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Mst1 and Mst2 maintain hepatocyte quiescence and suppress the development of hepatocellular carcinoma through inactivation of the Yap1 oncogene

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Abstract

Hippo-Lats-Yorkie signaling regulates tissue overgrowth and tumorigenesis in *Drosophila*. We show that the Mst1 and Mst2 protein kinases, the mammalian Hippo orthologs, are cleaved and constitutively activated in the mouse liver. Combined Mst1/2 deficiency in the liver results in loss of inhibitory Ser127 phosphorylation of the Yorkie ortholog, Yap1, massive overgrowth, and hepatocellular carcinoma (HCC). Reexpression of Mst1 in HCC-derived cell lines promotes Yap1 Ser127 phosphorylation and inactivation, and abrogates their tumorigenicity. Notably, Mst1/2 inactivates Yap1 in liver through an intermediary kinase distinct from Lats1/2. Approximately 30% of human HCCs show low Yap1(Ser127) phosphorylation and a majority exhibit loss of cleaved, activated Mst1. Mst1/2 inhibition of Yap1 is an important pathway for tumor suppression in liver relevant to human HCC.

Significance—The pathways that regulate quiescence and tumor suppression in the liver have not been fully elucidated. We show that the Mst1 and Mst2 kinases are tumor suppressors and regulators of liver size in adults and that negative regulation of the transcriptional coactivator, Yap1, is central to Mst1/2 tumor suppressor function. Loss of both Mst1 and Mst2 is sufficient to initiate hepatocyte proliferation, resulting in dramatic liver overgrowth, resistance to pro-apoptotic

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stimuli, and the development of HCC. Mst1 and Mst2 promote phosphorylation of Yap1 and thereby suppress its oncogenic activity. Mst1/2 regulation of Yap1 is tissue-specific and, in the liver, involves an Mst1/2-regulated Yap1 kinase distinct from Lats1/2. Significantly, the Mst-Yap1 pathway is disrupted in a substantial fraction of human HCCs.

Keywords

Mst1; Mst2; hepatocellular carcinoma; tumor suppressor; Yap1; hippo

Introduction

Mst1 and Mst2 are 56–60kDa class 2 GC kinases that share 76% identity in amino acid sequence (Dan et al., 2001). Mst1/2 are the closest mammalian homologs of the *Drosophila* Hippo kinase. Loss of Hippo function (in the fly eye) results in massive overgrowth, due to an acceleration of cell cycle progression and a failure of developmental apoptosis (Harvey et al., 2003; Udan et al., 2003; Wu et al., 2003); Mst2 can complement Hippo loss of function (Wu et al., 2003). The pathway downstream of Hippo has been extensively characterized (Reddy and Irvine, 2008; Zhao et al., 2008a); Hippo, when bound to the adaptor protein Salvador/Shar-pei, phosphorylates the Lats/Warts kinase. Hippo also phosphorylates the noncatalytic polypeptide, Mats (Mob1 as tumor Suppressor), enabling the latter to promote Lats/Warts autophosphorylation and activation. Active Lats/Warts in turn phosphorylates and inhibits the transcriptional coregulator Yorkie, by promoting its binding to 14-3-3 and nuclear exit (Dong et al., 2007). Yorkie's actions are pro-proliferative and anti-apoptotic, and elimination of Yorkie is epistatic to loss of function of all of the upstream elements named above (Huang et al., 2005). The regulation of Hippo kinase activity is less well defined, although elimination of the atypical cadherin, Fat, or both of the FERM domain proteins, Merlin and Expanded, results in Yorkie-dependent phenotypes resembling Hippo loss of function (Reddy and Irvine, 2008).

Essentially all of these components have mammalian orthologs, and the ability of Mst1/2 to phosphorylate Lats1/2 and Mob1, as well as Lats1 phosphorylation and inhibition of Yap1, the mammalian ortholog of Yorkie, have been observed in vitro and in cell culture (Chan et al., 2005; Dong et al., 2007; Hao et al., 2008; Praskova et al., 2008; Zhao et al., 2007). In addition, cell-cell contact of cultured mammalian cells induces phosphorylation and inactivation of Yap1, whereas overexpression of Yap1 or inactivation of either Lats2 or NF2/Merlin bypasses contact inhibition of growth (McPherson et al., 2004; Morrison et al., 2001; Zhao et al., 2007). Similarly, mouse keratinocytes lacking WW45, the ortholog of Salvador/Shar-pei, fail to activate Mst1, phosphorylate Yap1, and exit the cell cycle during differentiation in vitro (Lee et al., 2008). Consistent with a function as tumor suppressors, inactivating mutations in NF2 and WW45 have been observed in a number of human cancers and Lats1 knockout mice develop soft tissue sarcomas and ovarian cancers (McClatchey and Giovannini, 2005; McPherson et al., 2004; Tapon et al., 2002). Yap1 is amplified in a number of human tumor types and transgenic overexpression of Yap1 in mice leads to liver overgrowth and HCC as well as expansion of progenitor cells in multiple organs (Camargo et al., 2007; Dong et al., 2007; Overholtzer et al., 2006; Zender et al., 2006). Whether Yap1 is regulated by the mammalian Hippo pathway in vivo and whether loss of function of the growth inhibitory components of the pathway results in defects in organ size regulation has not been established.

Recent insight into the physiologic role of Mst1 has come from the phenotype of Mst1 deficient mice, and here a rather different picture emerges from that predicted from the Hippo paradigm (Katagiri et al., 2009; Zhou et al., 2008). Mst1 deficient mice exhibit greatly diminished numbers of mature, naïve T cells in peripheral lymphoid organs

associated with defects in adhesion and migration of the Mst1 null T cells. In addition, Mst1 null naïve T cells overproliferate upon stimulation of the T cell antigen receptor. T cell adhesion requires the chemokine-induced clustering and activation of integrins such as LFA-1; the latter in turn, requires the Rap1- GTP dependent recruitment and activation of Mst1 through its regulatory subunit, Nore1B (also called Rassf5B or RAPL), a Ras/Rap1-GTP binding protein (Avruch et.al., 2009; Katagiri et.al., 2006). The Mst1 substrate(s) mediating LFA-1 clustering/activation and the anti-proliferative effects in naïve T cells are unknown; although Mob1 phosphorylation is abolished in the Mst1 null T cells, Mst1 deficiency causes little or no change in the TCR-regulated phosphorylation of Lats1/2 and Yap1 (Zhou et.al., 2008). Taken together, these findings indicate that Mst1 and Mst2 participate in at least two signal transduction pathways, perhaps in a tissue-specific manner, each involving distinct upstream regulators and at least some different targets. Both pathways however generate anti-proliferative outputs, suggesting that Mst1 and/or Mst2 may serve to suppress tumor initiation. Here, we investigate the functions of Mst1/2 in growth control, tumorigenesis, and Yap1 regulation in vivo using genetically engineered mice.

Results

Mst1 and Mst2 are required for embryonic development and to suppress HCC

We generated mouse strains with germline deletion of Mst1 and Mst2 (Mst1- and Mst2- alleles)(Zhou et al., 2008 and Supplementary Fig. 1); all studies were performed on a mixed (75%–87.5% C57Bl/6) genetic background. Mst1^{-/-} mice were viable and fertile but had a reduced number of mature naïve T cells as reported previously (Zhou et al., 2008); Mst2^{-/-} mice exhibit no developmental or immunological defects and normal fertility. Over a period of 18–24 months, 2/23 Mst1^{-/-} mice developed lethal histiocytic sarcomas and 1/15 Mst2^{-/-} mice developed a mammary tumor, whereas all wildtype mice (N=15) remained healthy.

To evaluate the genetic interaction between Mst1 and Mst2 deficiency we intercrossed the Mst1 and Mst2 knockouts. The Mst1^{+/-}Mst2^{+/-}, Mst1^{-/-}Mst2^{+/-} and Mst1^{+/-}Mst2^{-/-} animals were viable, fertile and showed no developmental anomalies (Supplementary Fig. 2). The Mst1^{-/-}Mst2^{-/-} genotype however resulted in embryonic lethality with first signs of retardation evident at day 8.5 [E8.5] (Fig. 1A). By E9.5 Mst1^{-/-} Mst2^{-/-} embryos were severely compromised (Fig. 1A) and not detected thereafter (Supplementary Fig. 2). Hence, a single functional copy of Mst1 or Mst2 is both necessary and sufficient for normal mouse development.

Necropsy at 3 and 5 months of age did not reveal obvious defects in any of the viable genotypes. However, starting at 7 months of age, Mst1^{-/-}Mst2^{+/-} mice began to show signs of illness, including lethargy and presence of a palpable abdominal mass (the survival curve is shown in Fig. 1B). Mst1^{-/-}Mst2^{+/-} animals showing morbidity (N=13 of 16 animals by age 15 months) were euthanized and necropsies revealed that all mice harbored liver tumors (ranging from 0.5 cm–1.5 cm in greatest diameter; Fig. 1C). Some of these mice also displayed overgrowth (2–4 fold) of the apparently normal liver tissue.

Lethal liver tumors were also observed in 3/12 Mst1^{+/-}Mst2^{-/-} mice by 15 months of age. Histological examination revealed that the liver tumors in the Mst1/2 mutant mice were highly aggressive hepatocellular carcinomas (HCCs)(Fig. 1D). To further document the incidence of HCC, we performed necropsies on 7-month old asymptomatic animals. We observed HCCs in 6/7 Mst1^{-/-}Mst2^{+/-} mice, whereas none of the wild type (N=7) or Mst1^{+/-} Mst2^{-/-} mice (N=6) had visible tumors.

Dual inactivation of Mst1 and Mst2 in HCC

PCR analysis of HCCs from Mst1^{-/-}Mst2^{+/-} mice revealed the absence of wild type Mst2, while adjacent normal liver remained heterozygous for Mst2 (Fig. 1E). Similarly, tumor-specific loss of the wild type Mst1 allele was seen in HCCs from Mst1^{+/-}Mst2^{-/-} mice (Supplementary Fig. 3). Correspondingly, Mst1 and Mst2 proteins were undetectable in all HCCs analyzed, as assessed by western blotting (Fig. 1F, left and data not shown). In comparison, liver from wild type mice showed robust expression of Mst1 and Mst2. Unexpectedly, the majority of Mst1 protein—and a substantial fraction of Mst2—in extracts of normal liver is in the form of 34 kDa amino-terminal (NT) fragment (Fig. 1F, left). Previous studies have shown that truncated 34 kDa form of Mst1 can arise from caspase-dependent cleavage in response to apoptotic (Cheung et al., 2003; Graves et al., 1998; Ura et al., 2001) or cell differentiation (Fernando et al., 2002) signals, and that this form has increased kinase activity and altered substrate specificity (Anand et al., 2008). Notably we observe high levels of Mst1/2 activation in the normal liver (as judged by phosphorylation at the Ser183/180 site; Praskova, et al., 2004) and that the vast majority of the active Mst1/2 kinase is in the form of the 34 kDa fragment (Fig. 1F).

Although the 34Da polypeptide undergoes proteolytic degradation during Mst1 immunoprecipitation (seen as a ladder of polypeptides of faster mobility, Fig. 1F, right), the majority of the of the immunoprecipitated anti-pMst1(Ser183) immunoreactivity is retrieved with the 34kDa polypeptide, confirming its identity as the cleaved, activated form of Mst1. In the spleen, only full length Mst1 and Mst2 are observed (Fig. 1G). These results indicate that Mst1 and Mst2 are constitutively activated in the quiescent adult liver and that homozygous inactivation of both of these kinases in liver cells results in progression to HCC. Hence Mst1 and Mst2 have partially redundant functions and serve as critical HCC tumor suppressors. In addition, it appears that tissue-specific cleavage may be an important mechanism of Mst1/2 regulation in vivo.

Mst1 and Mst2 are critical regulators of quiescence and organ size in the adult liver

To explore in more detail the role of Mst1 and Mst2 in liver homeostasis we sought to generate mice with homozygous deficiency of both of these genes in the liver. To this end, we introduced an Mst2 conditional knockout allele (Mst2^F) onto an Mst1 and Mst2 germline null background (generating Mst1^{-/-}Mst2^{F/F} mice and Mst1^{-/-}Mst2^{F/-} mice). Adenovirus expressing Cre recombinase (Adeno-Cre) was injected into the tail vein of these mice at an age of 6 weeks to induce liver-specific deletion of Mst2; as controls, wild type and Mst1^{-/-}Mst2^{+/-} mice were also injected with Adeno-Cre. We confirmed the ~90% reduction of Mst2 in the livers of Adeno-Cre injected Mst1^{-/-}Mst2^{F/F} or Mst1^{-/-}Mst2^{F/-} mice (designated Mst1/2 null livers) compared to wild type livers (Fig 2A). Remarkably, analysis at serial time points revealed massive overgrowth of the Mst1/2 null livers. As early as 3 days after Adeno-Cre administration, a prominent increase in liver size was noted (data not shown). The Mst1/2 null livers were 2-fold larger than those from Adeno-Cre injected control animals at 8 days and 4-fold larger by 3 months (Fig. 2B and C). Ki-67 staining at day 8 showed that the Mst1/2 null livers had an ~ 4-fold increase in hepatocyte proliferation (Fig. 2D and E). These data establish Mst1 and Mst2 as critical regulators of quiescence and organ size in the adult liver.

The loss of the wild type Mst1 and Mst2 alleles in the spontaneous HCC models described above suggested that complete loss of Mst1/2 signaling in liver cells might be a rate-limiting step in HCC pathogenesis. Correspondingly, Mst1^{-/-}Mst2^{F/F} and Mst1^{-/-}Mst2^{F/-} mice developed lethal liver tumors with a mean latency of 10 weeks after Adeno-Cre injection (Fig. 2F and G). Histological examination revealed the presence of multifocal HCCs against the backdrop of hyperplastic, but untransformed hepatocytes (Fig 2G). To further validate

the Mst1/2 tumor suppressor function in hepatocytes, we crossed the Mst1/2 mutants with the Albumin-Cre transgenic strain that specifically targets hepatocytes. Whereas control mice showed no abnormalities, we found that 4/4 Albumin-Cre; Mst1^{-/-}Mst2 F/F mice developed massively overgrown livers and HCC by 3 months of age (Supplementary Fig. 4). The tumor-specific loss of heterozygosity in the spontaneous HCC models and the accelerated tumor development associated with homozygous inactivation of both Mst1 and Mst2 in hepatocytes together suggest that Mst1/2 suppress HCC at least in part through cell autonomous mechanisms.

Regulation of proliferative signaling pathways by Mst1/2 in the liver

To begin to address the downstream mechanisms associated with loss of hepatocyte quiescence following Mst1/2 inactivation, we profiled the expression of established cell cycle regulators in wild type and Mst1/2 null livers. Cyclin D1 expression was decreased and cyclin D2 levels were unchanged in the Mst1/2 null liver—as assessed by both immunoblot analysis and qRT-PCR—whereas there was an immediate upregulation of cyclin D3 and cyclin E, and a sharp decline in p21 levels (Fig 2H and data not shown). These expression alterations were also observed a series of 10 HCCs (Fig. 2I). Hence Mst1/2 inactivation, and the ensuing entry of hepatocytes into the cell cycle, is associated with an immediate and sustained alteration in regulators of the G1 checkpoint. We also analyzed the expression of a series of known regulators of hepatocyte proliferation and HCC pathogenesis. c-Myc protein and mRNA were modestly induced following acute Mst1/2 inactivation, whereas c-Myc levels were greatly elevated in all HCCs analyzed (Fig. 2H and I, and data not shown). The level of p70 S6 kinase phosphorylated at Thr389/412, a site phosphorylated directly by mTOR complex 1, was markedly elevated by acute inactivation of Mst1/2 and persisted at a high level in nearly all HCCs (Fig. 2I). In contrast, Akt(Ser473) phosphorylation, which has been proposed to be negatively regulated by Mst1/2 (Cinar et al., 2007), was not consistently altered by Mst1/2 inactivation or in the HCCs (Supplementary Fig. 5). Erk1/2 polypeptide levels were modestly increased after Mst1/2 inactivation, however, fractional Erk1/2 activation was not altered, and in the HCCs, Erk1/2 levels and activation were similar to wildtype liver (Supplementary Fig. 5). Thus inactivation of Mst1/2 gives selective activation of mTOR complex1 without apparent alteration of mTOR complex2 or in the Ras-MAPK pathway. Levels of activated β -catenin (i.e. unphosphorylated at Ser37 and/or Thr41) were unchanged by Mst1/2 inactivation but elevated in a subset of HCC. Finally, p53 expression levels were unchanged (Fig. 2I) whereas MDM2 overexpression was noted in 2/10 tumors (data not shown).

Mst1/2-independent regulation of Yap1 in MEFs

Previous studies in vitro studies have shown that cellular contact induces Ser127 phosphorylation and cytoplasmic retention of Yap1 and that this regulatory pathway is important for contact inhibition of proliferation (Zhao et al., 2007). The prevailing view has been that the core Hippo pathway components are required to regulate Yap1 phosphorylation and activity in mammalian cells (Dong et al., 2007; Zhao et al., 2008a). However, the specific requirement of individual pathway components in normal tissues has not been tested (apart from T cells, Zhou et al., 2008). Moreover, whether the pathway of Yap1 regulation is universal or has context-dependent circuitry has not been established. First, we sought to investigate the role of Mst1/2 in Yap1 regulation using immortalized mouse embryonic fibroblasts (MEFs). Consistent with published results we found that increasing cell density induced Yap1(Ser127) phosphorylation in wild type MEFs (Fig. 3A). Unexpectedly, basal and cell contact-induced Yap1(Ser127) phosphorylation were unimpaired in Mst1^{-/-}Mst2^{-/-} MEFs (generated by infection of MEFs from Mst1^{-/-}–Mst2F^{-/-} mice with a retrovirus expressing Cre recombinase) although these cells lost Mob1(Thr12) phosphorylation (Fig. 3A, C). Notably, in wild type MEFs, Mst1 and Mst2 are

present exclusively as full length polypeptides lacking detectable active site phosphorylation and confluence is not accompanied by detectable change in the phosphorylation of Mst1/2 or of Mob1(Thr12) (Fig. 3B). As with Yap1(Ser127) phosphorylation, Lats1/2 activation loop (AL) phosphorylation was induced at high cell density regardless of Mst1/2 status (Fig. 3C). Identical results were observed in primary (non-immortalized) MEFs except that confluence induced cleaved Lats1/2 carboxyterminal (CT) phosphorylation in the primary MEFs (Supplementary Fig. 6). In parallel with the contact-induced phosphorylation of Yap1(Ser127), both wild type and Mst1^{-/-}Mst2^{-/-} MEFs showed cytoplasmic translocation of Yap1 when grown to high cell density (Fig. 3D). Correspondingly, the Mst1^{-/-}Mst2^{-/-} MEFs exhibited normal contact inhibition of proliferation (data not shown). The present unexpected results demonstrate that in MEFs, Mst1/2 are not the upstream regulators of Lats1/2 and Yap1 in response to cell-cell contact.

Mst1/2 inactivation in the liver results in deregulation of Mob1 and Yap1 but not Lats1/2

Our results in MEFs indicate that either Mst1/2 do not have a conserved function in regulating Lats/Yap1 or that the pathway upstream of Yap1(Ser127) phosphorylation is operative in a tissue- or context-specific manner. The Adeno-Cre system provided an opportunity to study the early signaling events accompanying acute ablation of Mst1/2 in the liver. We found that the Mst1/2 null livers had sharp decreases in pMob1(Thr12) and pYap1(Ser127) at the 3 and 8 day time points as well as in the ensuing HCCs (Fig 4A). Levels of total Yap1 polypeptide were increased following Mst1/2 inactivation (Fig. 4A) whereas Yap1 mRNA levels were unchanged (data not shown) suggesting that the unphosphorylated form of Yap1 may be stabilized. Unexpectedly, phosphorylation of Lats1/2 at the carboxyterminal and activation loop was minimally altered in the Mst1/2 null livers and was at comparable levels in HCC and normal liver. Ndr1 and Ndr2 are the most closely related kinases to Lats1/2 and can also be activated by Mst1/2-mediated carboxyterminal phosphorylation (Vichalkovski et.al., 2008). Mst1/2 inactivation resulted in a modest decrease in Ndr1/2 carboxyterminal and activation loop phosphorylation (Fig. 4A). These patterns were also observed in livers and HCC from Albumin-Cre; Mst1^{-/-}Mst2^{F/F} mice indicating that such changes were not a secondary consequence of the Adeno-Cre infection (Fig. 4B).

Consistent with the role of Yap1(Ser127) phosphorylation in cytoplasmic retention of Yap1, cell fractionation experiments revealed that Yap1 is almost entirely cytoplasmic in wild type liver but present at high abundance in the nuclei of Mst1/2 null liver (Fig. 4C upper panel) and almost entirely nuclear in HCC (Fig. 4C lower panel). Immunohistochemical analysis confirmed the overexpression and nuclear translocation of Yap1 in HCC (Fig. 4D). Moreover, the expression of the candidate Yap1 target genes, CTGF and AFP (Dong et al., 2007; Zhao et al., 2008b), was significantly elevated in the Mst1/2 null liver and in HCC (Fig. 4E). Together, these data demonstrate that Mst1 and Mst2 are critical negative regulators of Yap1 activity in hepatocytes in vivo and moreover suggest that a kinase other than Lats1/2 may be responsible for Yap1 phosphorylation downstream of Mst1/2. Inasmuch as Yap1 overexpression has been shown to promote liver overgrowth and HCC, it is likely that the deregulation of Yap1 activity contributes to these phenotypes in the Mst1/2 mutant mice.

To gain further insight into the identity of the hepatic Yap1 kinase, we fractionated mouse liver extracts by anion exchange chromatography. The Yap1(Ser127) kinase activity of each column fraction was assayed against prokaryotic recombinant Yap1, using the phosphospecific anti-Yap1(Ser127-P) antibody to detect kinase activity (Fig. 4F). In addition to the elution of endogenous hepatic Yap1(Ser127-P), two peaks of Yap1(Ser127) kinase activity were observed in chromatograms from wild type mouse liver (arrow; fractions 13–15 and 20–23). Note that the later-eluting of these peaks of Yap1 kinase

activity co-migrates with Lats1, whereas neither peak co-elutes with Lats2. We next compared the chromatograms of wild type and Mst1^{-/-}Mst2F^{-/-} liver extracts prepared 8 days after injection of Adeno-Cre (Fig. 4G). We found that the earlier-eluting peak of Yap1(Ser127) kinase was entirely absent from the Mst1/2 null liver, whereas the later-eluting peak of Yap1(Ser127) kinase, which co-elutes with Lats1 (Fig. 4F) was unaltered. Hence, the liver contains an Mst1/2-dependent Yap1(Ser127) kinase that entirely distinct from Lats1 and Lats2. Furthermore, consistent with the insignificant changes in Lats1/2 phosphorylation (Fig. 4A, B), Lats1-catalyzed Yap1(Ser127) kinase activity is largely unaffected by Mst1/2 inactivation (Fig. 4G).

Mst1/2 deficiency in the liver leads to resistance to Fas-induced apoptosis

Deregulation of the Hippo pathway in *Drosophila* and Yap1 overexpression in mice results in both hyperproliferative and anti-apoptotic phenotypes (Zhao et al., 2008a). To test whether Mst1/2 deficiency affected hepatocyte apoptosis in addition to proliferation, we injected mutant mice and controls with anti-Fas antibody, Jo-2, 8 days after Adeno-Cre administration. Under these conditions, wild type livers showed extensive hepatocyte apoptosis, whereas Mst1/2 null livers were strongly protected from cell death as determined by histological examination as well as by TUNEL staining and western blotting for cleaved caspase-3 (Fig. 5A–C).

Yap1 is required for proliferation of Mst1/2 mutant HCC cell lines

Based on the activation of Yap1 in mice with Mst1/2 inactivation in the liver and the similar liver phenotypes of these mice to those with transgenic hepatic overexpression of Yap1, we sought to assess whether Yap1 is critical for the tumorigenicity of Mst1/2 mutant HCC. To this end, we first tested whether Yap1 is under Mst1 regulation in an HCC cell line derived from the mouse model (designated HCC-1). We introduced lentiviruses expressing either GFP alone or in combination with Mst1 into HCC-1 cells; infection rates were reproducibly 40–60% as reflected by microscopy for GFP. Western blot analysis—of both unsorted cells or following GFP FACS—showed that Mst1 restored detectable Mob1 phosphorylation and induced pronounced phosphorylation of Yap1(Ser127)(Fig. 6A). Lats1/2 phosphorylation was unaffected (Fig. 6A, B), a finding in line with our earlier observation that acute inactivation of hepatic Mst1/2 has little impact on Lats1/2 carboxy-terminal phosphorylation in vivo (Fig. 4A). Hence Mst1/2 appear to regulate Yap1 in a Lats1/2-independent manner in hepatocytes and in these HCC cells. Notably, Lats1/2 is probably capable of phosphorylating Yap1(Ser127) in HCC1 cells inasmuch as H₂O₂—which strongly activated Lats1/2 in the Mst1/2 null HCC cells—induced a modest increase in Yap1(Ser127) phosphorylation (Fig. 6B), presumably attributable in part to Lats1/2. The presence of Mst1, which was also activated by H₂O₂ (as demonstrated by the increased Mob1 phosphorylation), did not alter the extent of basal Lats1/2 activation, but did cause further phosphorylation of Yap1 (Fig. 6B). As expected, the increased Yap1 phosphorylation in the Mst1-expressing HCC-1 cells resulted in decreased Yap1 nuclear residence as reflected by cell fractionation studies (Fig. 6C). BrdU-DAPI labeling studies showed that Mst1 increased the proportion of HCC-1 cells in the G1 cell cycle phase from 23% to 43% (Fig. 6D). Mst1 restoration also led to a pronounced increase in the proportion of early apoptotic cells (13.97% versus 2.37%) and late apoptotic cells (5.78% versus 0.68%)(Fig. 6E) and induction of caspase-3 cleavage (Fig 6F). These cells also showed decreases in expression of c-Myc expression, and cyclins D3 and E (Fig. 6F), and a marked reduction cell proliferation, colony formation, and ability to grow in soft agar (Fig. 6G and data not shown). Hence Mst1 expression inactivates Yap1 and reverts the tumorigenic growth of HCC-1 cells.

To directly test whether Yap1 is required for tumor maintenance of Mst1/2 null HCC, we introduced lentiviruses expressing Yap1 shRNAs into HCC-1 cells. We find that Yap1

knockdown induced caspase 3 cleavage and a complete loss of c-Myc polypeptide as well as decreased expression of cyclin D3 and cyclin E (Fig. 7A). Yap1 shRNA expressing cells show a pronounced alteration in cell cycle distribution (Fig. 7B); cells in G1 increase from 28.6% to 44.8% whereas cells in S phase decrease from 56.8% to 36.55%. Yap1 depletion resulted in a high level of apoptosis (Fig. 7C; 28.47 versus 1.62% early apoptosis; 4.44% versus 1.02% late apoptosis). The Yap1-deficient cells are unable to form colonies at low density, to sustain proliferation or to grow in soft agar (Fig. 7D–F). These data indicate that Yap1 is necessary to both sustain proliferation and suppress apoptosis in Mst1/2 null HCC. Thus Mst1/2-dependent phosphorylation and inactivation of Yap1 is critical for tumor suppression in the liver.

pYap1, pMob1 and the cleaved, active Mst1 catalytic fragment are frequently lost in human HCCs

Previous studies have reported that Mst1/2 activity and expression of the candidate Mst1/2 regulators, Rassf1A and Nore1b, are reduced in most human HCCs compared to normal liver (Calvisi et al., 2006, Macheiner et al. 2009). Moreover, whereas Yap1 is exclusively cytoplasmic in the normal liver, it has been reported that ~50% of human HCC specimens exhibit nuclear staining for Yap1 (Dong et al., 2007; Zhao et al., 2007). Based on these data and our observation that loss of Yap1(Ser127) phosphorylation due to Mst1/2 inactivation results in nuclear translocation of Yap1 in the mouse liver, we sought to address whether p-Yap1(Ser127) is lost in human HCC and therefore may contribute to its pathogenesis. To this end, we analyzed expression of Mst1/2-Yap1 signaling components in a series of matched HCC and normal liver specimens by immunoblotting (Fig. 8). As in the mouse liver, all normal human liver specimens exhibited robust expression of total Yap1 and p-Yap1(Ser127). The Mob1 polypeptide immunoblot visualizes two closely spaced bands with the upper predominant (Fig. 8); on p-Mob1 immunoblot, the lower band is usually the predominant signal. Mst1 and Mst2 are readily visualized by immunoblot and in 19/21 normal livers and a substantial fraction of Mst1 is present as a cleaved 34kDa polypeptide (Fig. 8), corresponding to the pattern seen in normal mouse liver. These patterns are significantly altered in the majority of HCCs. First, Yap1(Ser127) phosphorylation is markedly reduced or absent in 7/21 HCCs (2,3,5,10,11,13,15); moreover, in four HCCs (4,6,12,15) the level of total Yap1 polypeptide is substantially elevated. Thus, in nearly half of these unselected HCCs, Yap1 is underphosphorylated and/or overexpressed, both circumstances likely to promote Yap1 nuclear localization as previously reported. In all but 6 of the HCCs (1,6,7,13,14,21), the level of p-Mob1 is markedly reduced as compared to the normal liver. Inasmuch as Mob1 is a highly specific Mst1/2 substrate, this argues for a marked reduction in Mst1/2 kinase activity (and/or increase in Mob1- phosphatase activity) in these HCCs. Expression of the Mst1 and Mst2 56–60kDa polypeptides is well preserved in the HCCs (except in 7 and 21), however in 13/21 HCCs, the 34kDa Mst1 polypeptide is absent despite its ready visualization in the paired normal liver. We have not assayed directly Mst1/2 kinase in these samples and immunoblot of these lysates with anti-Mst1/2(p-Ser183/180) exhibits numerous nonspecific bands near 55–60 and 34kDa, precluding this estimate of Mst1/2 kinase activity. Nevertheless, the lack of the 35kDa constitutively active catalytic fragment, which is the predominant active form of Mst1/2 in normal mouse liver, may contribute to a lowered Mst1/2 activity in these HCCs. It is notable that all but one (13) of the seven HCCs with diminished p-Yap1 exhibit diminished pMob1 and 5/7 lack expression of the Mst1 34kDa fragment (3,5,11,13,15). Hence, loss of negative regulation of Yap1, most often due to diminished Mst1/2 activity, is a common occurrence and likely pathogenetic factor in human HCC.

Discussion

We have demonstrated that Mst1 and Mst2 act in a redundant manner to maintain quiescence in the adult liver and that their dual inactivation leads to immediate Yap1 activation, liver overgrowth, resistance to Fas-induced apoptosis and rapid HCC development. We provide evidence that Yap1 nuclear residence and activation, resulting from loss of Ser127 phosphorylation, is critical for HCC development and maintenance in the setting of Mst1/2 deficiency. In addition, defects in Mst1/2-Yap signaling are present in a subset of human HCCs

The ability of Mst1/2 to regulate Yap1(Ser127) phosphorylation is anticipated by the “Hippo” paradigm. The present data indicate however that in the liver Mst1/2 controls Yap1 phosphorylation through a kinase other than Lats1/2. This and other recent work in Mst1/2 deficient mice illustrate two noteworthy features of Mst1/2 signaling downstream. First, in all cellular contexts examined thus far, loss of Mst1 (Zhou et al., 2008), or Mst1 plus Mst2, results in the loss of Mob1(Thr12/Thr35) phosphorylation. Thus Mob1 phosphorylation, which has been shown to mediate, in part, Mst1/2 inhibition of cell cycle progression (Praskova et al., 2008), is a reliable and apparently specific indicator of Mst1/2 activity in vivo. Second, it is apparent that Mst1/2 signaling and Yap1 phosphorylation exhibit an unexpected diversity, in addition to the relationships predicted by the *Drosophila* Hippo-Lats-Yorkie tumor suppressor pathway. Thus in mouse liver Mst1/2 signal through an unknown intermediary kinase(s) to control Yap1(Ser127) phosphorylation. In the mouse T cell Mst1 activated by anti-CD3 signals through Mob1 and unknown effectors (other than Yap1) to activate integrin clustering/activation and to inhibit naïve T cell proliferation (Zhou et al., 2008). MEFs present yet another arrangement; MEFs achieving confluence exhibit activation of Lats1/2, Yap1 phosphorylation and egress of Yap1 from the nucleus, however, these events are unaltered despite the deletion of Mst1 and Mst2 and the loss of Mob1 phosphorylation. Therefore, it is clear that Mst1/2-Lats1/2-Yap1 signaling varies according to cell type and cell context; there exist Lats1/2 activators other than Mst1/2 and Yap1(Ser127) kinases other than Lats1/2. It is also apparent that important Mst1/2 substrates apart from Mob1, Lats1/2 and Ndr1/2 (Vichalkovski et al., 2008) remain to be discovered.

Previous reports have shown that Yap1 overexpression in mice promotes the development of HCC (Dong et al., 2007; Zender et al., 2006) and that increased nuclear abundance of Yap1 is present in about 50% of human HCCs (Dong et al., 2007; Zhao et al., 2007). The mechanisms of Yap1 deregulation in human HCCs were not defined in these studies. Although amplification of the Yap1 locus may lead to Yap1 overexpression in some HCCs, such amplification appears to be relatively uncommon, restricted to ~5–10% of tumors (Zender et al., 2006). On the other hand, the present work points toward loss of Yap1(Ser127) phosphorylation as being an important mechanism for Yap1 deregulation in human HCC, as we have observed this alteration in ~30% of HCC specimens (Fig. 8). Significantly, we observed a close correlation between loss of pYap1(Ser127) and loss of pMob1(Thr12) in the human HCC specimens indicating that low Mst1/2 activity is likely responsible for most instances of low Yap1(Ser127) phosphorylation. Further studies will be required to demonstrate directly the functional contributions of Mst-Yap1 signaling defects to human HCC pathogenesis.

Although our work suggests that Yap1 is a major mediator of phenotypes downstream of Mst1/2 in the liver we cannot rule out the possibility that there are other critical effectors of the pathway that contribute to these phenotypes. In this regard, it is notable that the transcriptional coactivator Taz—a potential oncogene (Chan et al., 2008; Lei et al., 2008)—shares 45% homology to Yap1 throughout its sequence, and in vitro studies suggest that these proteins can be regulated through comparable mechanisms (i.e. by Lats-mediated

phosphorylation of a conserved motif resulting in cytoplasmic sequestration) (Lei et al., 2008). We find that Taz phosphorylation at this site is reduced following Mst1/2 inactivation in the liver (Supplementary Fig. 4A) suggesting that Taz is negatively regulated by Mst1/2. However, levels of Taz polypeptide are also decreased in the Mst1/2 liver and in HCC, indicating that Taz is unlikely to play a positive role in overproliferation or tumorigenesis in this setting.

Our data show that Mst1/2 are constitutively active in the quiescent liver and that they mediate sustained phosphorylation of Mob1 and Yap1. A key outstanding question is the nature of the upstream activating signals in this pathway. The importance of this question is emphasized by the finding that despite the frequent loss of pMob and pYap1 in human HCCs, the expression of the full length Mst1 and Mst2 polypeptides is maintained in nearly all these tumors. Consequently, it is likely that diminished upstream inputs are the basis for deficient Mst1/2 activity in these tumors. Three apparently independent mechanisms for upstream regulation of the Mst1/2 kinases have been identified thus far. In the *Drosophila* Hippo pathway, the activity of the Mst1/2 ortholog, Hippo, is controlled, at least in part, by cell-cell contact, mediated by the atypical cadherin, Fat, and the membrane-associated FERM-domain polypeptides, Expanded and Merlin (Reddy and Irvine, 2008). In mammals, NF2/merlin has been reported to regulate Yap1 cytoplasmic retention in neural tumor-derived cell lines (Zhao et al., 2007). Similarly, overexpression of FRMD6, the closest mammalian homolog of Expanded, can repress the activity of a Yap1 transcriptional reporter (Zhao et al., 2007). However, the physiological significance of this pathway and its role in the regulation of Mst1/2 in liver has not been defined. A second mechanism for Mst1/2 activation involves ligand-mediated receptor signaling and the Rassf1/Nore proteins that are found in a constitutive complex with Mst1/2 in cell lines that express these polypeptides (Avruch et al., 2009). In T cells Mst1 is activated by stimulation of antigen or chemokine receptors, which induce association of the Nore1B/Mst1 complex with activated Ras-like GTPases, specifically Rap1 and Rap2. The Nore1B/Mst1 complex is required in T cells for Rap1- GTP induced integrin clustering and in naïve T cells for restraint of proliferation. Hippo pathway components (e.g. a cadherin, ERM proteins or WW45) are not involved in the GTPase regulation of the Nore1/Mst1 complex. Inasmuch as Rassf1A, Nore1A and Nore1B have been shown to be epigenetically inactivated in wide range of human tumors including HCC (Calvisi et al., 2006; Donninger et al., 2007; Macheiner et al., 2009) it is plausible that their diminished expression might result in defective regulation of Mst1/2 signaling.

A third mechanism of Mst1/2 regulation, probably operational in the liver, appears to be at the level of caspase-dependent cleavage. Previous studies have shown that Mst1 and Mst2 exist as 56 kDa full-length polypeptides or as truncated 34–36 kDa amino-terminal forms that arise due to caspase-dependent cleavage during apoptosis (Graves et al., 1998; Lee et al., 2001). We find that the majority of Mst1 in normal mouse liver exists as a 35–40 kDa polypeptide, and a significant fraction of Mst2 is also found in a truncated form.

Moreover, the vast majority of the activated Mst1/2 kinase in liver (i.e., forms phosphorylated at the activation loop) are the short Mst1/2 polypeptides whereas spleen and MEFs exhibit only the presence of the full length forms. Hence the cleavage of Mst1/2 appears to be a regulated, tissue specific process. The truncated forms have lost their autoinhibitory and SARAH domains, are thus highly active and released from upstream regulation by the WW45 or Rassf family polypeptides. The truncated Mst1/2 polypeptides are preferentially localized to the nucleus and have an altered substrate specificity (Anand et al., 2008; Ura et al., 2001), however their potency in regulation of Yap1 as compared to the full length Mst1/2 polypeptides remains to be defined. The proportion of Mst1 exhibiting cleavage in the liver is much higher than that of Mst2. In addition, Mst1^{-/-} livers exhibit a

significant reduction in pYap1 and pMob1 compared to wild type livers, whereas there is only a modest decrease in pYap1 in pMob1 in Mst2^{-/-} livers (Supplementary Fig. 7). Hence, while both Mst1 and Mst2 are required for complete loss of Yap1 phosphorylation in the liver, Mst1 plays the more important role. Caspase-3 dependent cleavage of Mst1 is required for myoblast differentiation in vitro, in the absence of any detectable apoptosis (Fernando et.al., 2002, Murray et.al., 2008). This observation is in keeping with emerging data from embryonic stem cells and hematopoietic stem cells showing that caspase-3 activation may generally contribute to cellular differentiation through proteolysis of multiple targets (Yi and Yuan, 2008). Thus we surmise that the truncated, constitutively activated form of Mst1/2 may play an important role in maintenance of hepatocytes in a differentiated, nonproliferative state.

Our data also demonstrate the importance of endogenous Mst1/2 in apoptotic regulation in an intact mammalian organism, consistent with the loss of developmental apoptosis seen with *Drosophila Hippo* loss of function (Fig. 5). As to the mechanism Mst1/2 dependent apoptosis, the Jo-2 anti-Fas antibody does cause a small increase in Mob1 phosphorylation indicative of Mst1/2 activation, but surprisingly is accompanied by a reduction in Yap1 phosphorylation (Supplementary Fig. 8). Thus, whether and to what extent the modest activation of Mst1/2 by the Fas agonist contributes to the rapid activation of apoptosis by Fas is unclear. The protective effect of Mst1/2 ablation against Fas-induced apoptosis seems more likely to reflect enhanced expression of Yap1- dependent transcriptional outputs that confer resistance to apoptosis (Zhao et.al., 2008).

HCC is the fifth most common malignant neoplasm worldwide and third most frequent cause of cancer-related death (Llovet et al., 2003). Only a minority of patients are eligible for potentially curative surgery and conventional chemotherapeutic approaches are ineffective in this disease (Villanueva et al., 2008). While molecularly targeted agents have increased survival in patients with advanced HCC, only a subset of patients show responses and these agents do not achieve cures. It is clear that a better definition of the molecular pathogenesis of HCC and the molecular classification of subsets of tumors are important steps in the design of improved therapeutic strategies. Our work shows that loss of Yap1 phosphorylation specifies a subset of human HCC. In addition, we have observed that as in the murine HCCs, Yap1 knockdown in some human HCC cell lines blocks colony formation in vitro and promotes apoptosis (not shown). Hence elucidating the critical Yap1 target genes may point to effective therapeutic targets for a subset of HCC. In future studies, it will also be important to define whether loss of Yap1 phosphorylation is associated with other well-defined molecular alterations in HCC and to determine what is the prognostic significance of this subgroup of tumors.

Experimental Procedures

Animals

The generation of the Mst2^F and Mst2⁻ alleles and the analysis of the Mst1^{-/-}Mst2^{-/-} embryos is described in detail in the Supplementary Materials. All mice used in this study were maintained on a mixed genetic background (C57BL/6; 129/Sv). Mouse work was done with IACUC approval and in strict accord with good animal practice as defined by the Office of Laboratory Animal Welfare.

Materials and Reagents—Antibodies against Mst1, Mst2, pMst1 (Thr183P)/pMst2 (Thr180P), Lats1, pLats CT, pLats AL, Mob1 and pMob1(Thr12) were described previously (Praskova et.al., 2008). All other antibodies and assay reagents were obtained from commercial sources as described in the Supplementary Materials. Ad5CMVCre (Ad-Cre) virus was purchased from the Gene Transfer Vector Core at the University of Iowa.

Cell culture and molecular methods—Methods for cell culture, adeno- and lentiviral-mediated gene transfer, subcellular fractionation, immunoblotting, immuno-cyto- and -histochemistry, assays for cell proliferation, apoptosis and mRNA abundance are described in the supplementary materials.

Hepatic Yap1(Ser127) kinase activities—Fresh mouse livers were homogenized in Tris-HCl pH 7.6, 20 mM, Sucrose 0.3M, EDTA 1mM, EGTA 5 mM, DTT 1 mM, sodium pyrophosphate 2.5mM, beta-Glycerophosphate 1mM, containing a proteinase inhibitors tablet, Roche®. The supernatant of a 100,000g, 2 hours centrifugation was applied to a HiTrapHP Q column (GE Healthcare) equilibrated in Tris-HCl pH 7.6, 20 mM, EDTA 1mM, NaF 1mM. Elution was carried out with 4 column volumes of an NaCl gradient from 0.1 M to 0.6 M. Forty fractions were collected and an aliquot of each was assayed for Yap1(Ser127) kinase activity by incubation with prokaryotic recombinant GST-Yap, Mg 10mM, and ATP 100 uM for 30 min. at 30C. The kinase reactions were stopped by adding SDS sample buffer and subjected to SDS-PAGE and immunoblot as indicated.

Human liver and HCC samples—Human samples were obtained under informed consent from the Foundation for Human Tissue and Cell Research (HTCR) Regensburg, Germany. Experiments were performed under HTCR and MGH IRB approval and are considered exempt. Biopsies from specimens of normal liver tissue (distant from the tumor) and HCC were collected snap frozen. Patients receiving hepatotoxic medication, chemotherapy or patients with systemic disease known to affect the liver were excluded. Diagnosis of HCC and normal liver was confirmed based on histological findings by independent pathologists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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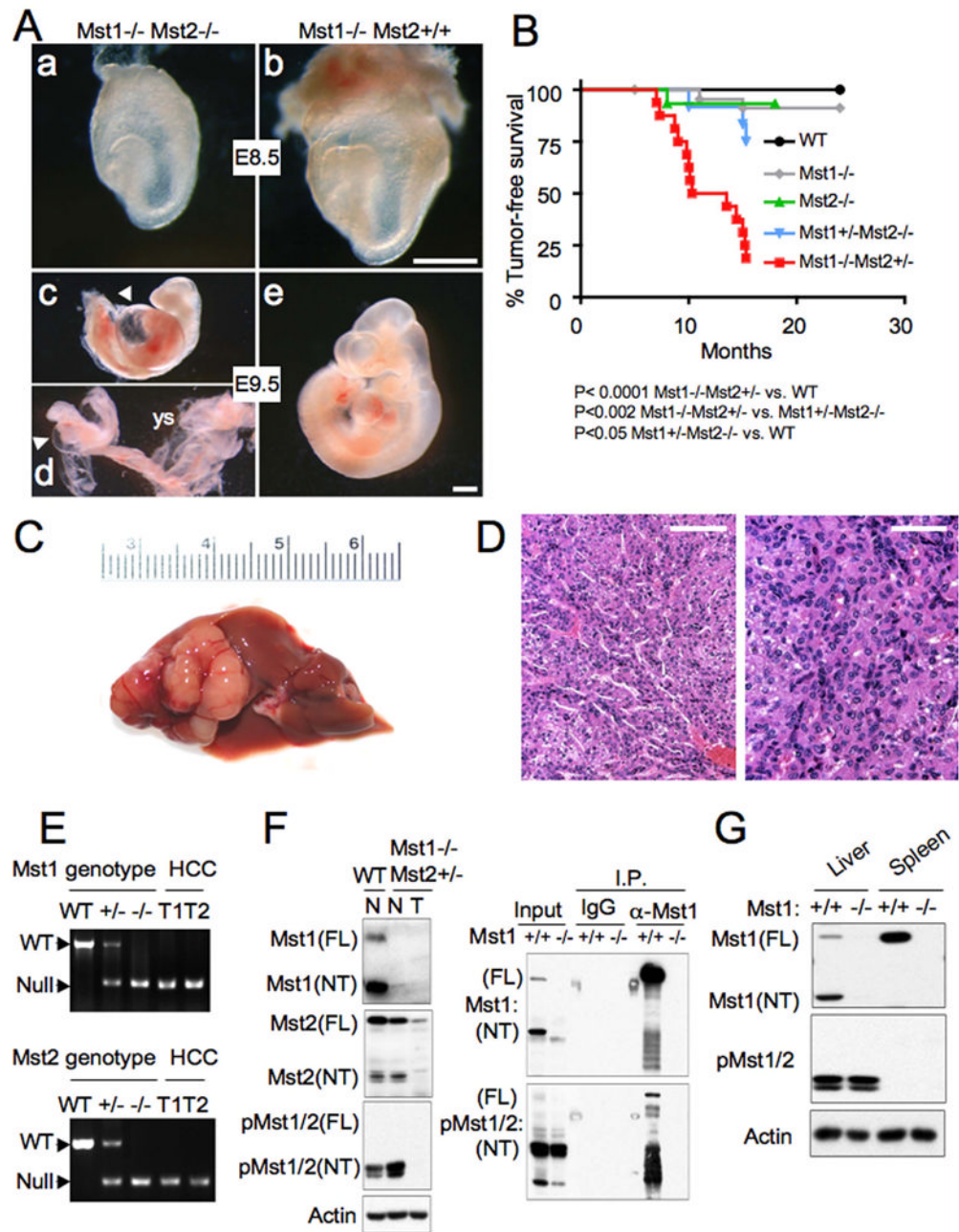
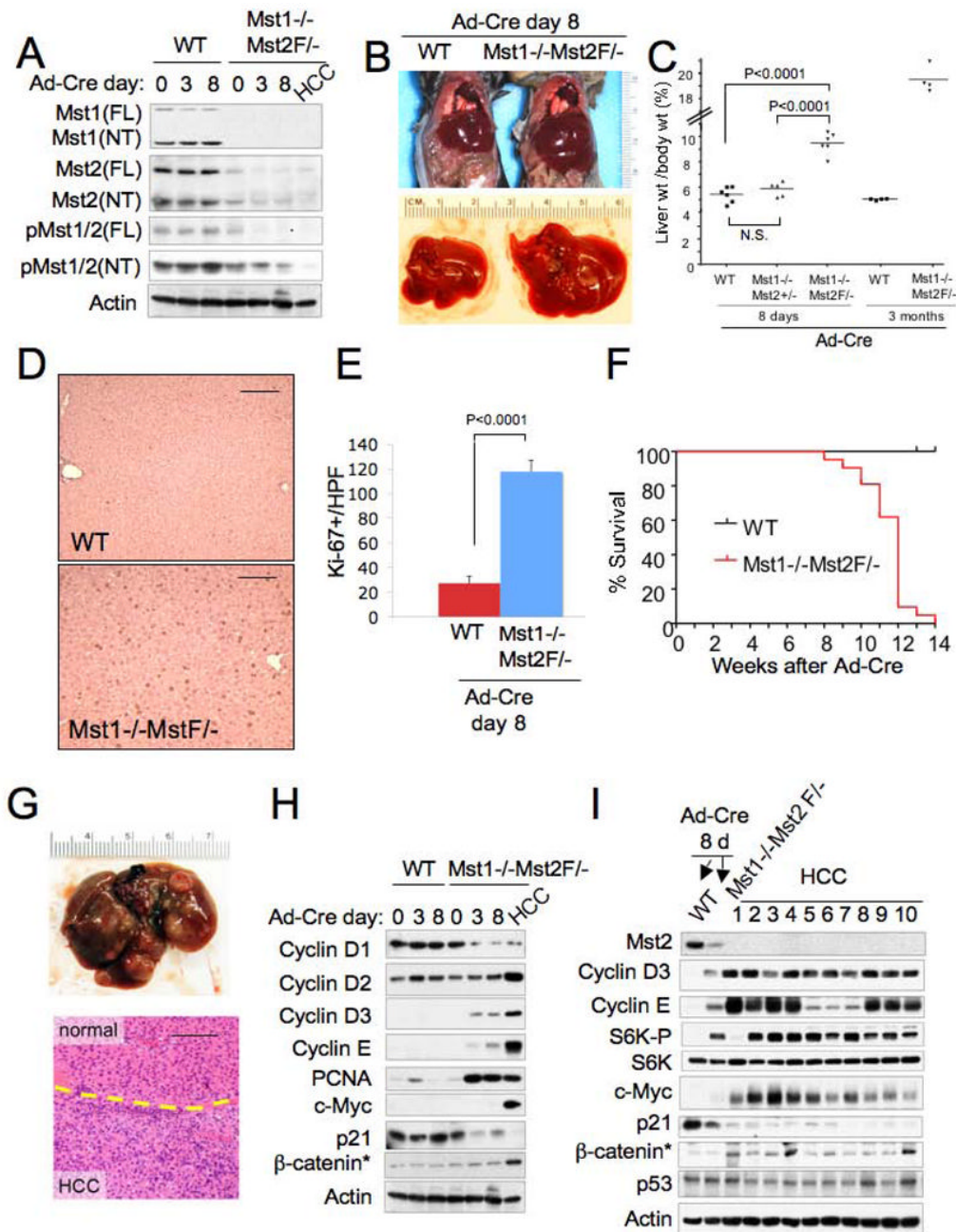


Figure 1. Phenotypes of $Mst1/2$ mutant mice. A) E8.5 $Mst1^{-/-} Mst2^{-/-}$ embryos (a) are smaller than littermate controls with one or more functional $Mst1$ or $Mst2$ alleles (b). By E9.5 $Mst1^{-/-} Mst2^{-/-}$ embryos (c, d) show various degrees of developmental abnormalities [compare with (e)] including stunted growth, failure to turn and close the neural tube (d). Also, embryos and yolk sacs (ys) appear pale and undervascularized and abnormally large pericardial cavities are observed (arrowheads in c, d). Scale bars 500 μ m. (B) Tumor-free survival curve of mice of the indicated genotypes. All deaths in the $Mst1^{-/-} Mst2^{+/-}$ and $Mst1^{+/-} Mst2^{-/-}$ cohorts were due to HCC. Statistical significance by the log-rank test is indicated. (C) Gross appearance of HCC arising in an $Mst1^{-/-} Mst2^{+/-}$ mouse. (D) H&E stained sections of murine HCC. Scale bars: left panel 500 μ m, right panel 125 μ m. (E) PCR

analysis of the wild type and null Mst1 (upper) and Mst2 (lower) alleles in normal liver tissue from the indicated genotypes and of HCC from Mst1^{-/-} Mst2^{+/-} mice (T1 and T2). Note that the HCCs lack the wild type Mst1 and Mst2 alleles. (F) Left: Western blots of liver from a wild type mouse (N) and the normal liver from an Mst1^{-/-}Mst2^{+/-} mouse (N) that bore an HCC (T). Note the absence of total Mst1 and Mst2 and phospho-Mst1/2 in the HCC. In the wild type liver, the majority of Mst1 and a significant proportion of Mst2 is present as an N-terminal truncated form (NT); Mst1/2 activity (as reflected by p-Mst1/2 levels) is restricted to the truncated Mst1/2 forms. Right: an Mst1 immunoprecipitate from wildtype and Mst1^{-/-} liver, demonstrating immunoprecipitation of the 34kDa polypeptide and the pMst1(Ser183) 34kDa polypeptide. (G) Western blot showing that Mst1 is exclusively full length in the spleen.

**Figure 2.**

Acute inactivation of Mst2 in Mst1 null liver initiates hepatocyte proliferation and HCC development. (A) Western blot showing expression of Mst1 and Mst2 in the liver of mice of the indicated genotypes before (day 0) or at intervals after Adeno-Cre injection in the tail vein. An HCC is shown, far right. (B) Gross images of livers 8 days after Adeno-Cre administration. (C) Liver weight to body weight ratios at various time points after Adeno-Cre delivery. Statistical significance (unpaired T test) is indicated. (D) Ki-67 staining at day 8; scale bar = 500 μ m. (E) Quantitation of Ki-67 staining. Statistical significance (unpaired T test) is indicated; error bars show standard deviation. (F) HCC-free survival curve of mice of the indicated genotypes treated at 6 weeks with Adeno-Cre. (G) Gross and H&E images of an HCC from an Adeno-Cre treated Mst1^{-/-}Mst2F^{-/-} mouse; scale bar = 500 μ m. (H)

Western blot analysis of a series of cell cycle regulators in liver tissue from WT and Mst1^{-/-} Mst2F^{-/-} mice before, or at the indicated number of days after Adeno-Cre administration and in an HCC. (I) Western blot analysis of liver extracts from wild type and Mst1^{-/-} Mst2F^{-/-} mice 8 days after AdenoCre, and of a set of 10 HCC's arising after AdenoCre treatment. β -catenin* = β -catenin dephosphorylated at Ser37 or Thr41.

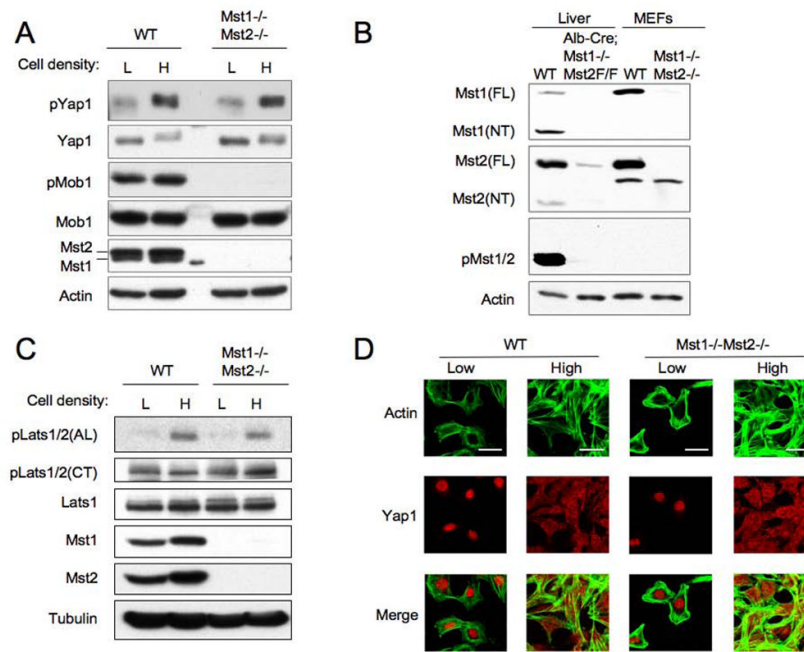
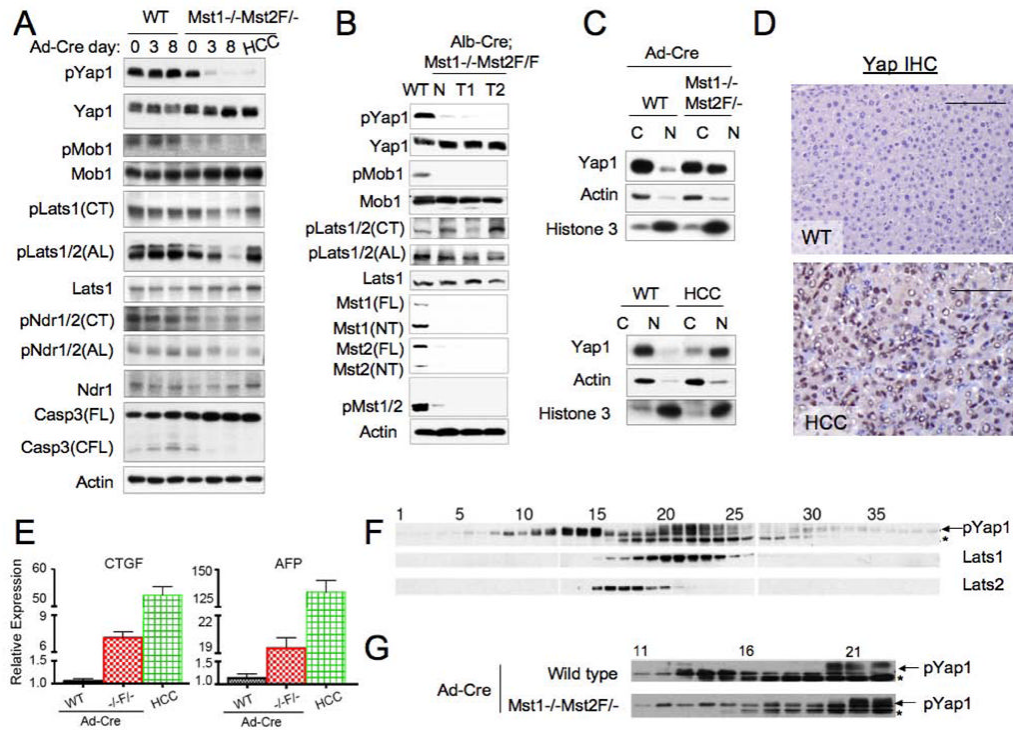


Figure 3. Analysis of the Hippo pathway in *Mst1*^{-/-}*Mst2*^{-/-} MEFs. (A) Western blot of immortalized MEFs grown to low (L) and high (H) density. Note that high density induces pYap1 regardless of *Mst1/2* status. In contrast, pMob1 strictly requires *Mst1/2*. The MEFs were generated from wildtype or *Mst1*^{-/-}*Mst2*^{F/F} mice and infected with a retrovirus expressing Cre recombinase. The cells were then immortalized by infection with a retrovirus expressing SV 40 T-Antigen. (B) Western blot showing expression of full length (FL), N-terminal truncated (NT), and activated phosphorylated forms of Mst1 and Mst2 in liver and MEFs. (C) Western blot showing that the induction of Lats1/2 activation loop (AL) phosphorylation in MEFs at high cell density does not require *Mst1* or *Mst2*. (D) Immunofluorescence showing that Yap1 (red) translocates out of the nucleus to the cytoplasm upon cellular confluence both in wild type and *Mst1/2* null MEFs; scale bar = 30 μ m.

**Figure 4.**

Mst1/2 regulate Yap1 phosphorylation and cytoplasmic retention in the liver. (A) Western blot of liver lysates prior to (day 0) or at 3 and 8 days after Adeno-Cre injection of mice with the indicated parental genotypes; an HCC is shown at the far right. Note that elimination of Mst1/2 leads to immediate loss of pYap1 and pMob1. The Mst1/2 site at the C-terminus (CT) of pLats1/2 is minimally affected whereas there is a delayed and transient decrease of Lats1/2 activation loop (AL) phosphorylation, however both Lats1/2 sites in the HCC are phosphorylated at WT levels. Mst1 and Mst2 are shown in Fig. 2A. (B) Western blot analysis of WT liver as well as normal liver (N) and HCC (T1, T2) from an Alb-Cre; Mst1^{-/-}-Mst2F/F mouse. (C) Western blots of cytoplasmic [C] and nuclear [N] fractions from WT and Mst1^{-/-}-Mst2F/F livers 8 days after Adeno-Cre injection (left) and from a murine HCC (right). (D) IHC analysis of Yap1 expression in WT liver and in HCC; scale bar = 250 μm. (E) qPCR analysis of CTGF and AFP mRNA expression in WT and Mst1^{-/-}-Mst2F/F livers 8 days after Adeno-Cre delivery, and in murine HCC (data is mean for 3 specimens per group; standard error of the mean is shown). (F) Hepatic Yap1(Ser127) kinases. An extract of normal mouse liver was separated by anion exchange chromatography and each fraction was assayed for the ability to catalyze the phosphorylation of a prokaryotic recombinant GST-Yap1 at Ser127 (arrow) as detected by immunoblot (upper panel). (*) Endogenous hepatic Yap1. The middle and lowest panels show the elution of the endogenous Lats1 and Lats2. Note that two peaks of Yap1(Ser127) kinase activity are present, eluting in fractions 13–15 and 20–23; Lats1 co-elutes with the latter peak of Yap1(Ser127) kinase whereas Lats2 elutes in the interval between the Yap1 kinase peaks. (G) Yap1(Ser127) kinase activity in fractions from livers from adeno-Cre treated wild type and Mst1^{-/-}-Mst2F/F mice. Recombinant Yap1 (arrows) and endogenous Yap1 (*) are indicated. Note that the earlier eluting peak of Yap1(Ser127) kinase activity (fractions 13–15) is absent in the Mst1/2-deficient liver whereas the later eluting activity (fractions 20–22) is unaffected by Mst1/2 loss.

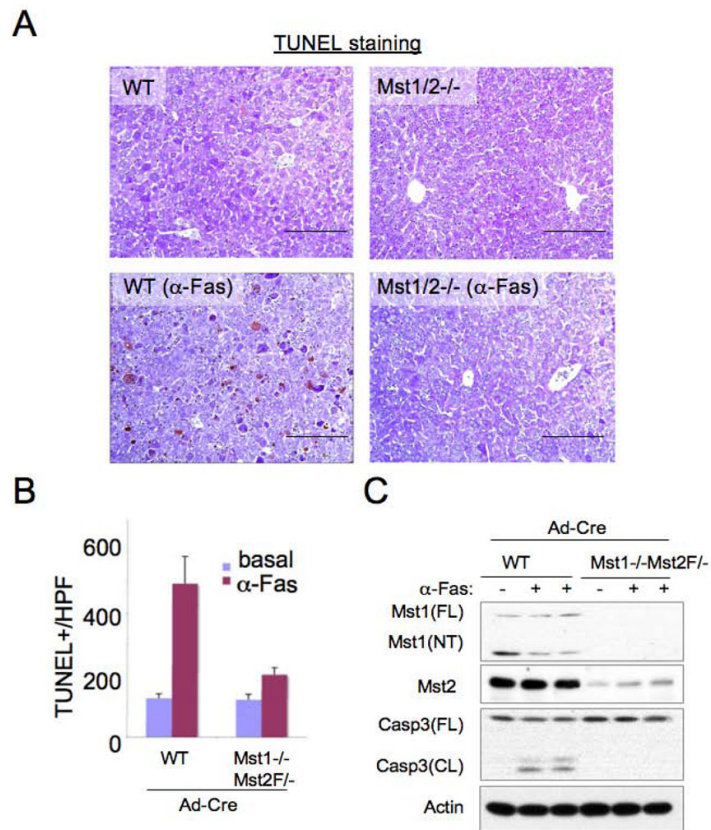
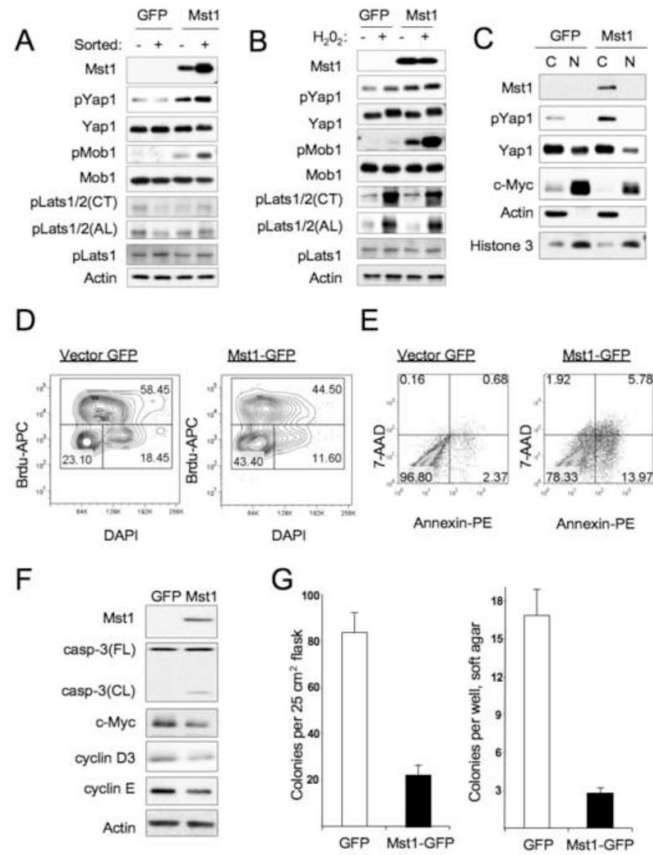
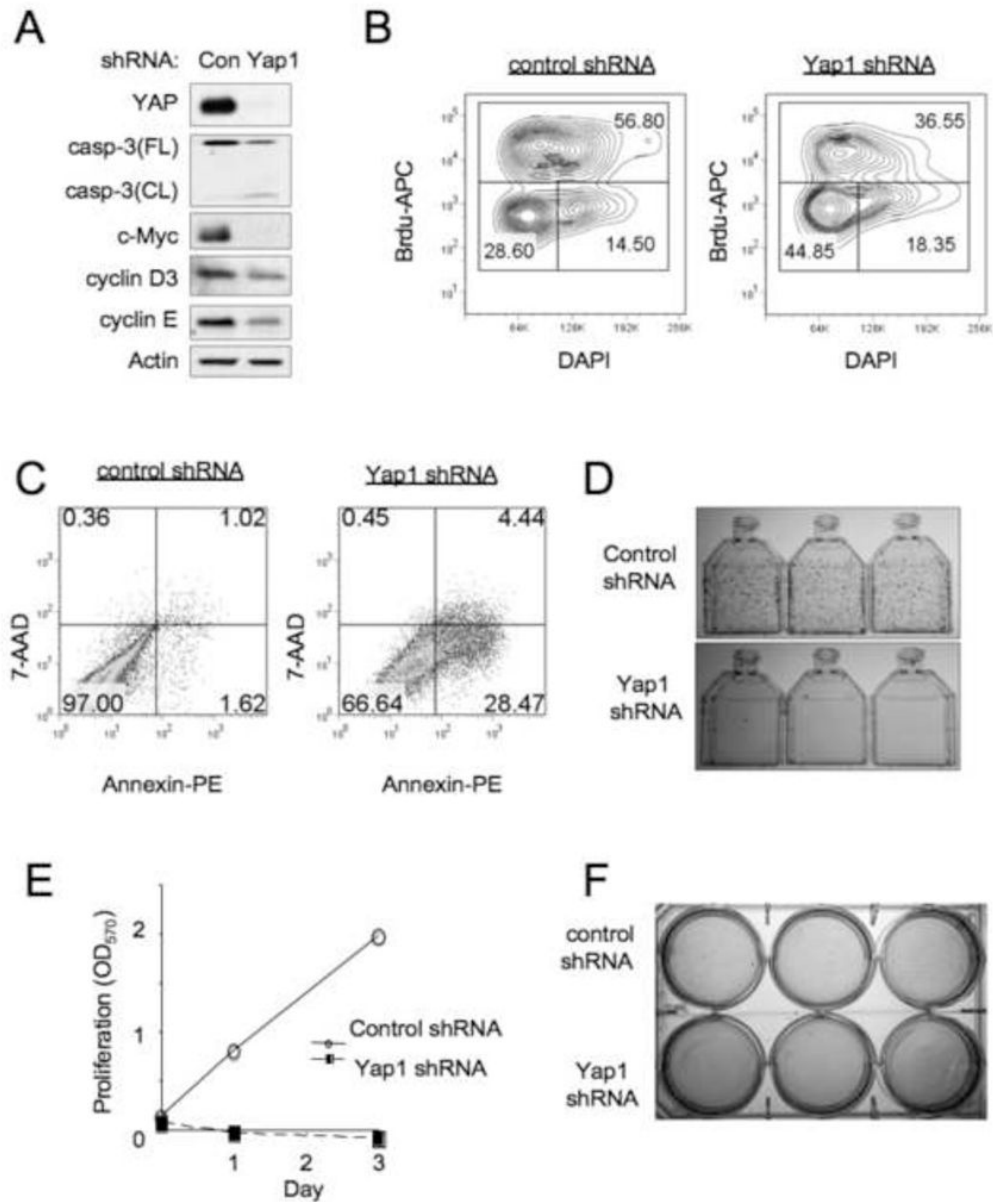


Figure 5. Mst1/2 deletion protects against Fas-induced apoptosis. (A) WT and Mst1^{-/-} Mst2F^{-/-} mice were injected with Adeno-Cre and 8 days later were treated with either vehicle or anti-Fas antibody (Jo-2). Livers were harvested 3 hrs after treatment and analyzed by TUNEL staining. The Mst1/2 deficient livers (Mst1/2^{-/-}) were resistant to Fas-induced apoptosis. Scale bar = 125 μ m. (B) Quantitation of TUNEL staining, n=3 (error bars show standard deviation). (C) Western blot analysis of liver lysates shows the upregulation of cleaved caspase-3 (CL) in anti-Fas-treated mice but not Mst1/2-deficient mice.

**Figure 6.**

Mst1 restoration inactivates Yap1 and reverts tumorigenicity of Mst1/2 deficient HCC cells. (A) The HCC-1 cell line, derived from an HCC arising in an Mst1^{-/-}Mst2F^{-/-} mouse, was infected with lentiviruses that express GFP alone or in combination with Mst1. HCC-1 cells were either FACS sorted for GFP (+) or were unsorted (-). The western blot of cells harvested 96 hours post-lentiviral infection shows that Mst1 induces Yap1 and Mob1 phosphorylation, whereas Lats1/2 carboxyterminal (CT) and activation loop (AL) phosphorylation are unaffected. (B) Western blot of sorted HCC-1 cells that were either untreated (-) or exposed to H₂O₂ (+), 0.5mM. H₂O₂ induces strong Lats1/2 phosphorylation independent of Mst1/2 whereas Yap1 phosphorylation is increased more by Mst1 than by H₂O₂ in the absence of Mst1/2. (C) Western blots of cytoplasmic (C) and nuclear (N) fractions of unsorted HCC-1 cells showing that Mst1 promotes increased phosphorylation and cytoplasmic retention of Yap1. (D) BrdU/DAPI staining and flow cytometry of HCC cells 72 hours post-infection. Mst1 induces an increase in G1 content (lower left quadrant) and a decrease in S phase (upper quadrant) cells. (E) Annexin V/7-AAD staining and flow cytometry of HCC-1 cells 48 hours post-infection. Mst1 induces early apoptosis (lower right quadrant) and late apoptosis (upper right quadrant). (F) Western blot expression of unsorted HCC-1 cells analyzed 96 hours post-infection showing that Mst1 induces cleavage of caspase-3 (CL) and a reduction in expression of c-Myc, cyclin D3 and cyclin E. (G) Mst1 reexpression suppresses HCC1 colony formation (left) and growth in soft agarose (right). Error bars show standard deviation.

**Figure 7.**

Yap1 is required for tumorigenicity of Mst1/2 mutant HCC cells. (A) Western blots of lysates from HCC-1 cells infected with lentiviruses expressing scrambled or Yap1 shRNAs and analyzed at day 6 post-infection. Yap1 shRNA silences YAP expression and results in caspase-3 cleavage (CL) and reduction in full length (FL) caspase-3. (B) BrdU/DAPI staining and flow cytometry. Yap1 shRNA increases the proportion of HCC-1 cells in G1 (lower left quadrant) and decreases cells in S phase (upper quadrant). (C) Annexin V/7-AAD staining and flow cytometry. Yap1 shRNA induces early apoptosis (lower right quadrant) and late apoptosis (upper right quadrant). (D) HCC-1 cells expressing Yap1 shRNA are unable to form colonies in clonogenic assays. (E and F) Yap1 knockdown prevents the proliferation of HCC-1 cells as determined by MTT assay (E) and suppresses growth in soft agarose (F).

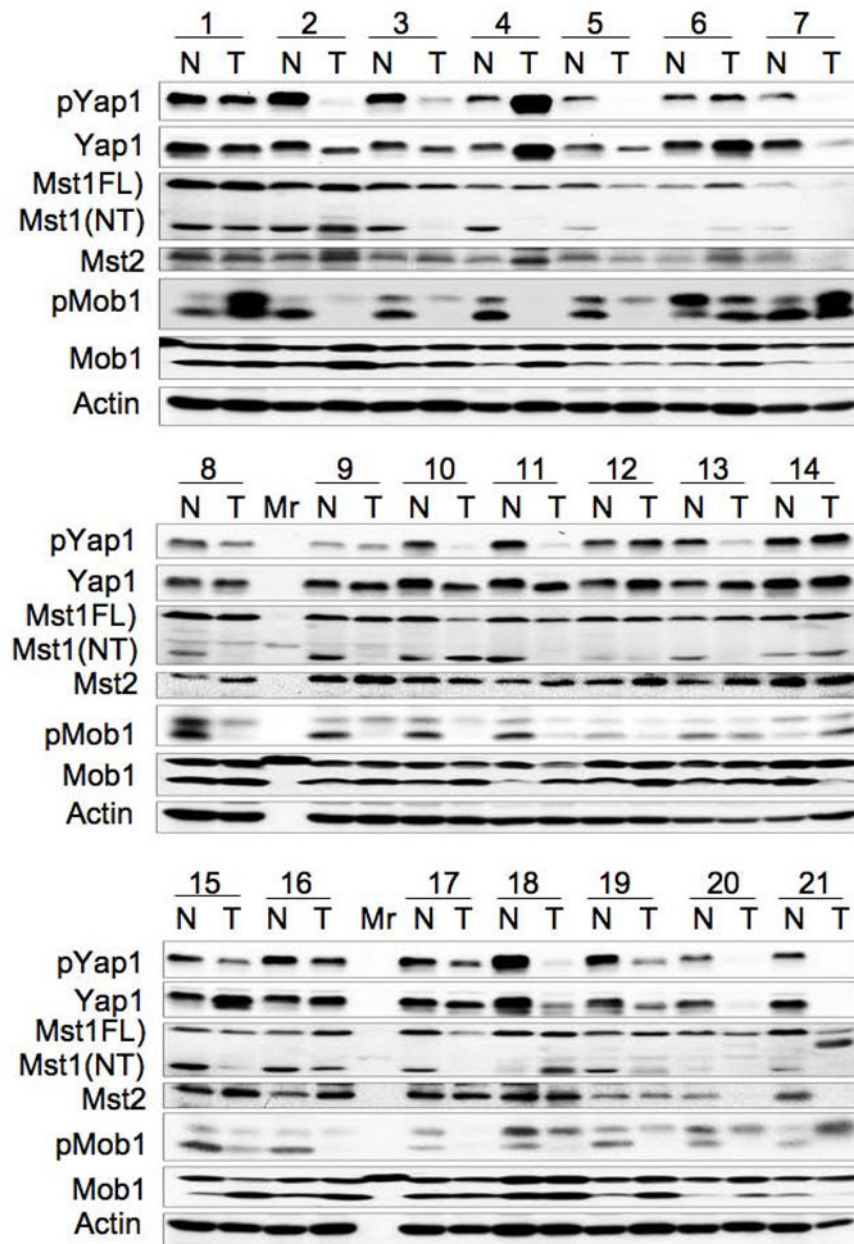


Figure 8.

Immunoblot analysis of Yap1, Mob1, and Mst1/2 in human HCC. Western blot analysis of 21 paired samples of non-tumorous liver (N) and HCC (T) from the same patient. Patients 2,3,5,10,11,13 and 15 exhibit substantially lower pYap1 in the tumor relative to the non-tumorous liver. Patients 4,6,12, and 15 exhibit Yap1 polypeptide overexpression in the tumor. The majority of HCCs exhibit much lower pMob1 relative to the paired non-tumorous liver. The Mst1 34kDa polypeptide fragment is lacking in the tumors while present in the non-tumorous livers of patients 3,4,5,7,8,9,11,13,15,17,19,20,21 whereas only patients 6 and 18 exhibit detectable 34kDa Mst1 in their tumors but little or none in their non-tumorous livers. The associated clinical information is presented in Supplementary Table 1.