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Identification and Validation of Oncogenes in Liver Cancer Using an Integrative Oncogenomic Approach

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SUMMARY

The heterogeneity and instability of human tumors hamper straightforward identification of cancer-causing mutations through genomic approaches alone. Herein we describe a mouse model of liver cancer initiated from progenitor cells harboring defined cancer-predisposing lesions. Genome-wide analyses of tumors in this mouse model and in human hepatocellular carcinomas revealed a recurrent amplification at mouse chromosome 9qA1, the syntenic region of human chromosome 11q22. Gene-expression analyses delineated *cIAP1*, a known inhibitor of apoptosis, and *Yap*, a transcription factor, as candidate oncogenes in the amplicon. In the genetic context of their amplification, both *cIAP1* and *Yap* accelerated tumorigenesis and were required to sustain rapid growth of amplicon-containing tumors. Furthermore, *cIAP1* and *Yap* cooperated to promote tumorigenesis. Our results establish a tractable model of liver cancer, identify two oncogenes that cooperate by virtue of their coamplification in the same genomic locus, and suggest an efficient strategy for the annotation of human cancer genes.

INTRODUCTION

Tumorigenesis results from a progressive sequence of genetic and epigenetic alterations that promote the malignant transformation of the cell by disrupting key processes involved in normal growth control and tissue homeostasis (Hanahan and Weinberg, 2000). Since complex signaling networks control these processes, mutations in many genes can provide the cell with a specific aberrant capability. Consequently, the combination of genetic alterations that can occur during tumor evolution is enormous, perhaps underpinning the substantial heterogeneity in tumor behavior that occurs even within a particular tumor type. In addition, genomic instability is a common, if not universal, feature of advanced tumors. This instability provides tumor cells with the ability to adapt to new environments but may also increase the rate of bystander mutations that do not contribute to the malignant phenotype.

The completion of the human genome project has enabled new approaches for studying cancer genetics and cancer genomes. For example, gene-expression profiling using microarrays has improved the classification of some tumor types (Segal et al., 2005). Moreover, DNA resequencing has identified unanticipated mutations in oncogenes such as *BRAF* and *EGFR*, thus suggesting new drug targets or therapeutic strategies (Davies et al., 2002; Lynch et al., 2004). Finally, genome scanning for gene copy-number alterations has identified many loci harboring candidate cancer genes (Kallioniemi et al., 1993; Lucito et al., 2003). Because of these advances, efforts to catalog all of the mutational events that contribute to human cancer can now be envisioned. Nevertheless, for such information to be efficiently translated into improvements in cancer diagnosis and therapy, cancer-causing mutations must be distinguished from irrelevant alterations linked to complex cancer genotypes. Furthermore, without in vivo validation, there is little stimulus for therapeutic development efforts. Integrative strategies to identify and validate genes with functional relevance for tumor initiation and progression are clearly needed.

Hepatocellular carcinoma (HCC) represents a tumor type where a more complete understanding of the underlying

genetics could have a major impact on treatment of the disease. HCC is the fifth most frequent neoplasm worldwide but, owing to the lack of effective treatment options, represents the third leading cause of cancer death (Parkin et al., 2001). The only curative treatments for HCC are surgical resection or liver transplantation, but most patients present with advanced disease and are not candidates for surgery. To date, systemic chemotherapeutic treatment is ineffective against HCC, and no single drug or drug combination prolongs survival (Llovet et al., 2003).

The development of HCC is invariably associated with liver damage caused by chronic hepatitis, extensive alcohol intake, or toxins, sequentially resulting in liver cirrhosis, dysplastic lesions, and finally invasive liver carcinoma. Recent studies suggest that these agents can target liver progenitor cells ("oval cells" in rodents and "hepatic progenitor cells" in humans), leading to their expansion and transformation (for review, see Alison and Lovell, 2005). One key target in liver carcinogenesis is p53, which is functionally attenuated by hepatitis B virus X protein (Wang et al., 1994) and is a mutational target of aflatoxin B1 (Aguilar et al., 1994). Other established lesions in liver cancer include activation of the c-*myc*, *CCND1* (cyclin D1), or c-*met* oncogenes, as well as mutations in components of the Ras/PI3 kinase pathways (Lee et al., 2005; Feitelson et al., 2002; Suzuki et al., 1994). Still, the molecular genetics of liver cancer, and how specific lesions interact to produce its aggressive characteristics, remain poorly understood.

Mouse models of human cancer provide powerful tools to investigate cancer biology, genetics, and therapy. Here we used a flexible mouse model of hepatocellular carcinoma to search for spontaneous mutations arising in tumors initiated by different oncogenic lesions and then compared these to alterations observed in human cancers. This approach enabled us to pinpoint "driver genes" that might contribute to human liver carcinogenesis and, using our model, to validate these changes in an appropriate in vivo context. Consequently, our study not only identified two oncogenes in the same focal amplification that cooperate during tumorigenesis but, more broadly, highlights the utility of integrating mouse models and cancer genomics for the functional annotation of cancer genes.

RESULTS

Generation and Transplantation of Genetically Altered Liver Progenitor Cells

Most mouse models for liver cancer are based on germline transgenic approaches that direct expression of an oncogene to the liver using a tissue-specific promoter (Sandgren et al., 1989; Murakami et al., 1993). Although these models continue to provide important insights into the pathogenesis of liver cancer, they express the oncogene throughout the entire liver, a situation that is distinct from spontaneous tumorigenesis. Moreover, incorporation of additional lesions, such as a second oncogene or loss of a tumor suppressor, requires genetic crosses that are time consuming and expensive. Finally, traditional transgenic and knockout strategies do not specifically target liver progenitor cells, one proposed "cell of origin" of the disease (Alison and Lovell, 2005).

Based on our previous work in the hematopoietic system (e.g., Schmitt et al., 2002), we reasoned that the study of genetic interactions in liver tumorigenesis would be facilitated by ex vivo genetic manipulation of liver progenitors followed by their retransplantation into the livers of recipient mice (Figure 1A). Embryonic hepatoblasts express high E-cadherin levels, enabling these cells to be isolated to high purity from E12.5–15 fetal livers using magnetic bead selection (Nitou et al., 2002). These cells also expressed markers characteristic of bipotential oval cells (see Figure S1A in the Supplemental Data available with this article online), one presumed target of transformation in the adult liver (Alison and Lovell, 2005). Although they proliferated poorly in initial experiments, the introduction of defined medium (Block et al., 1996), feeder layers, and gelatin-coated plates to the culture conditions enabled the hepatoblasts to be expanded without loss of their defining characteristics (data not shown). These conditions also allowed efficient gene transfer using MSCV-based retroviral vectors expressing green fluorescent protein (GFP) (Figure S1B) or short-hairpin RNAs (shRNAs) capable of suppressing gene expression through RNA interference (Zender et al., 2006).

To determine whether genetically modified hepatoblasts could colonize recipient livers, we used a protocol that optimizes engraftment of transplanted cells (Guo et al., 2002). Animals were pretreated with retrorsine, an alkaloid that exerts a strong and persistent block of native hepatocyte proliferation and increases the competitive advantage of transplanted cells (Laconi et al., 1998). Ten days after the last retrorsine treatment, 2×10^6 GFP-tagged E-cadherin$^+$ liver progenitor cells were delivered to the liver by intrasplenic injection. One week later, immunohistochemical analysis of liver sections revealed that approximately one percent of the host liver consisted of "seeded" GFP-positive cells that were embedded within the normal liver architecture (Figure 1B).

Generation of Liver Carcinomas from Transplanted Liver Progenitor Cells that Resemble Human HCC

We next tested whether this approach could produce liver carcinomas in situ. Owing to the importance of p53 inactivation in this disease, we isolated hepatoblasts from *p53*$^{-/-}$ fetal livers and transduced these cells with retroviruses coexpressing *myc* (c-*myc*), activated *Akt* (*Akt1*), or oncogenic *Ras* (*H-Ras*^{V12}) (each of which affects signaling pathways altered in human liver cancer) and GFP. As above, these transduced cell populations were transplanted into retrorsine-treated mice. To further facilitate expansion of the transplanted cells, recipient mice were treated with CCl_4 (Guo et al., 2002) and monitored for signs of disease by abdominal palpation and whole-body fluorescence imaging. Although *p53*$^{-/-}$ hepatoblasts were not tumorigenic during the analysis period, each of the cell populations that

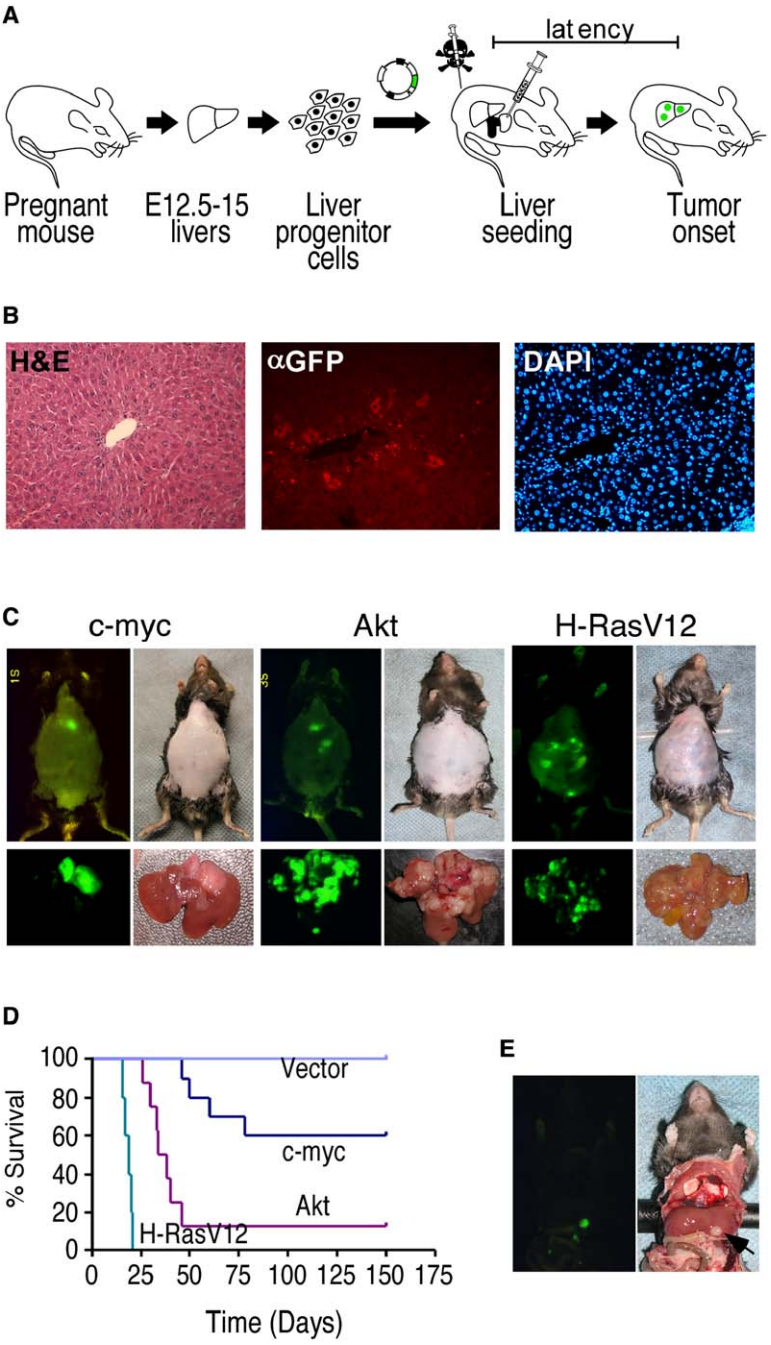

Figure 1. Development and Characterization of a Genetically Tractable, Transplantable Mouse Model of HCC

(A) Schematic diagram showing the generation of in situ liver carcinomas following retroviral transduction of purified E-cadherin^{+} hepatoblasts (see Figure S1).

(B) Analysis of liver sections from mice seeded with GFP-expressing hepatoblasts 1 week postreconstitution. Left, H&E; middle, anti-GFP immunofluorescence; right, DAPI.

(C) External GFP tumor imaging (top panels) or direct imaging of the respective explanted tumor-bearing livers (bottom panels) of mice reconstituted with *p53*$^{-/-}$ hepatoblasts transduced with the indicated oncogene.

(D) Survival curves of mice after intrahepatic seeding of *p53*$^{-/-}$ liver progenitor cells transduced with the indicated oncogene or control vector.

(E) Explanted murine liver carcinomas (*p53*$^{-/-}$; *myc*) were grown briefly in culture and then directly injected into the left liver lobe. Shown is a GFP-expressing (left) in situ tumor (right) 42 days postinjection.

also expressed an oncogene eventually produced GFP-positive tumors in the livers of recipient mice (Figure 1C, top).

Gross pathological analysis of explanted livers revealed that Myc-expressing tumors differ significantly from those expressing Akt or Ras (Figure 1C, bottom). First, Myc-expressing tumors grow primarily as unilocular tumors, whereas Akt- and Ras-derived tumors show aggressive, multilocular, and infiltrative intrahepatic growth. Second, the intrinsic tumorigenicity of *p53*$^{-/-}$ liver progenitor cells expressing Myc was significantly lower than those expressing Akt or Ras (Figure 1D). Of note, *p53* loss clearly contributed to tumorigenesis since tumors arising in mice reconstituted with *p53*$^{+/-}$ hepatoblasts showed further delayed tumor onset and loss of the wild-type *p53* allele (Figure S1D). In most instances, GFP-positive cells derived from established tumors could be grown in culture and subsequently formed secondary tumors upon subcutaneous injection into immunocompromised mice (data not shown) or direct intrahepatic injection into syngeneic recipients (Figure 1E).

An experienced liver pathologist (P.F.) examined the hematoxylin and eosin (H&E) stained sections derived from

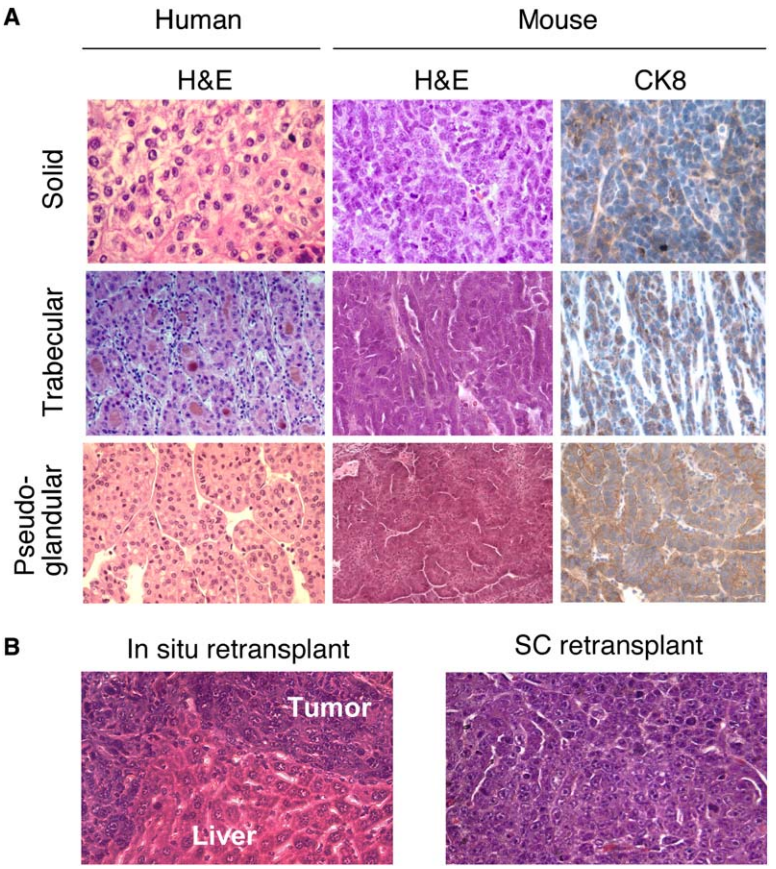

Figure 2. Murine Liver Carcinomas Derived from E-Cadherin^{+} Liver Progenitor Cells Histopathologically Resemble Human HCC

(A) H&E-stained sections of human HCC with the indicated histopathological variegation (left panels) shown adjacent to corresponding histopathologies in murine liver cancers arising from *p53*$^{-/-}$*;myc* hepatoblasts (center panels). Anti-CK8 immunohistochemistry of the murine tumors is shown at right.

(B) H&E staining of in situ and subcutaneously retransplanted HCCs derived from *p53*$^{-/-}$*;myc* hepatoblasts.

Myc-induced murine hepatomas (Figure 2A) and classified most as moderately well to poorly differentiated HCCs with a mostly solid, sometimes mixed solid/trabecular growth pattern. A smaller proportion of tumors displayed growth patterns resembling trabecular or pseudoglandular HCC. All tumors examined stained positive for cytokeratin 8, confirming their liver origin. However, despite their derivation from cytokeratin 19-positive liver progenitor cells, most HCCs lost this marker during tumorigenesis (Figure S1C). The tumors also expressed high albumin levels and, similar to the situation in human HCC, about half were positive for α-fetoprotein. Most also expressed moderate levels of vimentin (Figure S1C), a marker linked to aggressive tumor behavior (Hu et al., 2004). Transplanted hepatomas retained their HCC histology when injected orthotopically into the liver or subcutaneously into immunocompromised mice (Figure 2B). Therefore, ex vivo-manipulated liver progenitor cells can produce tumors that recapitulate the histopathology of human HCC.

ROMA Identifies Spontaneous Mutations in a Subset of Murine Liver Carcinomas Including a Recurrent Amplicon at Chromosome 9qA1

To further molecularly characterize the murine HCCs described above, we searched for spontaneously acquired lesions in these cancers using representational oligonucleotide microarray analysis (ROMA), a genome-wide scanning method capable of identifying copy-number alterations in tumor cells at high resolution (Lucito et al., 2003; B. Lakshmi, I.M. Hall, C. Egan, J. Alexander, J. Healy, L.Z., W.X., M.S.S., S.W.L., M.W., and R.L., unpublished data). Each human or mouse ROMA array consists of 85,000 oligonucleotide probes, allowing genome scanning at a theoretical resolution of ~35 kb. Although we did not detect focal genomic alterations (<5 Mb) in liver cancers induced by Akt, a number were detected in those initiated by Myc or Ras (Table S1). For example, a Ras-expressing tumor harbored two focal amplifications on chromosome 15 (Figure 3A), one containing *Rnf19* and the other containing c-*myc* (Figure 3B). While *Rnf19* has not been linked to tumorigenesis, c-*myc* alterations are common in human liver cancer (Peng et al., 1993), and *myc* cooperates with oncogenic *Ras* in transgenic models of HCC (Sandgren et al., 1989). These observations underscored the relevance of our model and suggested that further analyses would reveal other genes involved in human cancer.

ROMA analysis of seven independent Myc-expressing HCCs identified a focal amplicon on mouse chromosome 9qA1 in four of these tumors (Figures 3C and 3D; Figure S2 and Table S1). The minimal overlapping region is approximately 1 Mb and contains genes encoding for several matrix metalloproteinases (MMPs), *Yap*, *cIAP1 (Birc2)*, *cIAP2*

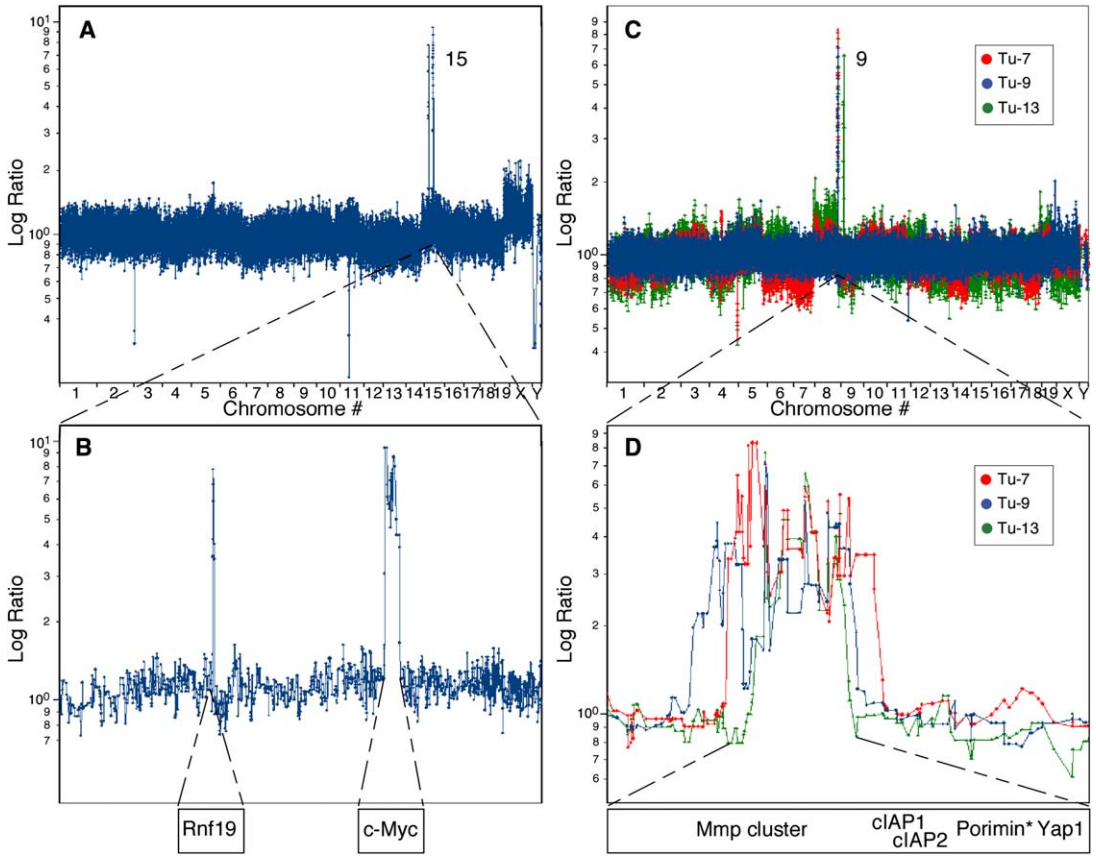

Figure 3. ROMA Identifies Focal DNA Amplifications in Murine HCCs

(A) ROMA profile of a tumor derived from *p53*$^{-/-}$*;Ras* embryonic hepatoblasts. Data plotted are the normalized log ratio for each probe (*EST).

(B) Single-probe resolution of chromosome 15 corresponding to the tumor described in (A).

(C) Genome-wide profiles of three independent HCCs (Tu-7, Tu-9, and Tu-13) derived from *p53*$^{-/-}$*;myc* embryonic hepatoblasts.

(D) Single-probe resolution of chromosome 9qA1 from the tumors described in (C). The minimal overlap region contains the indicated genes.

(Birc3), and *Porimin* (Figure 3D; see Table S1 for breakpoints). Amplification was confirmed by genomic qPCR using a probe within the *cIAP1* gene (data not shown). Interestingly, 9qA1 was never found amplified in *Ras*- or *Akt*-driven liver carcinomas as assessed by either genomic qPCR analysis or ROMA (n = 21; Table S1 and data not shown). These observations suggest that at least one of the genes in the 9qA1 region cooperates with *myc* and *p53* loss to promote hepatocarcinogenesis.

Comparative Oncogenomics Reveals Lesions in Common between Murine and Human Cancers

In parallel to our analysis of murine HCCs, we initially conducted ROMA on 25 human HCC samples. Although these tumors contained more alterations than their murine counterparts, using a strict cutoff of <5 Mb, we detected copy-number alterations affecting genes previously linked to HCC (Table S2). For example, three tumors had a chromosome 11 amplification containing *CCND1*, two had a chromosome 7 amplification containing c-*met*, two had a focal deletion on chromosome 10 containing the *PTEN* tumor suppressor, and one had a deletion of chromosome 9 harboring the *CDKN2A* (*INK4a/ARF*) locus.

We also detected a focal amplification on chromosome 11q22, a region that is syntenic to the murine 9qA1 locus. This tumor contained a c-*met* amplification (left peak) on chromosome 7 and three sharply delineated amplifications on chromosome 11 (Figure 4B), including *CCND1*, B′ (containing no known genes), and 11q22. A second HCC harboring the 11q22 amplicon was identified in a set of 23 additional human HCCs (Figures S2C and S2D), as well as in 4 of 53 human esophageal cancers (data not shown), indicating that it occurs in gastrointestinal malignancies derived from developmentally related organs. ROMA results were verified by genomic qPCR analysis using probes to the *cIAP1* and *cIAP2* loci (data not shown). Much like the chromosome 9 amplicon in murine HCCs, the boundaries of the 11q22 amplicon in human HCCs and esophageal cancers include genes encoding several matrix metalloproteinases, *Porimin*, *Yap*, *cIAP1*, *and cIAP2*.

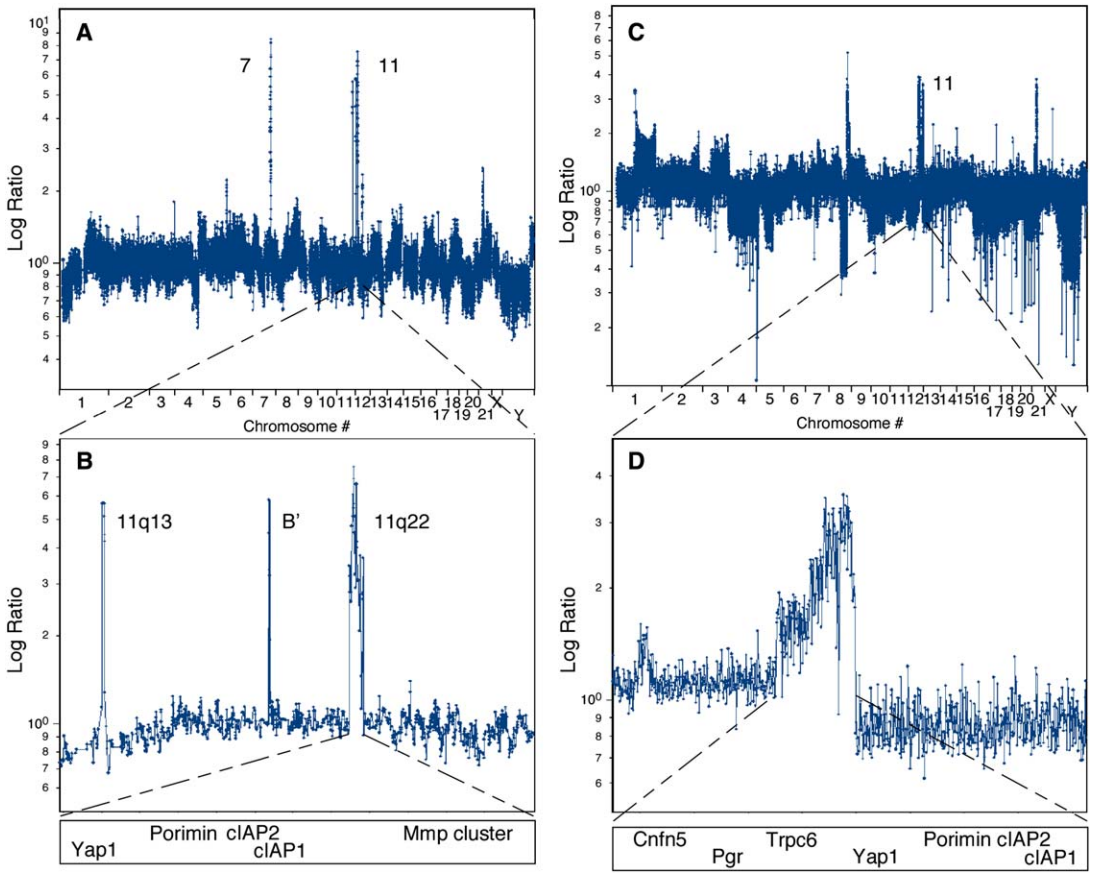

Figure 4. ROMA Identifies Amplification of the Human Syntenic Region 11q22 in HCC and Ovarian Cancer

(A) Genome-wide profile of a human HCC harboring an amplification on chromosome 7 containing the c-*met* gene and three regions amplified on chromosome 11.

(B) Single-probe resolution of chromosome 11, with genes contained within each amplicon depicted below.

(C) Genome-wide profile of an ovarian carcinoma containing the 11q22 amplification.

(D) Single-probe resolution of the 11q22 amplicon, with genes contained within the amplified region depicted below.

Cross-Species Expression Analysis of Genes from the Human and Mouse Amplicons Reveals Consistent Overexpression of *cIAP1* and *Yap*

The human 11q22 amplicon is observed in other human cancers (e.g., Figures 4C and 4D), although no driver gene has been decisively identified (Imoto et al., 2001; Dai et al., 2003; Bashyam et al., 2005; Snijders et al., 2005). While it represents only one of many low-frequency events in these tumors and the HCCs evaluated here (Table S2), our cross-species comparison suggests that a gene (or genes) within this recurrent amplified region is crucial for tumorigenesis in some genetic contexts. An essential criterion for establishing whether an amplified gene might contribute to tumorigenesis is that it be overexpressed in the tumors where it is amplified; we further hypothesized this should hold across species. Therefore, we performed a comprehensive expression analysis of overlapping genes from the murine 9qA1 and human 11q22 amplicons.

First, messenger RNA levels for all genes in these regions were measured by real-time quantitative RT-PCR (Figures 5A and 5C; Table S3). All amplicon-positive mouse HCCs displayed elevated mRNA levels for the MMPs except *MMP7* and had high variability in maximum expression levels (Table S3). In marked contrast, the mRNA levels for *MMP1*, *MMP3*, *MMP8*, *MMP12*, *MMP13*, *MMP20*, and *MMP27* were below detection limit in 25 human HCCs, including a tumor with the 11q22 amplicon (Table S3). However, *MMP7* and *MMP10* mRNAs were moderately elevated in an 11q22-positive HCC. Therefore, with the possible exception of *MMP10*, the MMPs are not consistently overexpressed in amplicon-positive murine and human HCCs and probably are not responsible for the selective advantage conferred by this genomic amplification. Furthermore, ROMA analysis on various 11q22-positive human carcinomas identified an ovarian carcinoma harboring an 11q22 amplicon that decisively excluded all of the MMPs (Figures 4C and 4D).

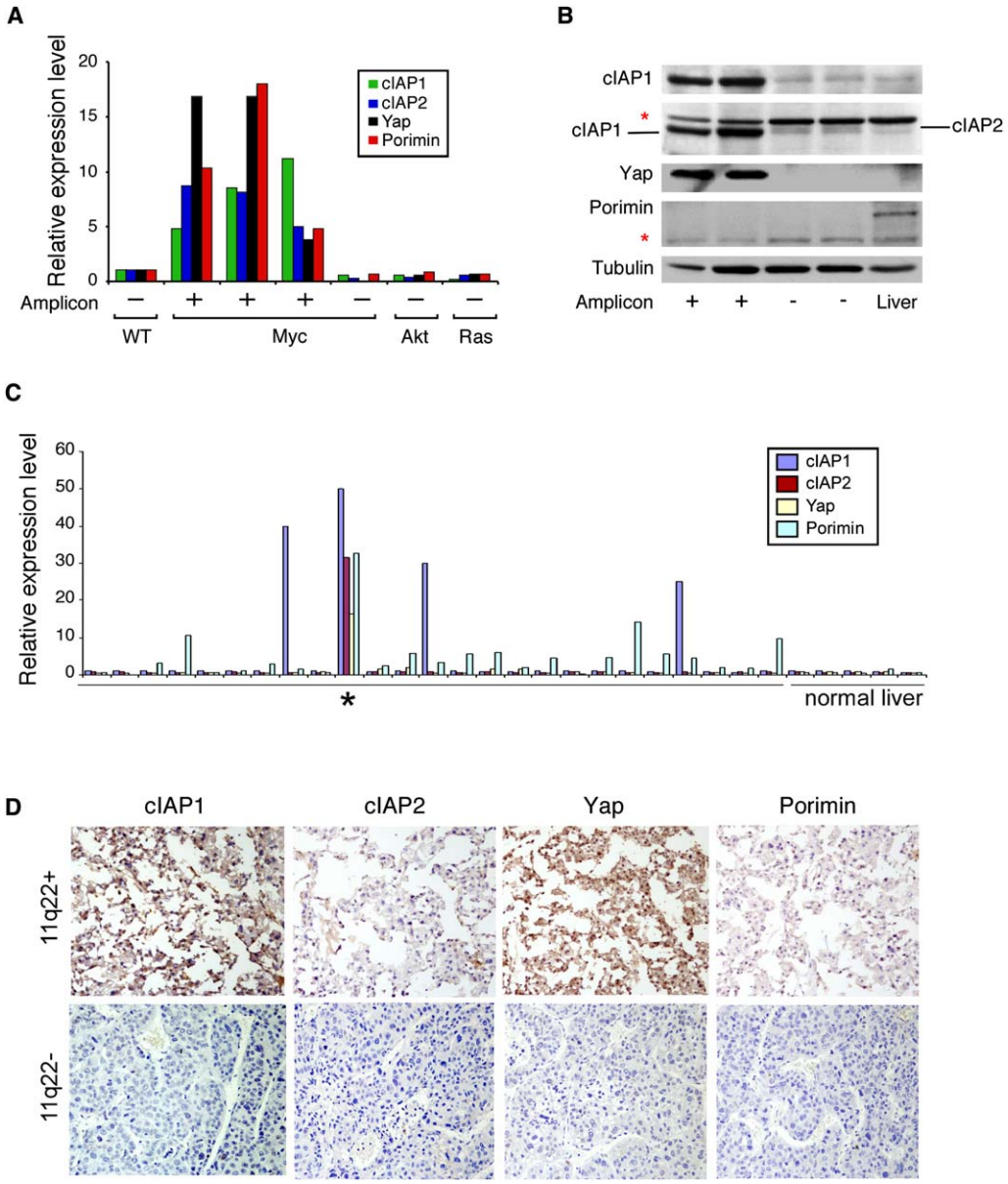

Figure 5. *cIAP1* and *Yap* Are Consistently Overexpressed in Mouse and Human Tumors Containing the 9qA1 or 11q22 Amplicon

(A) *cIAP1*, *cIAP2*, *Yap*, and *Porimin* mRNA levels in murine HCCs that contain the 9qA1 amplicon as determined by quantitative real-time RT-PCR analysis.

(B) Protein lysates from 9qA1-positive (+) or -negative (–) liver cancers and adult mouse liver were immunoblotted with antibodies against cIAP1, cIAP1/2, YAP, and Porimin. * denotes nonspecific bands. Tubulin was used as a loading control.

(C) Quantitative real-time RT-PCR analysis of cIAP1, cIAP2, Yap, and Porimin expression in human HCCs. * denotes a tumor with the 11q22 amplification. Cutoff for increased expression was >2-fold of the expression level in nonneoplastic liver (normal liver).

(D) Immunohistochemistry of an 11q22-positive (top) and -negative (bottom) human HCC using antibodies against the indicated protein.

Concordantly, low-resolution technologies excluded at least some MMPs from an 11q22 amplification in lung cancer (Dai et al., 2003).

In contrast, *cIAP1* and *Yap* mRNA and protein were elevated in all mouse and human amplicon-containing tumors examined (Figures 5A–5D). Both mRNAs were also found to be overexpressed in some 11q22-positive oral carcinomas (Snijders et al., 2005). Although *Porimin* and *cIAP2* mRNAs were elevated in all amplicon-containing tumors examined, we could not detect overexpression of either protein in 9qA1-positive mouse tumors (Figure 5B) or an 11q22-positive human tumor (Figure 5D). These observations may be explained by reports that many cells express *Porimin* mRNA without detectable protein

(Ma et al., 2001) and that cIAP1 promotes the ubiquitylation and degradation of cIAP2 (Conze et al., 2005; Silke et al., 2005). In fact, we observed that cIAP1 promoted the turnover of cIAP2 in a dose-dependent manner in vitro (Figure S3A) and showed that cIAP2 protein increases in 9qA1-positive murine HCC cells grown in the presence of a proteasome inhibitor (Figure S3B). Based on these aggregate observations, we considered *cIAP1* and *Yap* as the most likely candidate oncogenes in the region.

cIAP1 Has Oncogenic Properties and Is Required for Rapid Tumor Growth

Inhibitor of apoptosis (IAP) proteins were originally identified in baculovirus because of their ability to block cell death of infected cells (Crook et al., 1993). Overexpression of cellular IAPs can inhibit apoptosis induced by different stimuli (Lacasse et al., 1998). Although some IAPs bind and inhibit caspases, their contribution to apoptosis regulation and oncogenesis in mammalian cells is controversial (Wright and Duckett, 2005).

A significant advantage of profiling the genomes of defined murine tumors is that candidate genes can be evaluated in the genetic context where the mutation spontaneously arose. Our studies identified the 9qA1 amplicon in tumors derived from $p53^{-/-}$ hepatoblasts expressing Myc but not in other configurations, suggesting that these cells would be ideal for evaluating the oncogenic properties of *cIAP1*. Therefore, $p53^{-/-}$*;myc* liver progenitor cells expressing cIAP1 or a control vector were produced using retroviral-mediated gene transfer; as predicted, cIAP1 conferred a modest resistance to cell death triggered by serum starvation or confluence (Figure 6A).

To determine whether *cIAP1* could function as an oncogene in vivo, we injected the cells described above subcutaneously into nude mice to facilitate precise measurement of tumor growth. cIAP1 significantly accelerated the growth of $p53^{-/-}$*;myc* hepatoblasts (Figure 6B), reducing tumor onset times by half (24 ± 2.3 days for *myc* + *cIAP1* versus 45 ± 12.2 days for *myc* + vector [$p < 0.05$]) and greatly increasing tumor burden (*myc* + *cIAP1* versus *myc* + vector [$p < 0.005$] at 52 days). The resulting tumors stably overexpressed full-length cIAP1 protein (Figure 6C, compare lane 2 to lanes 8–13) and several degradation products (Silke et al., 2005) and displayed a histopathology that resembled moderately well to poorly differentiated HCC (data not shown). Interestingly, one control tumor that was harvested at a very small size already showed elevated levels of cIAP1 (Figure 6C, lane 7), consistent with a subset of cells acquiring a spontaneous alteration that upregulated the gene. In contrast, cIAP1 did not affect the onset or progression of tumors expressing Akt or Ras (Figures 6D and 6E), even though cIAP1 was efficiently expressed (Figures S4A and S4B). Thus, *cIAP1* is selectively oncogenic in the genetic context where its amplification occurs.

To determine whether the cIAP proteins help sustain tumorigenesis, we next examined the impact of reducing cIAP levels on tumor growth. We chose to suppress the expression of cIAP1 and cIAP2 since cIAP2 can be upregulated in response to downregulation of cIAP1 (Conze et al., 2005). shRNAs capable of suppressing cIAP1 and cIAP2 expression by RNA interference (Figure 6F, compare lanes 1 and 2) were cointroduced into outgrown murine hepatoma cells containing or lacking the 9qA1 amplicon. These cells were then injected subcutaneously into immunocompromised mice.

Tumors arising from 9qA1-positive cells expressing cIAP1 and cIAP2 shRNAs showed a reduced growth rate compared to controls (Figure 6G; $p < 0.005$ for tumor burden "vector; vector" versus *sh cIAP1;sh cIAP2* at day 18). Although tumor inhibition was incomplete, the efficiency of cIAP knockdown was greatly reduced in the outgrown tumors compared to the injected cells (Figure 6F, compare lane 2 and lanes 5 and 6), implying that cells retaining high cIAP levels were selected during tumor expansion. These same shRNAs had no impact on the growth of amplicon-negative tumors expressing either Myc or oncogenic Ras (Figure 6H; Figure S4D), suggesting that only cells selected for cIAP overexpression are sensitive to cIAP inhibition and ruling out off-target effects of these shRNAs on tumor growth (see also Figure S4C). Therefore, the *cIAP* genes are required for the efficient growth of tumors harboring the 9qA1 amplicon.

Yap Has Oncogenic Properties and Contributes to Rapid Tumor Growth

In addition to *cIAP1*, *Yap* was also overexpressed at the RNA and protein level in every tumor harboring the mouse 9qA1 or human 11q22 amplicon. *Yap* (synonyms *Yap65* or *Yap1*) was originally identified due to its interaction with the Src-family kinase Yes (Sudol, 1994) and acts as a transcriptional coactivator that can bind and activate Runx and TEAD/TEF transcription factors (Yagi et al., 1999). Inconsistent with an oncogenic role, mammalian Yap also interacts with the p53 family member p73 (Strano et al., 2001) and potentiates apoptosis in a manner that is suppressed by Akt (Basu et al., 2003). However, recent studies suggest that *yorkie*, the *Drosophila* homolog of *Yap*, promotes tissue expansion as an effector of the Lats/Warts pathway by activating *cyclin E* and the *Drosophila* inhibitor of the apoptosis gene *dIAP* (Huang et al., 2005). Interestingly, we also noted that murine tumors harboring the 9qA1 amplicon overexpressed cyclin E (Figure 7B).

To determine whether *Yap* could also contribute to the transformation of liver progenitor cells, we conducted functional studies that paralleled our analysis of *cIAP1*. $p53^{-/-}$*;myc* hepatoblasts expressing Yap grew more rapidly than vector-infected cells (data not shown), with a higher BrdU incorporation rate (Figure 7A). Furthermore, *Yap* significantly accelerated tumor onset and progression of $p53^{-/-}$*;myc* liver progenitor cells (Figure 7C) and greatly increased tumor burden (*myc;vector* versus *myc;Yap* at day 40 [$p < 0.005$]). In contrast, Yap did not accelerate tumorigenesis together with activated *Ras*, although it did enhance *Akt*-driven tumorigenesis, particularly at later times (Figures 7D and 7E).

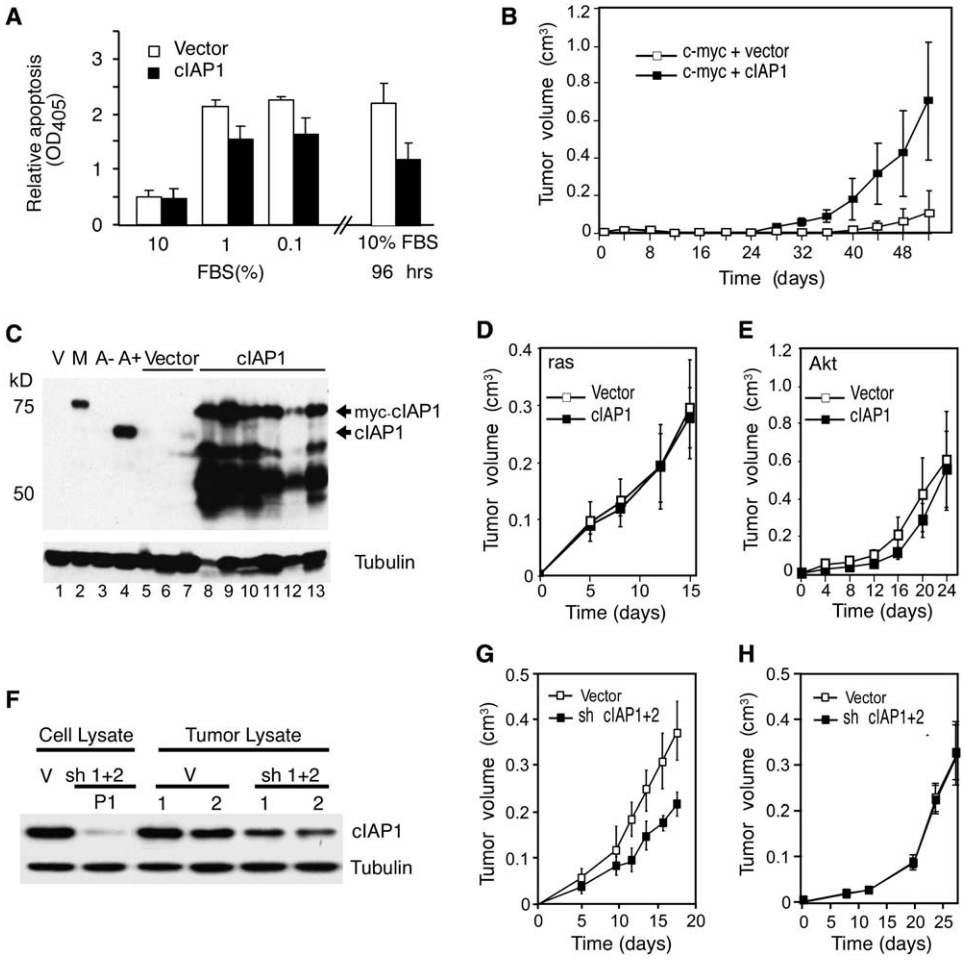

Figure 6. *cIAP1* Enhances the Tumorigenicity of *myc*-Overexpressing $p53^{-/-}$ Hepatoblasts

(A) Apoptosis measurements (Cell Death Detection ELISA^{PLUS} kit; Roche) from $p53^{-/-}$ hepatoblasts double infected with *myc* + *cIAP1* or *myc* + vector following culture under the indicated serum conditions for 48 hr (left panel). Cells grown to confluence were cultured for another 48 hr, and cell death was measured (right panel). Error bars represent mean ± SD (n = 3) per data point.

(B) Tumor volume measurements at various times following subcutaneous injection of $p53^{-/-}$ hepatoblasts double infected with *myc* + *cIAP1* or *myc* + vector into the rear flanks of nude mice (n = 6 for each group). Shown is a representative of three independent experiments, each showing a statistical difference between cIAP1 and control ($p < 0.05$). Error bars represent ±SD.

(C) Immunoblotting of tumors overexpressing *myc*-tagged *cIAP1* (lanes 8–13) or control vector tumors (lanes 5–7) using antibodies against cIAP1. Samples from cultured *myc*-tagged *cIAP1*-expressing hepatoblasts (M, lane 2) or vector alone (V, lane 1) and from 9qA1 amplicon-containing cells (A+, lane 4) were analyzed for comparison. Note that *myc*-tagged *cIAP1* migrates at 75 kDa and endogenous cIAP1 at 65 kDa. A− is lysate from amplicon-negative cells of the same genotype ($p53^{-/-}$;*myc*). Tubulin was used as a loading control.

(D and E) $p53^{-/-}$ hepatoblasts coexpressing *Ras* (D) or *Akt* (E) with *cIAP1* or a control vector were monitored for tumorigenicity following subcutaneous injection into nude mice (n = 6 per group). Error bars represent ±SD.

(F) Immunoblotting of lysates derived from hepatoma cells outgrown from a 9qA1-positive $p53^{-/-}$;*myc* tumor transduced with shRNAs targeting cIAP1 and cIAP2 (sh 1+2) or control vectors (V) using antibodies against cIAP1.

(G and H) 9qA1-positive (G) and -negative (H) hepatoma cells expressing cIAP1/2 shRNAs or a control vector were monitored for tumor growth following subcutaneous injection into nude mice. Error bars represent ±SD.

We also tested whether *Yap* was required for efficient tumor growth. Two distinct shRNAs were capable of suppressing Yap expression, and, interestingly, cells expressing either *Yap* shRNA downregulated cyclin E (Figure 7F). Despite the incomplete suppression of *Yap*, cells harboring the 9qA1 amplicon and expressing either *Yap* shRNA showed slower tumor progression compared to controls following injection into recipient mice (Figure 7G; $p < 0.05$ [0.013 (shYap 2)/0.018 (shYap 1)] at day 25 postinjection). Together, these data validate *Yap* as a potent oncogene.

cIAP1 and *Yap* Cooperate to Promote Tumorigenesis

As prevailing view in cancer genomics is that focal genomic amplifications contain a key driver gene that is selected for during tumorigenesis, it was surprising to identify two oncogenes in the same focal amplicon. To determine

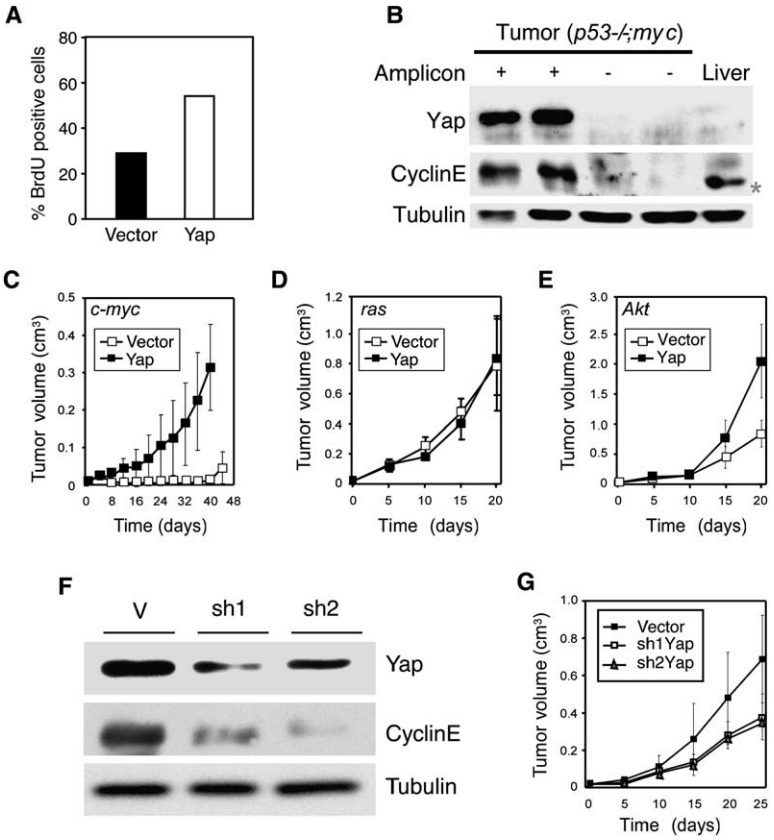

Figure 7. *Yap* Confers a Proliferative Advantage, Has Oncogenic Properties, and Is Required for Liver Tumor Progression

(A) The proliferation rates of $p53^{-/-}$;*myc* hepatoblasts expressing Yap or a control vector were assessed by the fraction of nuclei incorporating BrdU after a 1 hr pulse.

(B) Protein lysates from two 9qA1 amplicon-positive tumors (+), two amplicon-negative tumors (−), and adult normal mouse liver were immunoblotted with antibodies against Yap and cyclin E. Tubulin was used as a loading control. * denotes a nonspecific band in the liver lysate.

(C–E) The tumorigenicity of $p53^{-/-}$ liver progenitor cells coexpressing the indicated oncogene (upper left) with a control vector or Yap was assessed by caliper measurement following subcutaneous injection into the rear flanks of nude mice (n = 4 per group). Error bars represent ±SD.

(F) Protein lysates from hepatoma cells ($p53^{-/-}$;*myc*) stably overexpressing Yap were infected with control vector (V) or two different short-hairpin RNAs targeting Yap (sh1 and sh2) and were analyzed for Yap and cyclin E levels. Tubulin was used as a loading control.

(G) Tumorigenicity of 9qA1-positive cells infected with retroviral vectors expressing shRNAs targeting Yap (sh1Yap or sh2Yap) or control vector (n = 6 per group). Error bars represent ±SD.

whether *cIAP1* and *Yap* might cooperate during tumorigenesis, $p53^{-/-}$;*myc* liver progenitor cells were infected with either *Yap* and control vector or *Yap* plus *cIAP1* and assayed for their ability to form tumors in vivo. Tumors arising from $p53^{-/-}$;*myc* hepatoblasts coexpressing cIAP1 and Yap grew faster than those expressing either oncogene alone (Figure 8A; $p < 0.005$ and $p < 0.05$ [0.011] for cIAP1 + Yap versus cIAP1 or Yap alone, respectively). These effects were not merely additive: At time points when tumors expressing *Yap* alone were small and those harboring *cIAP1* alone were barely detectable, tumors coexpressing Yap and cIAP1 were so large that the animals had to be sacrificed (Figures 8A and 8B). Other gene combinations did not have these effects; for example, coexpression of cIAP2 and cIAP1 had no further impact on tumorigenesis compared to cIAP1 alone, and the combination of Porimin with Yap appeared to even delay tumorigenesis (data not shown). Thus, our study establishes that two adjacent genes from the same focal amplification can cooperate during tumorigenesis.

DISCUSSION

A New Mouse Model of Hepatocellular Carcinoma

Here we develop and utilize a genetically tractable mouse model of hepatocellular carcinoma that is based on the isolation and ex vivo genetic manipulation of mouse embryonic liver progenitor cells followed by their seeding into the livers of recipient mice. This model produces pathologies that resemble human liver carcinomas and has other features that make it amenable for studying liver cancer biology. First, the ability to manipulate liver progenitor cells—one presumed target of transformation in hepatocarcinogenesis—ex vivo allows the rapid production and analysis of tumors with complex genotypes without the cost and effort associated with genetic intercrossing of cancer-prone strains. Second, the fact that the system relies on transplantation of progenitor cells implies that the recipient mice could also have different genotypes, thereby facilitating studies of tumor/host interactions. Third, the model uses bipotential liver progenitors as the cancer-initiating cell, making it suitable for studying a potential "cell of origin" of different primary liver cancers. Finally, the ability to rapidly generate pathologically accurate liver tumors with different genotypes makes the model an ideal preclinical system for testing new drugs or drug combinations for HCC, a disease for which current therapies are ineffective.

Identification and Validation of *cIAP1* and *Yap* as Human Oncogenes

We noted that amplifications of chromosome 9qA1 occurred at a high frequency in murine tumors derived from Myc-expressing cells, and at a lower frequency at

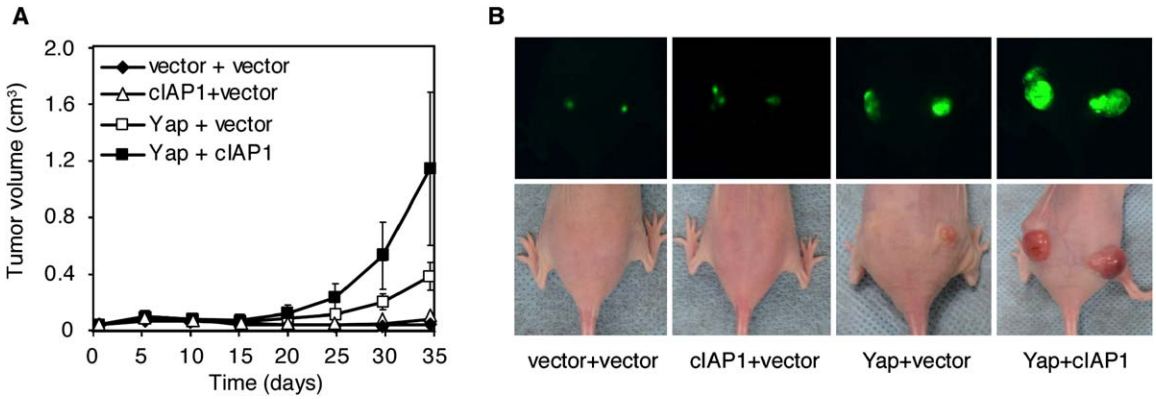

Figure 8. cIAP1 and Yap Synergize to Drive Tumorigenesis

(A) *p53*$^{-/-}$*;myc* liver progenitor cells were infected with control vector, *cIAP1*, or *Yap* or coinfected with *Yap* + *cIAP1* and then transplanted subcutaneously into nude mice (n = 6 per group). Error bars represent ±SD.

(B) GFP imaging of the tumors described in (A).

the syntenic region of chromosome 11q22 in human liver cancers. Although the 11q22 amplicon has been observed in several tumor types, previous studies have not agreed on the likely driver gene(s), in part, because validation was lacking (Dai et al., 2003; Snijders et al., 2005; Bashyam et al., 2005; Imoto et al., 2001). However, through comparative oncogenomics and expression analyses, we pinpointed *cIAP1* and *Yap* as the most likely driver genes in liver cancer; then, by returning to the mouse model, we rapidly demonstrated the oncogenic capacity of *cIAP1* and *Yap* in the genetic setting where spontaneous amplification occurred. Despite the detection of the 11q22 amplicon in only 5%–10% of human tumor types examined, its association with common malignancies including lung, ovarian, esophageal, and liver carcinoma suggest that the overall contribution of *cIAP1* and *Yap* to human cancer may be substantial.

The identification of *cIAP1* as an oncogene is interesting in light of the controversial role of IAPs in modulating apoptosis in mammalian cells (Deveraux et al., 1998; Liston et al., 2003; Salvesen and Duckett, 2002). In *Drosophila*, the IAP genes are important cell death regulators, with loss-of-function mutants displaying profound defects in developmental and stress-induced apoptosis (Goyal et al., 2000). In mammalian cells, a large body of work shows that IAPs can suppress apoptosis (Lacasse et al., 1998), although most studies observe relatively modest effects despite overexpression in transient assays, and mice with knockout of single IAP genes do not display substantial apoptotic defects (Conze et al., 2005). Similarly, IAP expression has been associated with various cancer phenotypes (Wright and Duckett, 2005), but no IAP has been decisively linked to tumorigenesis in vivo.

cIAP1 promoted murine HCC in cooperation with *Myc*, an oncogene that drives proliferation but also promotes apoptosis through both p53-dependent and -independent mechanisms (Lowe et al., 2004). As in other cell types, Myc sensitizes hepatoblasts to diverse proapoptotic signals, and dampening of such signals is linked to tumorigenesis in other contexts. In our system, the effects of *cIAP1* appeared more pronounced in vivo, suggesting that microenvironmental effects associated with tumor expansion may be critical for its prosurvival activities. Perhaps this explains the variable effects of cIAP1 observed in previous in vitro studies; alternatively, nonapoptotic cIAP1 activities may contribute to its oncogenic role. In any case, the antiapoptotic activity of *cIAP1* would be unlikely to cooperate with Akt or Ras, which already transmit prosurvival signals (Lowe et al., 2004).

The finding that *Yap* is also an oncogene in the 9qA1/11q22 amplicon is surprising considering its ability to potentiate the proapoptotic functions of p73, a p53 family member (Basu et al., 2003). However, *yorkie* (*yki*), the *Drosophila* homolog of mammalian *Yap*, links *warts/large tumor suppressor* (*wts/lats*) signaling and transcriptional regulation in the Hippo (*hpo*) pathway (Huang et al., 2005). This pathway also includes the genes *salvador* (*sav*), *mob as tumor suppressor* (*mats*), *NF2/Merlin*, and *expanded* (Hamaratoglu et al., 2006; Edgar, 2006) and functions to precisely control tissue expansion by coordinately regulating cell proliferation and cell death. This is ultimately achieved through transcriptional regulation of *cyclin E* and the *Drosophila* inhibitor of apoptosis protein, *dIAP*.

Interestingly, mammalian homologs exist for most Hippo pathway members (Chan et al., 2005; Tamaskovic et al., 2003), some of which have previously been linked to cancer development (e.g., *Lats2* and *NF2*) (St John et al., 1999; Takahashi et al., 2005; McClatchey and Giovannini, 2005). In addition, *Drosophila* mutants can be rescued by the human homologs for *Yap*, *LATS1*, *MATS1*, and *MSTS2*. Interestingly, although *Yap* may have additional targets in mammalian cells, we note that mammalian cells and tumors with high Yap levels proliferate rapidly and have high levels of cyclin E. It is intriguing that *cIAP1*—a gene with structural and functional similarity to *dIAP*—resides in an adjacent chromosomal location in

mice and humans and is coamplified with *Yap* in tumors. These data demonstrate a functional conservation and reveal the potential importance of the Hippo signaling network in hepatocellular carcinoma and perhaps other cancers. In further support of this possibility, Yap can also transform mammary epithelial cells in vitro (D. Haber and J. Brugge, personal communication).

Perhaps the most remarkable observation in our study was the demonstration that two genes embedded within the same focal amplification cooperate in cancer development. Thus, while *cIAP1* and *Yap* can act independently as oncogenes, they synergize in transforming hepatoblasts and promoting tumorigenesis. Such codriver effects may not be restricted to the 11q22 amplicon, as suggested in a recent study of the 20q13.2 breast cancer amplicon, where there is universal overexpression of two genes: *ZNF217*, which is capable of immortalizing human mammary epithelial cells in culture, and prefoldin 4 (*PFDN4*) (Collins et al., 2001). These results underscore the complexity of cancer genomes and should force a reexamination of well-characterized amplification events for the presence of additional oncogenic lesions and potential drug targets.

Comparative Oncogenomics to Accelerate Cancer Genome Annotation and Target Identification

Our study took advantage of cross-species comparisons to help prioritize candidate oncogenes and then used a relevant mouse model to validate their role in tumorigenesis. Thus, our genome-wide analysis of human HCC identified a large number of genetic changes including the 11q22 amplicon, but the fact we detected a syntenic lesion on chromosome 9qA1 in a specific type of murine HCC provided a filter that allowed us to prioritize this lesion for further study. Importantly, our analysis of mouse tumors identified an appropriate setting to study the oncogenic potential of *cIAP1* and *Yap*: a genetic background in which amplification of *cIAP1* and *Yap* spontaneously occurred. A similar comparative genomic approach has been applied by Chin and colleagues to identify *NEDD9* as a metastasis-promoting gene that allows escape from tumor cell dependence on Ras signaling (Kim et al., 2006 [this issue of *Cell*]).

Moving forward, our study suggests an integrative strategy to complement the Human Cancer Genome Project, whose goal is to catalog all of the mutations that contribute to human cancer (Garber, 2005). The advantages of using genomics to survey human tumors are obvious: These tumors harbor changes relevant to the human disease. However, spontaneous human cancers are heterogeneous and can display extreme genomic instability. Thus, it is often difficult to separate causative from passenger mutations or to identify the key gene (or genes) in a larger region of chromosomal gain or loss (Cox et al., 2005; Futreal et al., 2005). Even with a statistically good candidate, validation can be challenging—it is often necessary to survey many in vitro assays to link a particular lesion to cancer phenotypes. Furthermore, without in vivo validation, therapeutic development efforts are difficult to envision.

By integrating data from human cancer genomics with corresponding data from appropriate mouse models, it should be possible to expeditiously focus on the cancer lesions most likely to have translational potential. First, identifying common lesions through cross-species comparisons increases confidence that these lesions will prove important, a particularly relevant issue when studying relatively low-frequency events. In fact, owing to reduced selective pressure for multiple mutations and/or inherent differences in telomere biology (Artandi and Depinho, 2000), genetically defined murine tumors are often less complex than their corresponding human counterparts. Second, by incorporating murine tumors into the analysis, it is possible to increase the sample size and exploit synteny to exclude candidates outside the region of overlap. Third, genetically engineered mice, by definition, develop more defined cancers than humans. Thus, the identification of a particular lesion in murine cancers immediately provides information concerning the evolutionary context in which it arose and identifies a relevant setting in which to study its oncogenic potential. Finally, mouse models provide excellent settings to test the suitability of a cancer lesion as a therapeutic target and, at the same time, provide ideal preclinical models for subsequent drug testing. We believe that such integrative approaches will speed up the pace of discovery and help translate the promise of cancer genetics into improvements in cancer diagnosis, prognosis, and therapy.

EXPERIMENTAL PROCEDURES

Generation of Genetically Defined Liver Carcinomas, Tumor Retransplantation, Analysis, and Immunohistochemistry

Isolation, culturing, and retroviral infection of purified hepatoblasts as well as surgical procedures, retrorsine treatment of mice, tumor monitoring, and tumor retransplantation are described in the Supplemental Experimental Procedures and Zender et al. (2006). All retroviruses were based on MSCV vectors containing human cDNAs encoding for *myc*, *H-Ras*^{V12}, and *Akt* or the murine cDNAs encoding *Yap* and *myc*-tagged *cIAP1*. Short-hairpin RNAs against *cIAP1*, *cIAP2*, or *Yap* were expressed from the LTR promoter of MSCV retroviruses. Tumor volume (cm^3) was calculated as length × width × height. Paraffin-embedded liver tumor sections were stained with hematoxylin and eosin according to standard protocols or with α-GFP (Abcam 290). Standard Proteinase K antigen retrieval was used. Human hepatocellular carcinomas were analyzed using antibodies against Ck8 (RDI), cIAP1 (Silke et al., 2005), cIAP2 (sc-7944, Santa Cruz), YAP1 (sc-15407, Santa Cruz), and Porimin (IMG472, Imgenex).

Immunoblotting

Fresh tumor tissue or cell pellets were lysed in RIPA buffer using a tissue homogenizer. Equal amounts of protein (16 μg) were separated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. The blots were probed with antibodies against cIAP1 (Silke et al., 2005), cIAP1/2 (gift from P. Liston; 1:2000), YAP1 (sc-15407, Santa Cruz; 1:200), Porimin (IMG472, Imgenex; 1:300), cyclin E (#06-459, Upstate; 1:500), tubulin (B-5-1-2, Sigma; 1:5000), vimentin (Abcam; 1:1000), cytokeratin 19 (Biocare Medical; 1:1000), albumin (Biogenesis; 1:5000), or AFP (Dako; 1:1000).

Cell Proliferation Assay and Cell Death ELISA

Cells plated on gelatin-coated coverslips were incubated with 5-bromo-2′-deoxyuridine (BrdU; 100 μg/ml; Sigma) for 1 hr. Nuclei incorporating BrdU were visualized by immunolabeling using anti-BrdU antibody (Pharmingen; 1:400) as previously described (Narita et al., 2003). DNA was visualized by DAPI (1 μg/ml) after permeabilization with 0.2% Triton X-100/PBS. Cells were grown in various concentrations of serum, and apoptosis was measured using the Cell Death Detection ELISA^{PLUS} kit (Roche).

Representational Oligonucleotide Microarray Analysis

Human tumor samples were obtained from the NCI-sponsored Cooperative Human Tissue Network or the tissue bank of the University of Hong Kong. Genomic DNA was isolated from human or mouse tumors using the PureGene DNA Isolation Kit (Gentra). Hybridizations were carried out on 85K arrays (NimbleGen) (Lucito et al., 2003; B. Lakshmi, I.M. Hall, C. Egan, J. Alexander, J. Healy, L.Z., W.X., M.S.S., S.W.L., M.W., and R.L., unpublished data). The genome position was determined from the UCSC GoldenPath browser (freezes April 2003 for human and February 2003 for mouse). Focal gains or losses were defined as spanning <5 Mb.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed on a PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). Quantification of genomic copy number was based on standard curves derived from serial dilutions of normal human genomic DNA (Invitrogen). For quantitation of mRNA expression, mouse tumors were freshly homogenized in Trizol (GIBCO). RNA was isolated and treated with RNase-free DNase (QIAGEN) and purified over QIAGEN RNAeasy columns. Total RNA was converted to cDNA using TaqMan reverse transcription reagents (Applied Biosystems) and used in qPCR reactions with incorporation of Sybr Green PCR Master Mix (Applied Biosystems) done in triplicate using gene-specific primers. Quantification of mRNA expression of human tumor samples was performed using TaqMan probes (Applied Biosystems). Samples were normalized to the level of β-actin. Primer sequences are listed in Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, four figures, and three tables and can be found with this article online at http://www.cell.com/cgi/content/full/125/7/1253/DC1/.

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