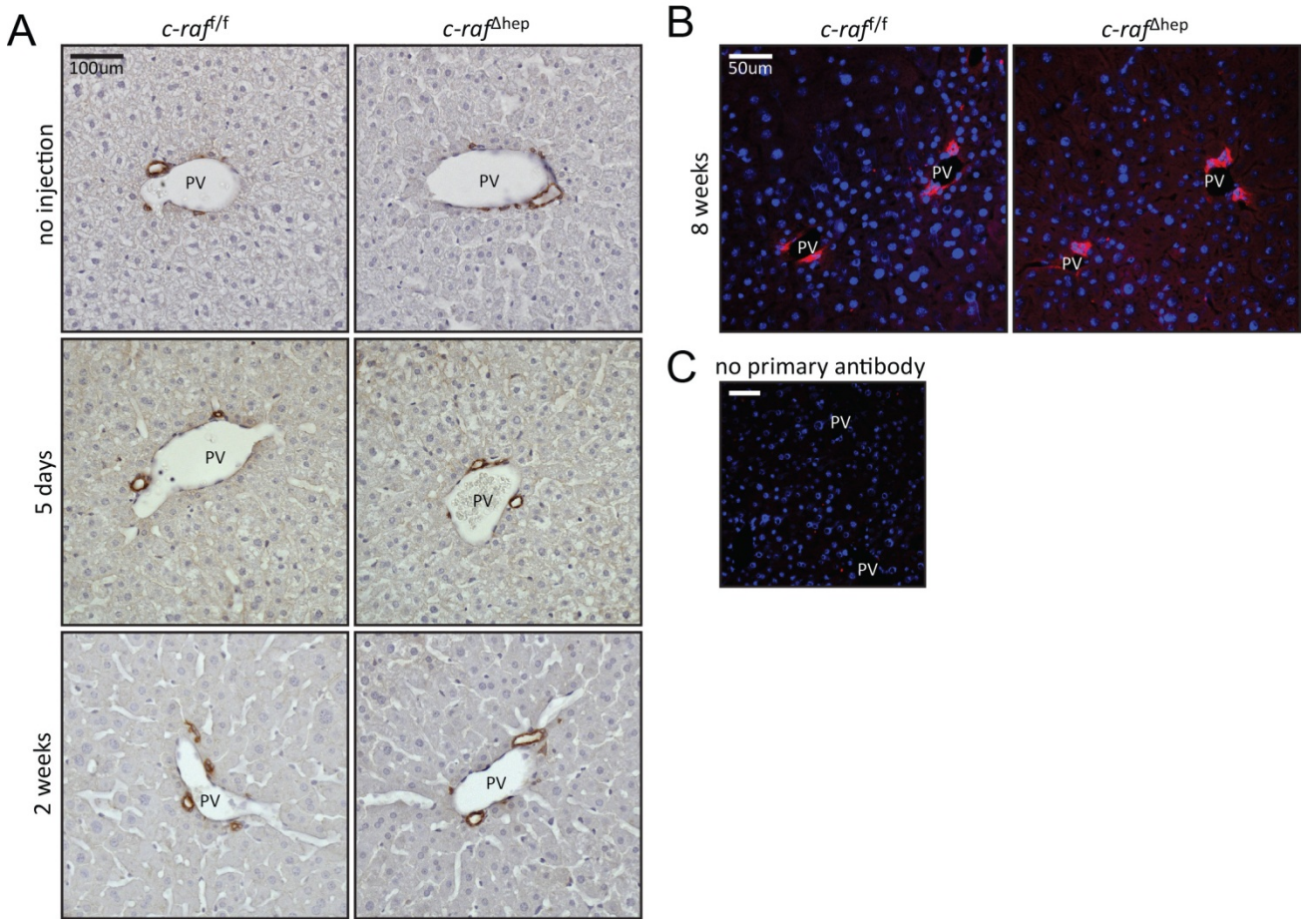

Figure 10. Oval cells are not mobilised during early liver carcinogenesis in mice.
(**A**) Immunohistochemical staining of pan-cytokeratins on paraffin-embedded liver sections from *c-raf*$^{f/f}$ and *c-raf*$^{\Delta hep}$ mice without, 5 days after and 2 weeks after DEN-treatment; (**B**) Immunofluorescence of MIC1 on frozen liver-sections from mice of same genotypes as in A, 8 weeks after DEN-treatment and 4 weeks on Pb-containing fodder; (**C**) Immunofluorescence of MIC1 on frozen liver section without primary antibody as negative control; PV - portal vein;

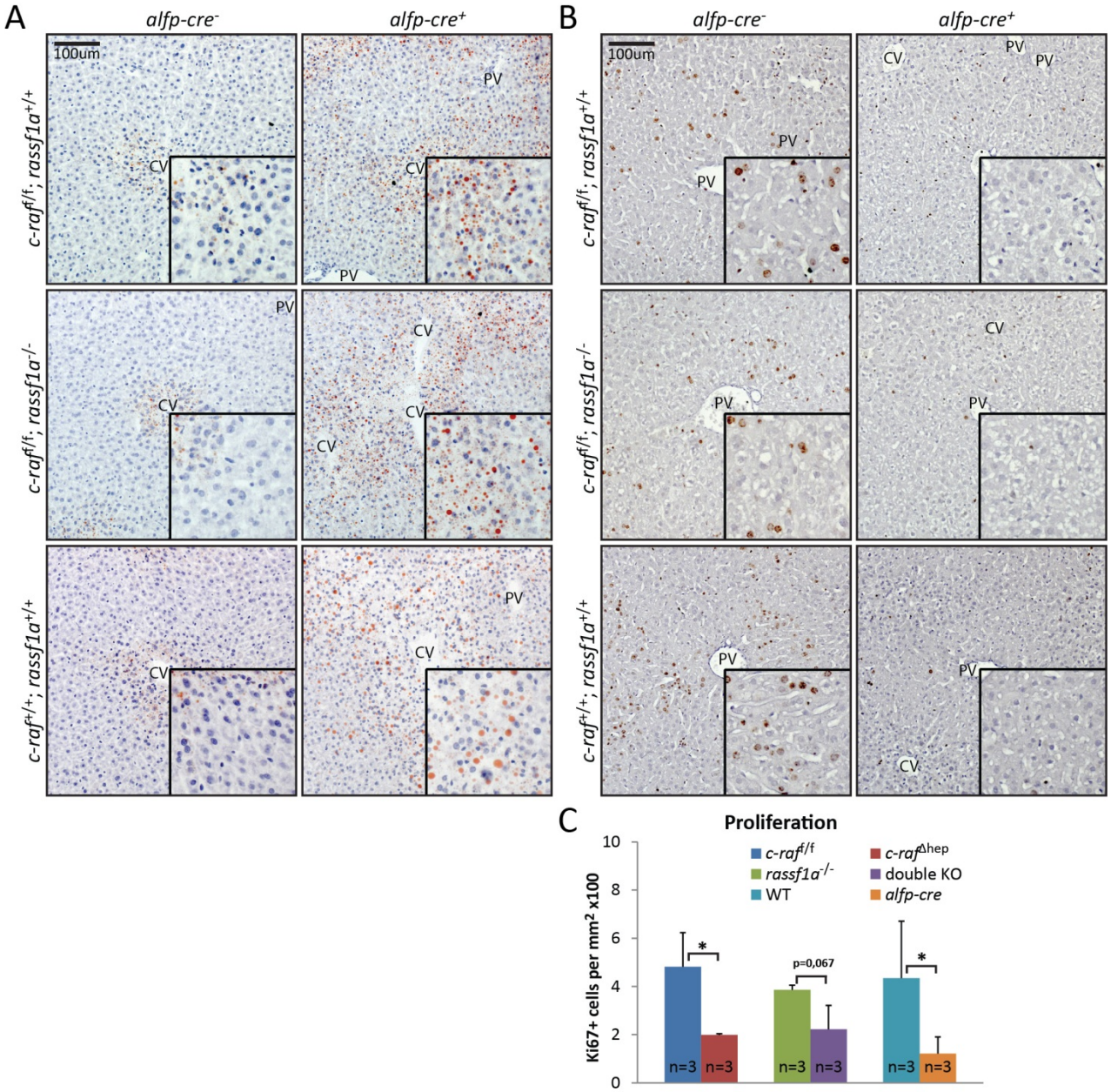

Figure 11. *alfp-cre* transgene sensitises mouse liver to acute effects of DEN-treatment.
(**A**) Histochemical staining of lipid droplets with OilRed on frozen liver sections from, *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *rassf1a*$^{-/-}$, double KO, WT and *alfp-cre* mice 48 hours after DEN-treatment;
(**B**) Immunohistochemical staining of Ki67 on paraffin-embedded liver sections and (**C**) quantification of Ki67-positive cells per 1mm^2 of tissue section from same animals as in A;
Data in graphs is expressed as mean values; error bars - 95% confidence interval; * - p-value<0,05 as calculated from two-tailed student t-test for samples of equal variation; insets on images are 2x magnified; PV - portal vein; CV - central vein;

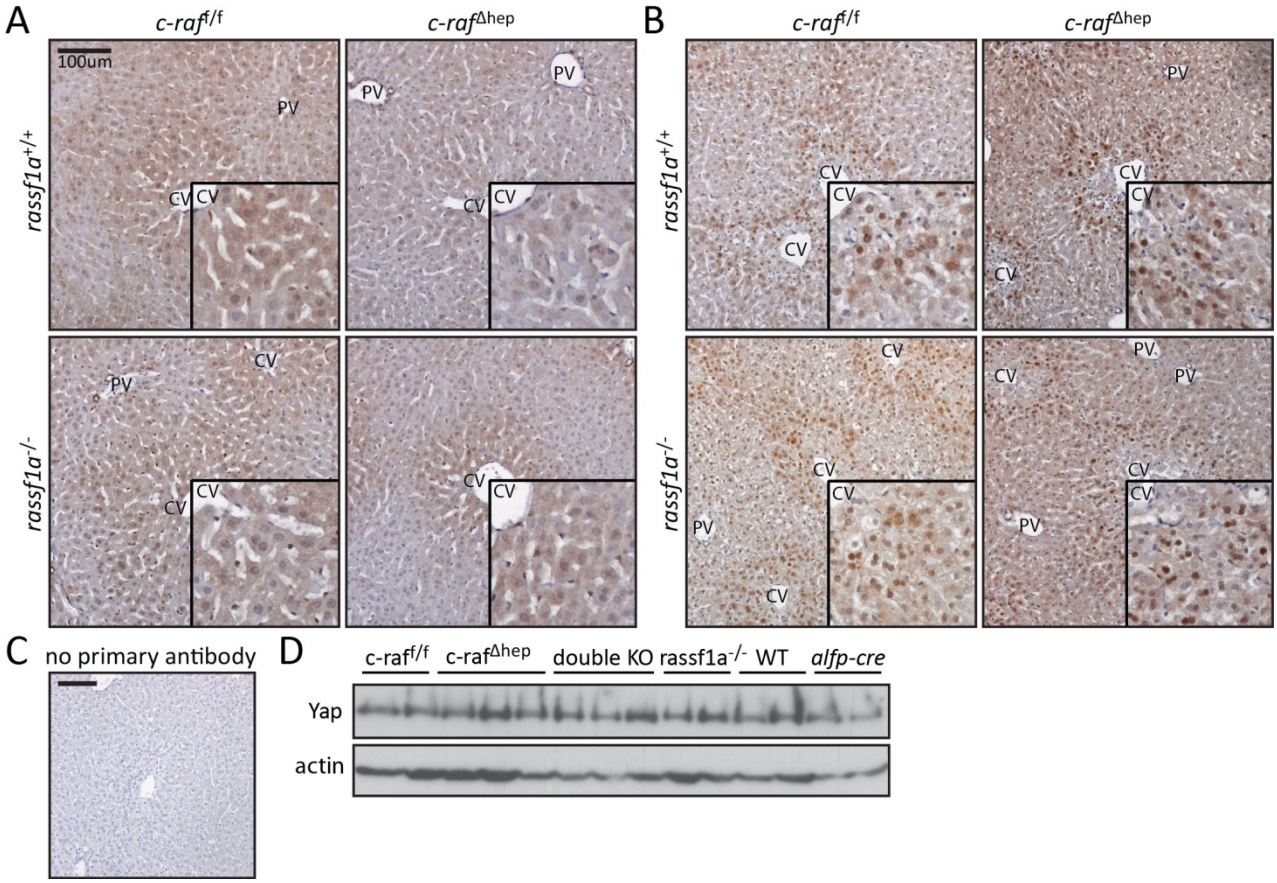

Figure 12. Yap nuclear accumulation in the liver shortly after DEN-treatment.
Immunohistochemical staining of Yap on paraffin-embedded liver sections from *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, *rassf1a*$^{-/-}$ and double KO animals (**A**) without and (**B**) 48 hours after DEN-treatment; (**C**) Immunohistochemical staining of Yap on paraffin-embedded liver section without primary antibody; (**D**) Western blot analysis of Yap and actin (as loading control) in livers from *c-raf*$^{f/f}$, *c-raf*$^{\Delta hep}$, double KO, *rassf1a*$^{-/-}$, WT and *alfp-cre* mice 48 hours after treatment;
Figure D with contribution of Ines Jeric.

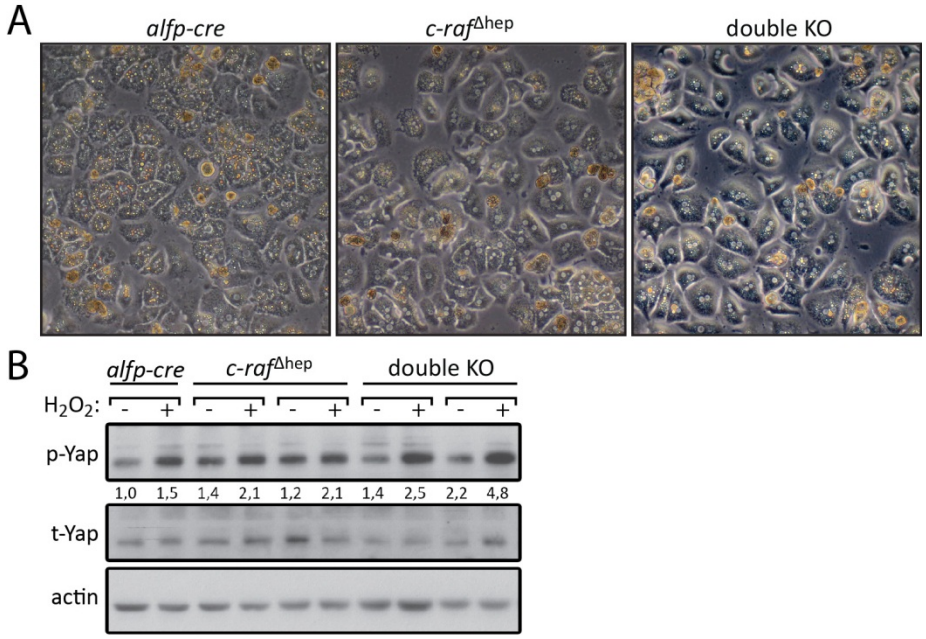

Figure 13. Increased Hippo pathway activation in double KO primary hepatocytes in response to oxidative stress. (**A**) Phase contrast images of *alfp-cre, c-raf*$^{\Delta hep}$ and double KO primary hepatocytes; (**B**) Western blot analysis of inhibitory Yap phosphorylation (S112), total Yap and actin (as loading control) in lysates of primary hepatocytes without and after H_2O_2-treatment (0,5mM for 20'); for *c-raf*$^{\Delta hep}$ and double KO genotypes cells were isolated from 2 different mice; numbers below p-Yap blot indicate band intensity relative to the first lane;

Discussion

Tumour suppressive function of C-Raf in the liver

Chemical liver carcinogenesis in mice deficient for C-Raf in parenchymal cells of the liver results in enhanced tumorigenesis (Figure 4A-D, 5C-D). This indicates surprising, not reported to date tumour suppressive role of C-Raf in the liver. C-Raf is one of 3 kinases of the first tier of the MAPK pathway core, involved in mediating proliferative and survival signalling in the cell. Many members of this pathway as well as upstream activators are therefore aberrantly activated in various cancers (Dhillon, Hagan et al. 2007; Roberts and Der 2007), HCC in particular (Whittaker, Marais et al. 2010). C-Raf is overexpressed in the tumours of HCC patients (Hwang, Choi et al. 2004) and sorafenib, broad-specificity kinase inhibitor designed primarily against C-Raf, is already admitted in Europe for treatment of patients with HCC (Llovet, Ricci et al. 2008).

On the other hand, it was suggested that main mediator of MAPK signalling in the cell is not C-Raf, but rather B-Raf, due to higher kinase activity of the latter (Emuss, Garnett et al. 2005) what is also the reason for rare *C-Raf* mutations in cancer in general. Moreover, work done mostly in our laboratory indicates that the main function of C-Raf *in vivo* is not the stimulation of Mek-Erk axis - quite the opposite - C-Raf is actually dispensable for Erk activation in many investigated cell types (Yamaguchi, Watanabe et al. 2004; Ehrenreiter, Piazzolla et al. 2005; Ehrenreiter, Kern et al. 2009) and (Reiner Wimmer, unpublished data). Therefore it is possible that the oncogenic signalling transmitted by the MAPK pathway is still mediated in the C-Raf deficient hepatocyte by B-Raf and could be investigated by western blotting for activatory phosphorylation sites on B-Raf. In such case role of C-Raf in the activation of Erk would be secondary, after its previously not described tumour suppressive role in the liver.

Most of the *in vivo* functions of C-Raf can be performed independently of its kinase activity and depend on its interaction and direct inhibition of Rock-2 kinase (Niault, Sobczak et al. 2009). Rho-Rock axis plays oncogenic roles in HCC (Xue, Krasnitz et al. 2008; Wong, Wong et al. 2009), mainly during progression by regulating migratory capabilities of cells (Itoh, Yoshioka et al. 1999; Yoshioka, Nakamori et al. 1999), EMT (Chen, Yuan et al. 2011) and secretion of MMPs (Xue, Takahara et al. 2008). Therefore it is possible that C-Raf deficiency in the hepatocyte leads to hyperactivation of Rock-2 what could facilitate transformation. This

could be investigated by probing phosphorylation status of direct Rock-2 targets like coffilin and myosin light chain phosphatase.

Furthermore, recently it was shown that upstream positive regulator of MAPK signalling, Shp2 phosphatase, might also play tumour suppressive functions in spontaneous and chemically-induced liver cancer, what is associated with decreased Erk but increased Stat3 activation (Bard-Chapeau, Li et al. 2011). Therefore mouse disease models begin to unravel previously unexpected roles of MAPK signalling in tumour suppression. This role might depend on inflammatory pathways inhibition, crucial for the development of HCC and maybe even more important than classical pro-proliferative circuits like RAF-MEK-ERK.

Suppression of HCC initiation by C-Raf

Deletion of C-Raf in the parenchymal cells leads to increased number of tumours after chemical liver carcinogenesis (Figure 4C-D), what suggests that frequency of transformation of C-Raf-deficient hepatocytes is increased. Consistent with this notion, when using inducible *mx-cre* system, C-Raf ablation before, but not shortly after carcinogen administration, leads to increased tumour number in the C-Raf-deficient livers (Figure 5A-H). Therefore C-Raf performs its tumour suppressive function in the hepatocyte during tumour initiation.

Acute effect of DEN-treatment can be divided into cytotoxic and genotoxic. DEN activation in the centrilobular hepatocytes causes their extensive death that in turn stimulates compensatory proliferation of periportal hepatocytes. Activated DEN also forms DNA-adducts introducing mutations in the hepatocyte genome that might be carcinogenic and lead to cellular transformation (Verna, Whysner et al. 1996). Theoretically each of these processes might be affected by C-Raf ablation. By participating in the activation of Erk, C-Raf might drive compensatory proliferation, and by inhibiting extrinsic (Piazzolla, Meissl et al. 2005; Galabova-Kovacs, Kolbus et al. 2006; Matallanas, Romano et al. 2007) or intrinsic apoptosis (Kebache, Ash et al. 2007; Polzien, Baljuls et al. 2009) it might restrict cell death. Apoptosis in the C-Raf-deficient hepatocytes is not affected shortly after DEN-administration (Gabriele Maurer, unpublished data), and compensatory proliferation is affected only by the presence of *alfp-cre* transgene, but not by the C-Raf ablation (Figure 11B). Similarly DNA damage, Tp53 induction, DNA-adduct formation and DNA-damage seem also not to be affected by C-Raf-deficiency during tumour initiation (Gabriele Maurer, unpublished data). Proteomic analysis of tissue after 30 weeks of chemical tumorigenesis revealed lower expression of liver carboxylesterase, enzyme involved in detoxification of xenobiotics (Gabriele Maurer,

unpublished data). This leaves open the possibility that C-Raf regulates expression of enzymes that could limit the amount toxic stress during DEN-challenge and in this way restrict the amount of initiated hepatocytes.

Hepatocellular carcinoma is a disease with a poor survival rate, mainly because it is most often detected in an advanced stage, when most available therapies, based on the local intervention, are unhelpful. Classical systemic chemotherapies are not beneficial for the patients with spread-out HCC and recently approved sorafenib treatment is very expensive and rather stabilises then cures the disease. Therefore the improvement in detecting early HCC and therapeutic intervention at this stage could improve patient health (Ferenci, Fried et al. 2010). Our results on the role of C-Raf in mouse liver carcinogenesis indicate that it might be important for the early events in HCC development.

Lack of C-Raf role in HCC maintenance and progression

Deletion of C-Raf in the mouse livers after development of macroscopic nodules did neither affect their maintenance nor progression (Figure 5E-K). Therefore we exclude possibility that DEN-induced HCC in mice is dependent on the presence of C-Raf. This is in contrast to the situation in Ras-induced epidermal tumours, where C-Raf is required for tumour maintenance (Ehrenreiter, Kern et al. 2009). In the skin keratinocytes undergo divisions only in the vicinity of the epithelium basal membrane. During their move to the more outer layers of epidermis they lose the ability to proliferate, and terminally differentiate, what is regulated by Rock-2 kinase (McMullan, Lax et al. 2003). The hepatocytes, although mostly quiescent in the adult organism, retain throughout their lifetime possibility to re-enter cell cycle, for instance in the case of extensive liver injury. Furthermore, the role of Rock-2 in hepatocyte differentiation has not been investigated, but its role in hepatocyte malignant conversion is well established (Grise, Bidaud et al. 2009). Therefore Rho-Rock axis in the liver seems to fulfil very different functions than in the skin, although in the latter it was also shown to be hyperactivated during squamous cell carcinoma development (Grossi, Hiou-Feige et al. 2005; Lefort, Mandinova et al. 2007). Anyways, even if C-Raf is also modulating Rock-2 activity in the hepatocyte, influence of C-Raf ablation on that process might lead to different responses depending on which function of Rock-2 C-Raf modulates in a certain cell type.

In the clinic, use of sorafenib appeared to be beneficial for patients with advanced HCC. Still, it is likely, that its primary target in this setting is not C-Raf, but rather RTKs that mediate

angiogenic signalling (Liu, Cao et al. 2006). Our results support the notion that C-Raf activity is not required for maintenance and progression of HCC.

Genetic interaction of C-Raf and Rassf1a in chemically-induced HCC

Deletion of *c-raf* in the hepatocyte in the WT, but not in the *rassf1a*$^{-/-}$ mice, leads to enhanced liver tumorigenesis (Figure 6A-D). Therefore *rassf1a*-deletion modifies hepatocyte-restricted *c-raf*-deletion phenotype, what indicates possible genetic interaction between these two genes and function of their products in the same pathway. Indeed, RASSF1A and C-RAF are both binding to pro-apoptotic kinase MST2 (O'Neill 2004; Guo, Tommasi et al. 2007), with C-RAF inhibiting its activity and RASSF1A relieving it from the inhibitory complex upon apoptotic stimuli (Matallanas, Romano et al. 2007). Therefore published biochemical data on *in vitro* mammalian cell cultures indicates that RASSF1A is upstream of C-RAF in the MST2-activation sequence. But our results indicate that, if in the mouse liver C-Raf and Rassf1a regulate tumorigenesis on the same pathway in the cell, Rassf1a is rather downstream of C-Raf, as it is required for manifestation of *c-raf*-deletion phenotype.

As *c-raf*-deletion leads to increased tumorigenesis in the liver in the Rassf1a-dependent manner, it suggests that Rassf1a plays oncogenic role in the hepatocyte that is neutralised by C-Raf. How Rassf1a could perform this oncogenic role in the hepatocyte is rather enigmatic, especially taking into consideration its tumour suppressive role in various other settings (Tommasi, Dammann et al. 2005; van der Weyden, Arends et al. 2008) and epigenetic silencing of its gene in many cancers (Dammann, Schagdarsurengin et al. 2003), including HCC (Dammann, Schagdarsurengin et al. 2003). One possible explanation is that proposed C-Raf-tumour suppressive control over Rassf1a plays role only during tumour initiation, and during advanced disease stages both proteins could perform other roles. In fact the published data about *Rassf1a* silencing in human HCC is derived from resected tumours, most likely advanced hepatocellular carcinomas. In our studies most observed nodules were benign foci of cellular alteration or adenomas, and hepatocellular carcinomas were minority. Therefore even if Rassf1a loss had given growth advantage to the advanced liver tumours, to observe this in our system we would have to allow for the further tumour progression and sacrifice our experimental animal after longer period of carcinogenic treatment. Interestingly, *rassf1a*$^{-/-}$ mice had more often HCC nodules than WT mice (Figure 6E), suggesting that indeed Rassf1a loss may promote liver tumour progression.

MST signalling cascade restricts liver tumourigenesis in mice (Zhou, Conrad et al. 2009). In mammalian cells RASSF1A was shown interact with WW45 and together induce MST2 activity (Guo, Tommasi et al. 2007). But in drosophila, dRassf protein rather inhibits activity of MST homologue (Polesello, Huelsmann et al. 2006), Hippo, by displacing it from the activatory complex with Sav (WW45 homologue). dRassf is able to do that as it contains evolutionarily conserved SARAH domain that is also present in the sequence of Sav and Hippo, and is mediating direct interactions between these 3 proteins (Hwang, Ryu et al. 2007). Therefore it would be interesting to check in hepatocytes the activity of MST in complexes with RASSF1A and WW45.

In our double knock-out studies we combined hepatocyte-restricted deletion of *c-raf* with conventional, germline deletion of *rassf1a*. Carcinogenesis depends not only on characteristics of tumour cells, but also their microenvironment composed of various cell types of tumour stroma, vasculature and various inflammatory cells (Hanahan and Weinberg 2011). Therefore we cannot exclude the possibility, that the modification of hepatocyte-restricted *c-raf* deletion phenotype on *rassf1a*$^{-/-}$ background is a result of function impairment of some other cell than hepatocyte. For instance Mst1 kinase restricts naïve T-cell proliferation and cell polarisation upon T-cell receptor ligation, what is regulated by close Rassf1 orthologue Nore1B/Rassf5/Rapl (Katagiri, Imamura et al. 2006; Zhou, Medoff et al. 2008). T-cells are present among non-parenchymal liver cells and might influence tumorigenesis either directly by lysing transformed cells and stimulating them with cytokines or indirectly by educating macrophages and activating B-cells (Grivennikov, Greten et al. 2010). Therefore it would be interesting to investigate lymphocyte numbers and their activation in livers of *rassf1a* KO mice. To determine the exact cell type in which Rassf1a performs its role in modifying *c-raf*-ablation phenotype it would be necessary to apply conditional ablation if its gene in specified cell types.

Influence of C-Raf and Rassf1a on proliferation and inflammation in livers with induced HCC

Neither C-Raf, nor Rassf1a, nor the deletion of both does influence apoptosis in tumours or tumour unaffected tissue (Figure 7C-D). This is very surprising result taking into consideration role of both proteins in the regulation of apoptotic pathways. C-Raf can counteract apoptosis by inhibiting activation of Mst2 (O'Neill, Rushworth et al. 2004), Ask-1 (Chen, Fujii et al. 2001) or Rock-2 that regulates availability of Fas receptor on cell surface (Piazzolla, Meissl et al. 2005). Rassf1a also binds to and regulates activity of Mst2 (Guo,

Tommasi et al. 2007; Matallanas, Romano et al. 2007), and moreover can modulate activation of Bcl-2-family protein Bax in response to Fas stimulation (Baksh, Tommasi et al. 2005). Nevertheless, regulation of these pathways by C-Raf and Rassf1a seems not to be important for survival of hepatocytes during chemical liver carcinogenesis, at least in the livers 30 weeks after carcinogen treatment.

Deletion of *c-raf* in hepatocytes leads to increased proliferation in tumour-unaffected tissue, and concomitant deletion of *rassf1a* attenuates this phenotype. We did not detect any differences in proliferation in the tumour tissue (Figure 7A-B). Therefore *c-raf*-deficient hepatocytes seem to have increased proliferative potential before transformation, what is not the case for double KO hepatocytes. It has been suggested that out of many initiated cells only a fraction is surviving long enough to form stable neoplastic foci (Boucher and Yakovlev 1997). Taking this into consideration it is possible that higher proliferative potential of *c-raf*-deficient untransformed hepatocytes allows them for more efficient formation of stable neoplastic foci shortly after acquiring oncogenic mutations induced by DEN. This could lead to increased number of nodules that we observe at later stages of carcinogenesis. The reason for the increased proliferation in *c-raf*$^{\Delta hep}$ mice is not clear, but it might be a consequence of increased numbers of inflammatory cells in the liver (Figure 9A-B). In the livers of double KO mice, where proliferation is not increased, there is also higher number of inflammatory cells. But the activation of MAPK pathway is decreased and Lats activation is increased (Figure 8A-B), what could explain attenuated proliferative capacity in double KO livers. Decreased activation of Erk would lead to lower activation of transcription of pro-proliferative genes. Increased activity of Lats can either inhibit activity of Yap transcriptional activity on promoters of pro-proliferative genes like cyclin D1. If in the liver activated Lats would not primarily target Yap, as it recently was suggested (Zhou, Conrad et al. 2009), it might also regulate activity of Tp53 tumour suppressor through phosphorylation of its partner ASPP1 (Aylon, Ofir-Rosenfeld et al. 2010).

In the livers of *c-raf*$^{\Delta hep}$ mice we detect increased numbers of Kupffer cells that is also the case in double KO livers compared to rassf1a$^{-/-}$ (Figure 9A-B). Kupffer cells (KC) can either proliferate in the liver or be derived from circulating mononuclear cells (Burt, Portmann et al. 2007). It would be interesting to investigate whether the increased numbers of KCs in *c-raf*$^{\Delta hep}$ and double KO livers result from their enhanced proliferation in the liver or increased recruitment of monocytes from the circulation. In first case the double staining for proliferation markers like Ki67, PCNA, or injected BrdU with markers of macrophages

(F4/80, CD68) could be performed. To investigate the migration one could reconstitute lethally irradiated mice with labelled, for instance with fluorescent protein, bone marrow and investigate recruitment of label-positive cells to the liver. Interesting is also what factors from the C-Raf-deficient hepatocytes do affect KC abundance in the liver. One of the candidates would be CCL2/MCP1, cytokine attracting macrophages. It might be that its expression in the *c-raf*-deficient hepatocyte is perturbed.

Irrespective of the factor that induces increased proliferation or recruitment of macrophages to the livers with *c-raf* ablated in the hepatocytes, increased number of KCs in these animals can have tumour promoting role. KCs are drivers of inflammatory responses in the liver and respond with cytokine production to the tissue damage by sensing factors released by dying hepatocytes (Sakurai, He et al. 2008). They are able to secrete Il-6 and TNF-α that can stimulate proliferation of hepatocytes and contribute to their neoplastic transformation (Maeda, Kamata et al. 2005). In fact, livers of *mx-cre*;*c-raf*$^{f/f}$ animals, that have increased numbers of tumours (Figure 5A-D), but are not as much more affected by the disease in comparison to livers from WT animals as are *alfp-cre*;*c-raf*$^{f/f}$ (Figure 4A-D), do not exhibit increased macrophage numbers (Gabriele Maurer, unpublished data). This also indicates that opposite to its role in the hepatocyte, C-Raf might have tumour promoting role in macrophages. To determine this one could apply macrophage-specific *c-raf* ablation with the help of LysMCre mouse line (Clausen, Burkhardt et al. 1999).

Classical activation of Kupffer cells is very limited in the livers of our experimental animals - we detected only 20-50 times less cells positive for classical macrophage marker, inducible nitric oxide synthase, as we did for general macrophage marker, F4/80 (Figure 9C-D). Classical macrophage activation is in general thought to have anti-tumour effects, by inducing their direct cytotoxicity towards transformed cells (Biswas and Mantovani 2010). On the other hand liver cancer development is largely dependent on inflammation that classically activated macrophages are supporting by secretion of pro-inflammatory cytokines. It might be that macrophages present in the tumour affected livers exhibit alternative activation type, that is more associated with tissue remodelling and might drive tumour promotion (Gordon and Martinez 2010). Therefore it would be also interesting to investigate expression of markers of other macrophage activation types in tumour-affected livers.

In the tumour unaffected tissue activation of Erk is decreased in the livers from double KO animals (Figure 8A). Although C-Raf is able to activate Erk through Mek, in most tissues it is

not required for Erk activation. In this view it is not surprising that Erk activation is not affected in *c-raf*$^{\Delta hep}$ hepatocytes. But the decrease in Erk phosphorylation in the livers of double KO animals is not easily explainable. Rassf1a is a putative Ras effector as it contains Ras-association domain, but detailed investigations raise doubts about Rassf1a binding to any of Ras proteins under physiological conditions (Ortiz-Vega, Khokhlatchev et al. 2002). Nevertheless, the decrease in Erk activation in the livers of the double KO animals could explain attenuation of increased proliferation in comparison to the C-Raf-deficient hepatocytes.

Yap protein phosphorylation is not affected in any of investigated genotypes, contrary to what we initially expected by genetically manipulating upstream regulators of Mst kinase. Yap phosphorylation induces change in its nuclear localisation but also induces proteasome-mediated degradation (Zhao, Li et al. 2010). Therefore it is possible that Yap phosphorylation status that we observe in the livers after carcinogenesis is just a snapshot resulting from continuous degradation of its phosphorylated form. In such case, even if the activity of Yap kinases is affected by C-Raf or Rassf1a ablation, we might not be able to observe it at steady state. In fact we observe increased level of Yap in unaffected and tumour affected tissue of *c-raf*$^{\Delta hep}$ and double KO mice, so wherever C-Raf is absent (Figure 8B). C-RAF is directly inhibiting MST2 kinase *in vitro*, what should lead to decreased YAP phosphorylation and increased stability. Therefore it seems surprising that ablation of C-Raf leads to increased expression of Yap in mouse livers and might be a result of some other mechanism. It would be therefore interesting to investigate Yap stability in *c-raf*-deficient hepatocytes, for instance in cycloheximide-treated cells, but also expression of Yap-encoding gene by investigating abundance of its mRNA transcript.

Double KO animals have also decreased activation of Lats1 kinase in the liver that also could contribute to the attenuation of proliferation increase resulting from *c-raf*-ablation in the hepatocyte (Figure 8C). Classically Lats kinases are considered primary Mst targets, but recent reports indicate that this view might hold true only for mouse embryonic fibroblast *in vitro* cultures. In the liver Lats activation is not dependent on Mst kinases, and also Lats kinases are not mediating phosphorylation of primary Hippo pathway target, Yap in response to Mst activity (Zhou, Conrad et al. 2009). Recently Lats kinases 1 and 2 were shown to interact with ASPP1 protein, regulator of TP53 activity, and be decoupled by ASPP1 from Yap (Vigneron, Ludwig et al. 2010), or induce through ASPP1 TP53 proapoptotic activity (Aylon, Ofir-Rosenfeld et al. 2010). This suggests the possibility that increased

phosphorylation/activation of Lats in the livers of double KO mice might lead to increased activity of Tp53. It would therefore be interesting to check the Tp53 stability, localisation and expression of its target genes in the livers of double KO animals.

Finally, we also observe decreased phosphorylation of p38 stress kinase in tumour unaffected tissue from double KO animals (Figure 8C). p38 is a MAPK activated in response to various stress stimuli and it plays tumour suppressive role in various cancers (Hui, Bakiri et al. 2007), including HCC (Hui, Bakiri et al. 2007). It is therefore surprising to see its lower activation in tissue of double KO animals, where tumorigenesis is compromised to WT levels. Recently it has been suggested that MST mutants that cannot be phosphorylated by AKT associate more with RASSF1A and induce p38 phosphorylation (Romano, Matallanas et al. 2010). It is then possible that lack of Rassf1a in our livers decreases activity of Mst towards p38, but why the deletion of C-Raf is also required for that to happen, is rather enigmatic.

Effects of C-Raf, Rassf1a and *alfp-cre* transgene on DEN acute effects in the liver

By using labelling for markers of oval cell, the liver stem cells, we could see no induction of this progenitor compartment 5 days, 2 weeks and 8 weeks after DEN administration (Figure 10A-B). Oval cells can be induced with various chemical treatments in rodents, but their induction after DEN or phenobarbital treatment was reported only once (He, Smith et al. 1994). DEN causes extensive liver damage, hepatocyte death and subsequent compensatory proliferation. Therefore the protocol itself is not inhibiting proliferation of hepatocytes, condition in which the induction of liver progenitors compartment is usually observed. On the other hand Hippo pathway was recently shown to limit expansion of liver progenitor compartment and in this way suppress tumorigenesis in this organ (Lee, Lee et al. 2010; Lu, Li et al. 2010). Nevertheless, in our protocols cells with markers expressed on oval cells were limited to the proximity of portal triads and their number was affected neither at any timepoints nor in the livers of the animals with any investigated genotype.

All mice with *alfp-cre* transgene have increased lipid accumulation (steatosis) in response to the DEN-treatment, irrespectively of the status of *c-raf* or *rassf1a* gene (Figure 11A). Liver steatosis can inhibit proliferation of hepatocytes (Vetelainen, van Vliet et al. 2007) and indeed in our system, mice with *alfp-cre* transgene have inhibited compensatory proliferation response irrespectively of *c-raf* or *rassf1a* deletion (Figure 11B-C). Integration site of *alfp-cre* transgene within mouse genome has not been mapped so far and it is possible that it disrupts

some gene or regulatory element responsible for this metabolic phenotype. Furthermore it was shown that prolonged expression of Cre recombinase in cells, including hepatocytes, may have genotoxic effects (Loonstra, Vooijs et al. 2001; Takami, Kaposi-Novak et al. 2007). Nevertheless, effects of the Cre recombinase might be pleiotropic and difficult to predict, and therefore it is advisable to control the experiments that employ Cre recombinases with Cre-positive, flox-negative animals instead of only Cre-negative littermates. It would be also interesting to check the site of the *alfp-cre* transgene integration that might help to gain some insight into other defects this strain might exhibit.

Yap expression seems to be zonated in the liver, with higher expression and cytoplasmic localisation in the centrilobular areas and lower, nuclear expression in the periportal areas. Main regulator of liver zonation that determines different function of centrilobular and periportal hepatocytes is Wnt-β-catenin pathway (Benhamouche, Decaens et al. 2006). Wnt-β-catenin pathway activity is regulated by proteasomal degradation of β-catenin triggered by ubiquitylation directed by β-TRCP protein. Lately, β-TRCP was found to recognise and direct for ubiquitylation also phosphorylated Yap, adding additional inhibitory mechanism on top of its cytoplasmic retention. It is also remarkable, that uncontrollable activation of β-catenin (Colnot, Decaens et al. 2004) and Yap (Camargo, Gokhale et al. 2007; Dong, Feldmann et al. 2007) in mouse liver both lead to similar phenotype - hepatomegaly and subsequent HCC development. It is interesting whether Yap also participates in the zonation of the liver especially taking into consideration recent reports about YAP responding to β-catenin-mediated contact inhibition signals from E-cadherin (Kim, Koh et al. 2011), controlling heart size by co-regulating expression of same genes as β-catenin, (Heallen, Zhang et al. 2011) and reports about TAZ inhibiting β-catenin signalling by hindering phosphorylation of DVL (Varelas, Miller et al. 2010). Carcinogen administration induces Yap nuclear translocation, which indicates its activation, in liver centrilobular areas (Figure 12B). The nuclear accumulation or amounts of total Yap are not affected by the deletion of the investigated genes (Figure 12B-D). This indicates that Yap activity is induced during compensatory proliferation after tissue injury caused by carcinogen, and might regulate hepatocyte survival and proliferation during that stage, but this is most likely not regulated by C-Raf or Rassf1a.

Primary hepatocytes react to the oxidative stress with the enhanced phosphorylation of Yap that is higher in double KO hepatocytes. Oxidative stress induced by addition of H_2O_2 in tumour-derived hepatocyte cell culture was previously shown to induce activation of Mst kinases, phosphorylation of Lats and Yap (Zhou, Conrad et al. 2009). Increased Yap

phosphorylation in the absence of Rassf1a and C-Raf compared to C-Raf-ablation alone indicates that in the hepatocyte, opposite to what it does in cell lines (Matallanas, Romano et al. 2007) and similarly to drosophila dRassf (Polesello, Huelsmann et al. 2006), Rassf1a might act as Mst inhibitor. Increased oxidative stress is associated with and required for efficient liver tumorigenesis in mice (Sakurai, He et al. 2008; He, Yu et al. 2010). Therefore by negatively influencing, in the condition of oxidative stress, activation of Mst kinases that are crucial for the tumour suppression in the liver, Rassf1a might act as a proto-oncogene in this organ.

Materials and methods

Mouse handling and maintenance

Mice of all strains were kept in SPF animal facility. They were mated from 6 weeks of age on; pups were marked by toe-clip on 10th day after birth and weaned on 20th day after birth.

Mouse strains

Following strains have been used in experiments:

- 129/Sv *c-raf*$^{f/f}$ - pure 129/Sv background mice with floxed exon 3 of *c-raf* gene (Mikula, Schreiber et al. 2001).
- 129/Sv *alfp-cre*$^{+}$ - pure 129/Sv background mice with *alfp-cre* transgene (Kellendonk, Opherk et al. 2000).
- 129/Sv *mx-cre*$^{+}$ - pure 129/Sv background mice with *mx-cre* transgene (Kuhn, Schwenk et al. 1995). Expression of Cre recombinase was induced by injecting mice intraperitoneally with 13µg per g body weight poly(I:C) diluted in sterile PBS 2g/l (Cat. No. 27-4732-01 Amersham, GE Healthcare, Waukesha, US).
- 129/B6 *rassf1a*$^{-/-}$ - mixed 129/Sv-C57BL/6 background mice with a germline disruption of exon 1α of *rassf1* gene (Tommasi, Dammann et al. 2005).

DNA methods

DNA isolation

Either tail or liver ~2-5µg tissue fragment was digested in 100µl of Direct PCR lysis reagent (Cat. No. 31-102-T, Viagen Biotech, LA, US) with 200µg/ml Proteinase K (Cat. No. P6556, Sigma) overnight at 55°C, followed by enzyme inactivation for 45' at 85°C.

PCR genotyping

For all PCRs crude tissue lysate was used. All primer stocks were diluted in water at 100nM concentration. After PCR whole reaction mixture was run 40' at 100V together with DNA marker (Cat. No. SM1331, Fermentas/Thermo, Waltham, US) on 2% agarose gel prepared with TAE buffer and ethidium bromide, and visualised under UV light.

Following PCR protocols were used:

c-raf

Mastermix - µl per reaction:	
2x PCR RedMix	12,5
Primer MPXR1	0,125
Primer MPXR2	0,125
Primer MPXR3D	0,125
H_2O	9,625
DNA	2,5
TOTAL:	25

PCR Program		
98°C	30”	
98°C	10”	30 cycles
65°C	30”	
72°C	45”	
72°C	1’	
21°C	inf.	

Primers:
MPXR1: 5’-tgt gcc ctt gga acc tca gca c-3’
MPXR2: 5’-aca acg aga tag atg agg aaa gca-3’
MPXR3D: 5’-cac tga aat gaa aac gtg aag acg-3’

Products:
WT: ~280bp (primers R1 and R2)
flox: ~330bp (primers R1 and R2)
- or Δ: ~450bp (primers R2 and R3D)

cre

Mastermix - µl per reaction:	
2x PCR RedMix	12,5
Primer MPXC1	0,125
Primer MPXC2	0,125
H_2O	9,75
DNA	2,5
TOTAL:	25

PCR Program		
98°C	30”	
98°C	10”	30 cycles
65°C	30”	
72°C	45”	
72°C	1’	
21°C	inf.	

Primers:
MPXC1: 5'-ctg cca cga cca agt gac agc a-3’
MPXC2: 5'-gcc aga tta cgt ata tcc tgg ca-3’

Products:
+: ~180bp (primers C1 and C2)
- : no product

rassf1a

Mastermix - µl per reaction:	
2x PCR RedMix	12,5
Primer UMIOAI	0,025
Primer LMIIAA	0,025
Primer Neo48U	0,025
H_2O	9,925
DNA	2,5
TOTAL:	25

PCR Program		
95°C	4’	
63°C	1’	2 cycles
72°C	1’	
95°C	1’	
63°C	1’	33 cycles
72°C	2’	
72°C	10’	
21°C	inf.	

Primers:
UMIOAI: 5’-ttg tgc cgt gcc ccg ccc a-3’
LMIIAA: 5’-tga cca gcc ctc cac tgc cgc-3’
Neo48U: 5’-ggg cca gct cat tcc tcc cac-3’

Products:
WT: 520 bp
KO: 380 bp

Chemical liver carcinogenesis

1g of DEN (Cat. No. N0258, Sigma) was diluted 1:9 in 0,9% NaCl to obtain stock solution and stored at RT protected from light up to 4 months. Before the injection the stock was diluted again 1:9 in 0,9% NaCl to obtain working solution.

4 weeks old male mice (±2 days) were injected intraperitoneally with working solution of DEN (100µg per g body weight). At 8 weeks of age (±2 days) normal fodder was exchanged for the one supplemented with 0,07% phenobarbital (Ssniff, Soest, DE) and mice were kept on it until they were euthanized.

Liver isolation and liver sample collection

Mice were weight and euthanized by cervical dislocation, peritoneal cavities were opened by V-incision, gall bladders were removed, livers were separated from diaphragm, removed from the body and washed in ice-cold PBS. After brief drying on paper towel, livers were photographed, weight and separated to single lobes. Big liver lobes were fixed in PFA and

embedded in paraffin; single smaller lobes were snap-frozen in TissueTek O.C.T. compound (Cat. No. 4583, Sakura, Alphen aan den Rijn, NL) for cryo-block, and from the rest of the tissue tumours were separated from tumour-unaffected tissue and tumour pools, single tumours and tumour unaffected tissues were snap-frozen for DNA and protein analysis.

Histology

Stainings on paraffin-embedded liver sections

After isolation big liver lobes were cut into 2 or 3 pieces for better penetration with the fixative and fixed overnight at 4°C in 4% PFA in PBS, following by 2x wash with PBS and 1x with EtOH 70%. After that they were dehydrated and paraffinised in Shandon Excelsior tissue processor (Thermo) by incubating 1x in each 70, 80, 90 and 96% EtOH; 3x in 100% EtOH; 3x in xylene (each for 1h); and 3x in wax at 55°C (each for 1h20'). Paraffinised tissue was embedded in paraffin blocks.

Paraffin embed-livers were cut on the microtome into 5µm-thick sections, straighten in water bath set to 45°C, transferred on coated microscope support slides (Cat. No. J4800AMNZ, Thermo) and allowed to dry and adhere overnight at 45°C.

H&E staining

Paraffin-embed liver sections were stained with hematoxylin and eosin with the help of ASS-1 staining unit (Pathisto, Georgsmarienhütte, DE), by incubating slides with liver sections in the following solutions:

- 2x 10' in xylene substitute
- 2x 5' in 100% EtOH
- 2' in 90% EtOH
- 2' in 70% EtOH
- 2' in H_2O
- 6' in hematoxylin
- 10' in H_20
- 5'' in 0,37% HCl in EtOH
- 10' in H_2O
- 30" in 70% EtOH
- 30" in 80% EtOH
- 2" in eosin solution
- 2x 1' in 90% EtOH
- 2x 1' in 100% EtOH
- 2x 5' in xylene substitute
- Wash with xylene and mounting with entellan

Immunohistochemistry

General protocol

Wash buffers:

- TBST: 10mM Tris-HCl, 150mM NaCl, Ph 7,4, Tween-20 0,1%;
- PBS: 9,1mM dibasic sodium phosphate, 1,7mM monobasic sodium phosphate, 150mM NaCl, pH 7,4;

Deparaffinisation: Slides were incubated 2x for 10' in xylene, 2x 10' in 100% EtOH, 2x 5' in 90% EtOH and 2x 5' in ddH_2O.

Endogenous peroxidase blocking: Slides were incubated in 1 or 3% H_2O_2 in either ddH_2O or ddH_2O: methanol 1:1 mixture or pure methanol and washed 2x 5' with ddH_2O**.**

Antigen unmasking:

- Heat-mediated: Slides in antigen unmasking solution were brought to boil in microwave and kept in steam cooker in sub-boiling temperature, cooled down at room temperature and washed 3x 5' with wash buffer;
  citrate buffer: 10 mM sodium citrate, pH to 6.0;
  basic antigen unmasking solution: 10mM Tris-base, 1mM EGTA; 0,05% Tween-20, pH 9,0;
- Proteolytic: slides were incubated in one of the following solutions in indicated conditions and washed 3x 5' with wash buffer:
  Proteinase K: 20ug/ml in 10mM Tris-HCl in 37°C;
  Protease type XIV (Cat. No. P5147, Sigma): 0,5mg/ml in PBS 8' at room temperature;

Protein blocking: Slides were blocked in diluted in wash buffer serum from the species that secondary antibody was raised in.

Primary antibody: Slides were incubated with primary antibody and washed 3x 5' in wash buffer.

Secondary antibody: Slides were incubated for 30' at room temperature:

- with HRP polymer-conjugated anti-rabbit secondary antibody solution (Cat. No. K4003, Dako, Glostrup, DK), and washed 3x 5' with wash buffer.
- with biotinilated anti-rat secondary antibody (Cat. No. BA-4000, Vector Labs, Burlingame, US) diluted 1:250 in wash buffer, washed 3x 5' with wash buffer, incubated with Vectastain Elite ABC kit (Cat. No. PK-6100, Vector Labs) (drop of each reagent A and B in 2,5ml PBS) 30' at room temperature and washed 3x 5' with wash buffer.

Detection: Slides were incubated in developing solution: 1 tablet DAB (Cat. No. D5905, Sigma), diluted in 50ml PBS, with 50µl of 30% H_2O_2 added directly before use and washed briefly in ddH_2O.

Couterstaining: Slides were incubated 30-60" in hematoxylin diluted 1:20 in ddH_2O and washed 10' under the running water tap.

Dehydration and mounting: Slides were incubated, 5" in EtOH 50%, 5" in EtOH 70%, 2x 5" in EtOH 90%, 2x 10' in EtOH 100%, 2x 10' in xylene and mounted with entellan.

Specific protocols

Ki67: wash buffer: TBST; peroxidase blocking: 10' with 3% H_2O_2 in ddH_2O; antigen unmasking: heat in citrate buffer 30', cool down 20'; protein blocking: 1,5% normal goat serum; primary antibody: rabbit polyclonal anti-mouse Ki67 diluted 1:1000 (Cat. No. NCL-Ki67p, NovoCastra/Leica, Wetzlar, DE) overnight at 4°C; secondary antibody: HRP-conjugated anti-rabbit; detection: incubation with DAB 8';

pan-cytokeratin: wash buffer: TBST; peroxidase blocking: 3% H_2O_2 in ddH_2O, 10'; antigen unmasking: proteolytic with Proteinase K, 2'; protein blocking: 1,5% normal goat serum; primary antibody: polyclonal anti-cow cytokeratin wide-spectrum screening diluted 1:500 (Z0622, Dako) 30' at room temperature; secondary antibody: HRP-conjugated anti-rabbit; detection: incubation with DAB 5';

INOS: wash buffer: PBS; peroxidase blocking: 1% H_2O_2 in ddH_2O, 5'; antigen unmasking: basic antigen retrieval solution 30', cool down 20'; protein blocking: 1,5% normal goat serum; primary antibody: rabbit polyclonal anti-human INOS diluted 1:500 (Cat. No. SC-651, Santa Cruz Biotechnology, Santa Cruz, US) overnight at 4°C; secondary antibody: HRP-conjugated anti-rabbit; detection: incubation with DAB 5';

Yap: wash buffer: TBST; peroxidase blocking: 3% H_2O_2 in ddH_2O, 5'; antigen unmasking: citrate buffer 30', cool down 20'; protein blocking: 5% normal goat serum; primary antibody: rabbit polyclonal anti-human YAP diluted 1:25 (Cat. No. 4912, Cell Signaling, Danvers, US) overnight at 4°C; secondary antibody: HRP-conjugated anti-rabbit; detection: incubation with DAB 10';

F4/80: wash buffer: PBS; peroxidase blocking: 1% H_2O_2 in ddH_2O:methanol 1:1, 10'; antigen unmasking: protease type XIV; protein blocking: 1,5% normal goat serum; primary antibody: rat polyclonal anti-mouse F4/80 diluted 1:50 (Cat. No. MCA497G, Serotec, Kidlington, UK) overnight at 4°C; secondary antibody: biotinilated anti-rat; detection: incubation with DAB 10';

TUNEL: conducted with TACS 2 TdT In situ Apoptosis Detection Kit (Cat. No. 4810-30-K, Trevigen, Gaithersburg, US); wash buffer: PBS; antigen unmasking: proteolytic with

Proteinase K, 15'; peroxidase blocking: 3% H_2O_2 in methanol, 5', washed 1'; protein blocking: 5% normal goat serum; labelling: wash slides in labelling buffer 5', label in labelling mix 1h at 37°C, wash slides with stop buffer for 5', wash 2x with ddH_2O 5'; POD signal conversion: incubate slides with Strep-HRP solution for 10' at 37°C; Detection: incubation with DAB 5';

Stainings on cryo-sections

Snap-frozen tissue in cryo-blocks was stored in -80°C and for staining cut into 7µm-thick sections that were transferred on coated microscope slides and before staining dried for 30' at room temperature.

MIC-1: Sections were fixed in ice cold acetone for 10', air dried for 30' washed in wash buffer (PBS with Triton X-100 0,1%) 3x 5'. Then sections were blocked in with 5% normal horse serum in wash buffer for 1h at room temperature and incubated with primary antibody (rat monoclonal anti-mouse, Cat. No. MIC1-1C3, Novus Biologicals, Littleton, US) overnight at 4˚C. After that slides were washed with wash buffer 3x 5', incubated with secondary antibody (donkey anti-rat-alexa-594, diluted 1:500 in wash buffer Cat. No. A-21209, Molecular Probes/Invitrogen) 30' at room temperature, washed with wash buffer 3x 5', counterstained with DAPI (200ng/ml in PBS) 5', washed with wash buffer 5' and mounted with Mowiol with DABCO.

OilRed: Sections were fixed in 4% PFA in PBS for 10' at room temperature. After that they were washed in ddH_2O 5', incubated in 60% isopropanol 5', stained with filtered Oil Red 10', washed in 60% isopropanol 5' followed by brief wash in ddH_2O. Finally sections were counterstained with hematoxylin diluted in 1:20 ddH_2O 30", washed under the running water tap for 10', mounted with glycerol and sealed with nail polish.

Image processing

Tumour-affected area and number of tumours on H&E sections was quantified either on images acquired with microscopes Axio Imager.M1 and SteREO Discovery.V12 equipped with AxioCam MRc5 camera (Zeiss, Oberkochen, DE) (*alfp-cre*-mediated single KOs) or on scans acquired at 20x magnification with digital slide scanner Pannoramic SCAN (3DHISTECH/Zeiss) (Paweł Pasierbek, IMP, Vienna) (*mx-cre*-mediated single KOs and *alfp-cre*-mediated double KOs).

Quantifications of immunohistochemical stainings were performed on images acquired at 20x magnification with Axio Imager.M1 microscope equipped with AxioCam MRc5 camera (F4/80, Ki67 48hrs after DEN) or regions of interests from scans acquired at 20x magnification with Pannoramic SCAN (Ki67 30 weeks after DEN, TUNEL, INOS). Images were processed in ImageJ: first colour deconvolution plugin was used to isolate DAB-signal, which was then thresholded and positive cells were automatically counted with the help of particle analyser function.

Hepatocyte primary cultures

Portal vein catheterisation and liver perfusion: Mice were euthanized by cervical dislocation and their abdomen and thorax were disinfected with EtOH 70% before opening peritoneal cavity by V-incision. Further, intestine and stomach were moved aside and liver was flipped up to visualise portal vein. Slight incision in the portal vein was made, capillary connected to rubber tube with perfusion solution (Cat. No. 17701, Gibco/Invitrogen, Paisley, UK) was introduced into the portal vein, vena cava was cut open, and the liver was perfused with the help of peristaltic pump at ~8ml/min for 3'. After that time pump was stopped, rubber tube was moved to liver digest medium (Cat. No. 17703, Gibco/Invitrogen), and perfusion was continued for 5' at the same speed. Perfusion solutions were warmed up so at the end of the capillary their temperature reached 37°C.

Hepatocyte isolation: Gall bladder was removed, perfused and digested liver separated from diaphragm and transferred to sterile petri dish, cut into pieces for better hepatocyte release from the liver capsule and transferred to 15ml ice-cold hepatocyte isolation medium. Cells were filtered through 70µm cell strainer into fresh 50ml falcon, centrifuge at 50g for 5', re-suspended in 10ml fresh hepatocyte isolation medium, and centrifuged at 20g for 2' twice, changing medium in between. After that cells were re-suspended in 10ml 37°C-warm hepatocyte growth medium and their viability and number was estimated in trypan blue with Neubauer chamber. Hepatocytes were plated 35000 alive cells per $1cm^2$ culture dish surface on dishes coated with collagen $5\mu g/cm^2$ (Cat. No. 2586-C02-0206, Inamed, Santa Barbara, US).

Hepatocytes isolation medium: RPMI, 10% FCS, pen-strep, amphotericin; 20mM HEPES;

Hepatocyte growth medium: RPMI, 10% FCS, pen-strep, amphotericin, growth hormones (40ng/ml TGF-α (Cat. No. T7924, Sigma), 30ng/ml IGF-II (Cat. No. I2526, Sigma), 1,4nM insulin (Cat. No. I5500, Sigma));

Protein methods

Protein isolation from mouse livers

Around 5-10 volumes of RIPA tissue lysis buffer with inhibitors was added to 5-10µg frozen liver tissue that was subsequently homogenised in FastPrep-24 (MP Biomedicals, Irvine, US) with~8 ceramic beds in tube, power 5.0, 2x 20" bursts with 2' pause on ice in between. Lysates were centrifuged 2x at 20000xg for 15' with supernatant transferred into a fresh tube. Protein concentration was estimated with Bradford method (Cat. No. 500-0006, Bio-Rad, Hercules, US) with samples diluted in ddH_2O 5-10x. To achieve desired protein concentration in the final sample (usually 1-2 g/l) 1x RIPA buffer with detergents and inhibitors was added together with 3x Laemmli sample buffer to achieve final 1x concentration.

RIPA buffer: 50mM Tris-HCl pH 8,0; 150 mM NaCl; 1% TX-100; 0.1% SDS; 5 mM EDTA; 1 mM EGTA;

Detergent and inhibitors added just before use: 0.5% sodium deoxycholate; 1x PIC; 1ug/ml pepstatin; 10mM NaF; 1mM PMSF; 1mM β-glycerophosphate; 2,5 mM sodium pyrophosphate;

3x Laemmli sample buffer: 187,5mM Tris-HCl, 6% SDS, 30% glycerol, 15% β-mercaptoethanol, 0,03% bromophenol blue;

Protein isolation from cell cultures

Cell culture plates were put on ice, medium was aspirated and plates were washed 3x with ice-cold PBS. 1x Laemmli buffer (without β-mercaptoethanol and bromophenol blue) was added on plates (200µl per confluent 10cm plate), cells were scraped with cell scraper and lysate was transferred to fresh eppendorf tube. Lysate was incubated 5' at 95°C with shaking and 2x Leammli buffer was added to achieve final 1x concentration. Protein concentration was measured with BCA method (Cat. No. P23228, Thermo) after diluting samples 5x in 1x Laemmli buffer. To achieve desired protein concentration in the final sample (usually 0,5-1 g/l) 1x Laemmli buffer was added and β-mercaptoethanol:bromophenol blue mixture in ratio 5:2 was added to achieve final 7% concentration.

1x Laemmli buffer: 62,5mM Tris-HCl, 2% SDS, 10% glycerol;

SDS-PAGE

SDS-polyacrylamide gels were casted and run with SE250 Mighty Small II (small gels) or SE600 (large gels) systems (Hoefer, Holliston, US). Gels were composed of resolving and 1cm stacking part, each allowed to polymerise in the casting apparatus after adding APS and TEMED for 15'. Samples (10-50µg protein per 3-7mm-wide well) were run at 60V through stacking gel and 90V through resolving gel until bromophenol blue exited the gel.

SDS-PAGE running buffer: 25mM Tris, 192mM glycine, 0,1% SDS

resolving gel - 10ml	6%	8%	10%
ddH_2O	5,3ml	4,6ml	4,0ml
30% acrylamide mix	2ml	2,7ml	3,3ml
1,5M Tris (pH 8,8)	2,5ml	2,5ml	2,5ml
10% SDS	100µl	100µl	100µl
10% APS	100µl	100µl	100µl
TEMED	8µl	6µl	4µl

stacking gel - 3ml	
ddH_2O	2,1ml
30% acrylamide mix	0,5ml
0,5M Tris (pH 6,8)	380µl
10% SDS	30µl
10% APS	30µ
TEMED	3µl

Immunoblotting

After the run gels were washed 5 minutes with transfer buffer and assembled in immunoblotting sandwich with nitrocellulose membrane Hybond-C Extra (Cat. No. RPN303E, Amersham/GE Healthcare) in Mini Trans-Blot (small gels) or Trans-Blot (big gels) electrophoretic transfer cell (Bio-Rad). Transfer was run either 1h at 350mA, cooled with an ice block and in the 4°C cold room (small gels) or overnight at 30V cooled with heat exchanger set to 4°C (large gels). Quality of transfer was checked by staining transferred proteins on membranes with Ponceau Red.

Transfer buffer: 25mM Tris, 192mM glycine, 20% MetOH

Protein detection

Membranes were blocked in TBST with 5% BSA 30' at room temperature and incubated with primary antibodies diluted in blocking solution overnight at 4°C. Then membranes were washed with TBST 3x 5' and incubated with respective secondary antibody in TBST with 5% non-fat dry milk 1hr at room temperature, and washed again with TBST 3x 5'. Detection was performed by soaking films in enhanced chemiluminescence substrate (Cat. No. 32106, Pierce/Termo) and recording signal with Hyperfilms ECL (Cat. No. 28-9068, Amersham/GE Healthcare).

Primary antibodies					
Antigen	Dilution	MW (kDa)	Source	Company	Cat. No.
p-ERK1/2 (T202/Y204)	1:1000	42/44	rabbit polyclonal	Cell Signaling	9101
ERK1/2	1:1000	42/44	rabbit polyclonal	Cell Signaling	9102
Tubulin	1:20000	55	mouse monoclonal	Sigma	T9026
C-RAF	1:500	74	rabbit polyclonal	Cell Signaling	9422
p-YAP (S127)	1:1000	75	rabbit polyclonal	Cell Signaling	4911
YAP	1:500	75	rabbit polyclonal	Cell Signaling	4912
MST2	1:2000	55	rabbit monoclonal	Epitomics	1943-1
p-LATS1 (S909)	1:500	126	rabbit polyclonal	Cell Signaling	9157
LATS1	1:500	126	rabbit monoclonal	Cell Signaling	3477
Actin	1:2000	43	goat polyclonal	Santa Cruz	SC-1616
p-P38α (T180/Y182)	1:1000	41	rabbit polyclonal	Cell Signaling	9211
P38α	1:1000	41	rabbit polyclonal	Cell Signaling	9212
IκBα	1:1000	35	mouse monoclonal	Cell Signaling	4814

Secondary antibodies		
Antigen	Company	Cat. No.
mouse IgG	Amersham/GE Healthcare	NA931V
rabbit IgG	Amersham/GE Healthcare	NA934V
goat IgG	Santa Cruz	SC-2020

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19/09/2006 - 28/01/2007	Erasmus Project Student in Prof Robert Ford lab, Faculty of Life Sciences of the **University of Manchester**
18/06 - 29/08/2006	FEBS Summer Fellow in Dr Rainer Pepperkok lab, Cell Biology and Biophysics Program of **EMBL Heidelberg**
05/12/2005 - 04/07/2007	Diploma Student in Prof Krzysztof Staron lab, Institute of Biochemistry, Faculty of Biology of the **Warsaw University**
01 - 31/07/2005	Trainee in Dr Michał Dadlez lab, **Institute of Biochemistry and Biophysics of Polish Academy of Sciences in Warsaw**
30/08 - 02/10/2004	Trainee in Prof Krzysztof Staron lab, Institute of Biochemistry, Faculty of Biology of the **Warsaw University**

EDUCATION

11/11/2007 - 30/09/2011	PhD Student in the **Vienna Biocenter International PhD Program**
19/09/2006 - 28/01/2007	Socrates-Erasmus EU program exchange student at the Faculty of Life Sciences of the **University of Manchester**
01/10/2005 - 04/07/2007	MSc program in Biotechnology, speciality: Molecular Biology at Faculty of Biology of the **Warsaw University** (graduated with academic distinction)
01/10/2002 - 19/07/2005	BSc program in Biotechnology, speciality: Molecular Biology, at Faculty of Biology of the **Warsaw University** (graduated with academic distinction)

ADDITIONAL TRAINING

03 - 04/2011	Biostatistics Workshop, **Medical University of Vienna**
31/01 - 04/02/2011	Flow Cytometry Practical Course by Austrian Society for Cytometry at the **General Hospital Vienna**
15 - 16/07/2010	Communication Workshop by Punkt International GmbH at **MFPL Vienna**
6 - 14/09/2008	EMBO Practical Course: Anatomy and Embryology of the Mouse at the **University of Zagreb**
25/06 - 02/07/2006	EMBO Practical Course: Microinjection and Detection of Probes in Living Cells at **EMBL Heidelberg**

COMPETENCES

LABORATORY SKILLS	**Microbiology**: molecular cloning; heterologous protein expression in bacteria;
	Biochemistry: pull-down protein interaction assay; DNA, RNA isolation from cells, tissues; directed mutagenesis; genotyping; RT-PCR;
	Cell biology: cell culture (cell lines, primary); fixed and live-cell imaging with bright-field and fluorescence microscopy (wide field, confocal); FACS; microinjection;
	Animal models: mouse handling and dissection; intraperitoneal, intravenous, intradermal, intrasplenic injections; partial hepatectomy; chemical mouse cancer models; histology; immunohistochemistry; basics of mouse embryology and embryotransfer;
	Bioinformatics: use of web resources for analysis of protein and nucleic acid sequences and genomes (ExPASy, SRS of EBI, GenomeBrowser);
LANGUAGE COMPETENCY	Polish (native), English (fluent), German (very good)
COMPUTER LITERACY	ImageJ, Axiovision, SPSS Statistics, Photoshop, Ilustrator, Corel DRAW, Microsoft Office

PUBLICATIONS	
2011	Maurer G, Tarkowski B, Baccarini M: *"Raf kinases in cancer - role and therapeutic opportunities"*. **Oncogene** (Epub ahead of print). Review
2005	Tarkowski B, Girstun A: *"Application of mass spectrometry for discovery of cancer biomarkers"*. **Kosmos** 54, 331-343. Review. In Polish
MEETINGS	
25 - 28/04/2011	The Biology of Cancer Meeting in **Cold Spring Harbor Laboratory**; poster: *"Interplay between C-Raf kinase and Rassf1a tumour suppressor in liver cancer"*
5 - 19/03/2009	EMBO/FEBS/ISF Workshop: Spatial 2009 - Overcoming Distance in Signaling Networks at **Jerusalem Hills**; poster: *"C-RAF interaction with tumor suppressors in liver carcinogenesis"*
25 - 28/09/2008	FEBS/ESF Workshop: 16th Protein Kinase Meeting - Dynamics of Cell Signal Systems in **Oslo**; poster: *"C-RAF interaction with tumor suppressors in liver carcinogenesis"*
6 - 14/09/2008	EMBO Practical course: Anatomy and Embryology of the Mouse at the **University of Zagreb**; presentation: *"Raf-1 interaction with tumor suppressors in liver carcinogenesis"*
13 - 24/08/2007	International Summer School in Functional Genomics at the **University of Copenhagen**; presentation: *"Interaction of adenoviral protein E4orf4 with RNA-binding protein domain"*
06 - 09/04/2006	3rd International Biology Students Conference at the **University of Latvia** in Riga; presentation: *"Interactions between adenoviral E4orf4 and proteins containing RRMs"*
02 - 05/03/2005	International Natural Sciences Student Conference at Faculty of Natural Sciences of the **Vilnius University**; presentation: *"Role of proteomics in protein biomarker discovery"*
HONOURS, AWARDS	
06/2007	**FEBS Summer Fellowship Prize** for the best summer fellowship report in 2006
10/2006 - 07/2007	**Scholarship of Minister of Science and Higher Education** for scientific achievements
10/2006 - 07/2007	**Award scholarship** from Faculty of Biology of Warsaw University for excellent study results
09/2006 - 01/2007	**Socrates-Erasmus Scholarship** for student exchange at The University of Manchester
18/06 - 29/08/2006	**FEBS Summer Fellowship** for an internship at EMBL Heidelberg
10/2005 - 07/2006	**Scholarship of Minister of Education and Science** for scientific achievements
10/2003 - 07/2004	**Award scholarship** from Faculty of Biology of Warsaw University for excellent study results
ADDITIONAL ACTIVITIES	
11/2008 - 11/2009	Member of Organising Committee of the **Vienna Biocenter PhD Symposium 2009**: *"Android and Eve: Bridging Biology, Medicine and Technology"*
03/2008 - 06/2009	**Representative of PhD students** of Vienna Biocenter PhD Program
since 02/2008	Member of **Austrian Association of Molecular Life Sciences and Biotechnology, FEBS Constituent Society**
03/2006 - 12/2007	Member of **Polish Biochemical Society, FEBS Constituent Society**
07/2005 - 06/2006	Voluntary worker at non-profit organisation **School of Science Festival**
10/2004 - 07/2007	Member of **Student Society of Molecular Biology of the Warsaw University**
INTERESTS	
	molecular biology, travelling, sports, history of 20th century, music, cinematography

Acknowledgements

First of all, I would like to thank Manuela for giving me the opportunity to work in her lab.

Further, I would like to thank all the past and present Baccarini lab members for great working atmosphere, tons of help during work and some of the beers we had after it. In detail, I would like to thank Florian for taking me for my first trips to Viennese IKEA, helping to manage through all the bureaucracy at the University and working at the desk literally arm in arm for first two years of our PhDs, Gabriele for showing how to work on liver and sharing experiments, Theo for introducing me to VBC social life and her enthusiasm for science, Katrin for explaining me things even when she was in the middle of the experiment that could make her whole PhD, Geri for all the tips about histology and all the chats over the drinks, Federica for all her temperament and for providing a bit more drama to everyday lab-life, Reiner for throwing hoops after work and thinking of science the way he does, Ines for sharing the work, discussing results, sharing sweets and cheering me up, Josipa for being so light-hearted and sharing all the gossip and sweets too, half-Italian Matthias for being such a diva and giving us all good laughs about it, Botond for all the vigorous discussions over the beer and ensuring me about my work, Eszter for all the technical tips and her constant good mood even when working until late, Christian for his huge help with a German intro to this thesis and laughing all the time through his infinite pessimism, Andrea for allowing me to invade her bench-space from time to time, Karin for all her assistance and making lab more ordered place, Clemens, Thomas, Isabella, Kuba and Viennese Matthias for all the help with genotyping, Anna, Barbara, Christine, Claudia, Katka, Karina, Veronika, for their help, smile and chats, my first two students Martin and Zeynep for helping with animal dissections, Lisi, Birgit, Pia, Miriana, Uli, Sabine, Angela, and Sabina for the help with all office stuff, and Maria and Fati for all their work in lab kitchen.

I thank the neighbouring labs of Thomas Decker, Pavel Kovarik, Emanuelle Charpentier, Kristin and Florian Raible, Denise Barlow and Heribert Hirt for all their help and chats on the corridors, especially Asia, Ivana, Iwona, Florian, Marton, Nina, Franz, David, Sebastian, Isabella, Orest, Andrea, Karine, Eli, Krzysiek, Ruth, Benjamin, Federica, Martha, Paulina, Stefan, Sarah and Alessandro.

I would also like to thank all the scientists that helped me in conducting my project in Vienna, especially Bettina Grasl-Kraupp, Josef Penninger and Maria Sibilia for all the discussions and suggestions during my PhD committee meetings, Pawel Pasierbek for scanning histology slides what made quantifications so much faster, Stella Tommasi and Gerd Pfeifer for sharing rassf1a knock-out strain, Stratigoula Sakellariou, Vassilis Gorgoulis and Athanassios Kotsinas for performing nodule staging, and Franziska Van Zijl from Wolfgang Mikulits lab for showing hepatocyte isolation.

I would like to thank all the other VBC colleagues that made my 4 years in Vienna so enjoyable with all the activities that were possible thanks to their participation. Especially I would like to thank first two guys I met here, Mircea and Manuel, for introducing me to Sturm on the evening after the PhD selection and exploring all the clubs and bars on the weekends that came later, Tanya for all the sports we did together, Pedro for being so easy-going, Federico for organising so much stuff in the VBC and outside of it, Brooke for all entertainment he provided, and all the others that were so often around: Spyros, Mark, Oksana, Natalia, Justyna, Sophia, Anton, Andrea, Gerald, Leo, Sven, Tomasz, Sofia, Renping, with special thanks to Nico, who showed me that keeping the distance is all what I really need to make it.

Last but not least, I want to thank my family for being always there for me when I needed them, especially my Mom, Krzysiek and my fiancée Magdalena Monika Leonowicz for all her support.