Mouse Double Minute homolog 4 (MDM4) gene up-regulation often occurs in human hepatocellular carcinoma (HCC), but the molecular mechanisms responsible for its induction remain poorly understood. Here we investigated the role of the phosphoinositide-3-kinase/v-akt murine thymoma viral oncogene homolog/mammalian target of rapamycin (PI3K/AKT/mTOR) axis in the regulation of MDM4 levels in HCC. The activity of MDM4 and the PI3K/AKT/mTOR pathway was modulated in human HCC cell lines by way of silencing and overexpression experiments. Expression of main pathway components was analyzed in an AKT mouse model and human HCCs. MDM4 inhibition resulted in growth restraint of HCC cell lines both in vitro and in vivo. Inhibition of the PI3K-AKT and/or mTOR pathways lowered MDM4 protein levels in HCC cells and reactivated p53-dependent transcription. Deubiquitination by ubiquitin-specific protease 2a and AKT-mediated phosphorylation protected MDM4 from proteasomal degradation and increased its protein stability. The eukaryotic elongation factor 1A2 (EEF1A2) was identified as an upstream inducer of PI3K supporting MDM4 stabilization. Also, we detected MDM4 protein up-regulation in an AKT mouse model and a strong correlation between the expression of EEF1A2, activated/phosphorylated AKT, and MDM4 in human HCC (each rho > 0.8, P < 0.001). Noticeably, a strong activation of this cascade was associated with shorter patient survival. Conclusion: The EEF1A2/PI3K/AKT/mTOR axis promotes the protumorigenic stabilization of the MDM4 proto-oncogene in human HCC by way of a posttranscriptional mechanism. The activation level of the EEF1A2/PI3K/AKT/mTOR/MDM4 axis significantly influences the survival probability of HCC patients in vivo and may thus represent a promising molecular target. (HEPATOLOGY 2014;59:1886-1899)
carcinoma (HCC) shows a substantial geographical variation. Indeed, HCC display a surprisingly low frequency of p53 mutations in Western countries, ranging between 10 to 20%, whereas up to 50% of HCC samples from Southeast Asia harbor p53 mutations. Such a different frequency is mainly due to the exposure to certain etiological agents (e.g., aflatoxin B1). Nevertheless, although point mutations represent the most common mechanism responsible for p53 inactivation in cancer, additional mechanisms achieving the same effect in the absence of p53 mutations have been described. Functional inactivation of wild-type p53 by posttranscriptional mechanisms such as phosphorylation and proteasomal degradation has been demonstrated in various human cancers, including HCC. In mammalian cells, the activity of wild-type p53 is mainly under control of its negative regulator protein, Mouse Double Minute homolog 2 (MDM2), which maintains p53 at low levels, thus allowing the growth of normal cells. MDM2 is able to bind to the transcription domain of p53 and promotes its proteasomal degradation by functioning as an E3 ligase. Another p53 binding protein, Mouse Double Minute 4 (MDM4, MDMX, or HDMX), has been identified in mammalian cells. Due to its sequence homology to (MDM4, MDMX, or HDMX), has been identified in p53 binding protein, Mouse Double Minute 4 mostly those harboring a wild-type p53 gene. HCCs harboring wild-type p53, and corresponding peritumorous nonneoplastic liver tissues (PT) from a previous study were used. Patient characteristics are shown in Table 1. Liver tissues were kindly provided by Snorri S. Thorgeirsson (National Cancer Institute, Bethesda, MD). Institutional Review Board approval was obtained from participating hospitals and the National Institutes of Health.

Materials and Methods

Human Tissue Samples. Five normal livers, 48 HCCs harboring wild-type p53, and corresponding peritumorous nonneoplastic liver tissues (PT) from a previous study were used. Patient characteristics are shown in Table 1. Liver tissues were kindly provided by Snorri S. Thorgeirsson (National Cancer Institute, Bethesda, MD). Institutional Review Board approval was obtained from participating hospitals and the National Institutes of Health.

Hydrodynamic Injection and Mouse Treatment. Wild-type FVB/N mice were subjected to hydrodynamic injection as described previously. Briefly,
10 µg of the pCMV/SB and pT3-EF1x-HA-myr-AKT constructs in a ratio of 1:25 were diluted in 2 mL of 0.9% NaCl, filtered, and injected into the lateral tail vein of 7-week-old mice in 7 to 9 seconds. Injected mice were monitored and sacrificed in groups after 12 weeks and 28 weeks. An additional group of AKT-injected mice was subjected, 4 weeks after hydrodynamic injection, to administration of either vehicle (n = 4) or Sirolimus (Rapamune; 5 mg/kg, n = 5) by oral administration for 5 days. Liver tissue was harvested 5 hours after the last dose. Sirolimus was obtained from the UCSF Pharmacy. Mice were housed, fed, and treated in accordance with protocols approved by the Committee for Animal Research at the University of California, San Francisco.

**Cell Lines, Transfection, Xenograft Model, and Treatments.** Culturing conditions, transfections of human HCC cell lines, xenograft model, and treatment with specific inhibitors were performed as described in the Supporting Materials.

**Western Blot Analysis and Immunoprecipitation.** Liver tissues were processed as reported in the Supporting Materials. The primary antibodies used are shown in Supporting Table 1.

**Tissue Microarrays and Immunohistochemistry.** The tissue microarray (TMA) and the immunohistochemical analyses are described in the Supporting Materials.

**Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR).** Quantitative real-time RT-PCR was performed as reported previously. Primer sequences are listed in Supporting Table 2.

**Statistical Analysis.** Statistical analyses were performed as reported in the Supporting Materials.

**Results**

**MDM4 Sustains the Growth of HCC Cells by Way of p53-Dependent and Independent Mechanisms.** Previous findings indicated that the oncogenic activity of MDM4 is due to its ability to inactivate the transcriptional function of the p53 tumor suppressor.
gene. To test the relevance of MDM4 for HCC cell growth in vitro, HuH6 and HepG2 (harboring wild-type p53) as well as HuH7 and Hep3B cells (harboring mutated or deleted p53) were subjected to MDM4 silencing by way of small interfering RNA (siRNA) (Fig. 1A-C). Suppression of MDM4 led to growth restraint in all cell lines tested, due to decreased proliferation and increased apoptosis (Fig. 1B,C). The in vivo relevance of these findings could be confirmed using a xenograft mouse model (Fig. 1D). In addition, double inhibition of MDM4 and p53 using genespecific siRNAs partially rescued the effect of MDM4 knockdown in HepG2 cells, while HuH7 cells remained largely unaffected (Supporting Fig. 1). Suppression of MDM4 resulted in a strong increase of p53 target genes, including p21 and PUMA, in p53 wild-type cell lines, with little or no changes in expression of the same genes in p53 mutant cell lines (Fig. 1A). Treatment with the MDM4 inhibitor SJ-172550, which disrupts the binding between p53 and MDM4, resulted in a dose-dependent growth inhibition and up-regulation of p53 target genes only in cell lines with wild-type p53, with no appreciable effects on the same parameters in cell lines with mutant p53 (Fig. 1A; Supporting Figs. 2-5). On the other hand, transient overexpression of MDM4 in the SNU423
Fig. 2.
cell line, harboring a mutant p53 gene, resulted in growth acceleration in vitro (Supporting Fig. 6; $P < 0.01$). Altogether, these data indicate that MDM4 promotes the growth of HCC cells in a p53-dependent and -independent manner.

**PI3K-AKT Signaling Is Involved in the Regulation of the MDM4 Protein Levels in HCC.** Since it has been shown that the AKT serine/threonine kinase can stabilize MDM4 in various cancer cell lines, we assessed the role of the PI3K-AKT pathway in the regulation of MDM4 in human HCC cell lines. For this purpose, we performed a time-course experiment using the PI3K inhibitor LY294002 in the same cell lines. MDM4 protein decrease started 4 hours after treatment and MDM4 protein levels remained low compared to dimethyl sulfoxide (DMSO)-treated (control) cells for at least 24 hours. This effect was observed in all analyzed cell lines independently of the p53 gene status and was paralleled by a decrease in MDM2 protein in HepG2 and HuH7 cells (Fig. 2A; Supporting Fig. 7). Gene-specific siRNA-mediated targeting of AKT1 and/or AKT2 isoforms (Fig. 2C; Supporting Figs. 8, 9) confirmed the involvement of the PI3K-AKT axis in the regulation of the MDM4 protein levels. LY294002 treatment did not affect MDM4 mRNA levels (Fig. 2B; $P > 0.05$), suggesting that the PI3K pathway regulates MDM4 expression through a posttranscriptional mechanism. Importantly, the decrease in MDM4 protein levels was associated with the reactivation of p53-dependent transcriptional activity, as determined by quantitative RT-PCR of several p53 target genes in HepG2 cells (p53 wild-type; Fig. 2B). Indeed, a strong and significant increase of p21 mRNA was recorded in LY294002-treated cells as early as 4 hours after treatment ($2.27 \pm 0.57$ versus $1.0 \pm 0.02$ in DMSO-treated cells; $P < 0.05$). Similar effects were observed for the BCL2-associated X protein (BAX; at 8 hours: $1.26 \pm 0.07$ versus $1.0 \pm 0.02$, respectively; $P < 0.01$), the BCL2 binding component 3 (PUMA; at 4 hours: $1.44 \pm 0.02$ versus $1.0 \pm 0.07$, respectively; $P < 0.01$), and the MDM2 (at 8 hours: $1.55 \pm 0.10$ versus $1.0 \pm 0.03$, respectively; $P < 0.01$).

**mTOR Pathway Cooperates With PI3K/AKT Signaling to Stabilize MDM4.** Since AKT activation can lead to induction of mTOR signaling, we next analyzed whether the mTOR pathway contributes to the regulation of MDM4. Treatment with the mTOR complex 1 (mTORC1) inhibitor rapamycin resulted in a significant reduction of MDM4 protein levels in HCC cells. The specificity of the rapamycin effect was confirmed by the decreased phosphorylation of the downstream mTORC1 target, ribosomal protein S6 kinase (RPS6K) (Fig. 2D; Supporting Fig. 10). In addition, treatment with PI103 (a PI3K-, mTORC1-, and mTORC2-multikinase inhibitor) efficiently knocked down the MDM4 expression in HCC cell lines for at least 48 hours (Fig. 2E; Supporting Fig. 11) and significantly reduced the cell viability of HepG2 and Hep3B cells after 24 hours (40% and 42% reduction compared to DMSO-treated controls, respectively; $P < 0.01$ and 48 hours (62% and 71% reduction compared to DMSO-treated controls, respectively; $P < 0.01$; Fig. 2F) following treatment. The influence of both branches of the mTOR pathway (mTORC1 and mTORC2) on the regulation of the MDM4 protein level was further confirmed by siRNA-mediated silencing of regulatory associated protein of mTORC1 (Raptor) and Raptor independent companion of mTORC2 (RICTOR; Supporting Fig. 12).

**EEF1A2 Oncogene Induces MDM4 Up-Regulation by Way of AKT Activation.** Recently, we have found that EEF1A2 is frequently up-regulated concomitant with MDM4 in human HCC and exerts oncogenic functions. Since EEF1A2 has been described to promote its protumorigenic effect at least partly through activation of the AKT proto-oncogene, we assessed whether the same applies to HCC. Noticeably, siRNA-mediated silencing of EEF1A2 in human HCC cells resulted in decreased phosphorylation of AKT and
Fig. 3.
RPS6K. Additionally, MDM4 levels were markedly decreased (Fig. 3A). As a consequence, transcription of p53 target genes was significantly up-regulated (p21: 2.9-fold; BAX: 1.9-fold; PUMA: 1.5-fold, and MDM2: 2.8-fold compared to scrambled-treated cells, each \( P < 0.05 \), while the MDM4 messenger RNA (mRNA) level was even lowered (Fig. 3B). Conversely, forced overexpression of EEF1A2 in HuH6 cells by transient transfection led to growth acceleration and reduction of apoptosis (Fig. 3D,E; \( P < 0.01 \)), which were paralleled by activation of AKT and up-regulation of MDM4 (Fig. 3C). The changes induced by EEF1A2 overexpression were almost completely reverted by treatment with the AKT1/2 inhibitor (Fig. 3C-E; \( P < 0.01 \)). Altogether, the present data indicate that EEF1A2 promotes MDM4 up-regulation by way of AKT-dependent mechanisms in HCC cells.

**AKT Stabilizes MDM4 and Prevents Its Degradation.** To explore the molecular mechanism responsible for MDM4 down-regulation, we analyzed its protein stability following inhibition of PI3K (by using the PI3K inhibitor LY294002), with and without simultaneous inhibition of protein biosynthesis using cycloheximide. A significant decrease in MDM4 protein levels was detected in HepG2 cells after combined treatment with LY294002 and cycloheximide, when compared to the administration of either LY294002 or cycloheximide alone after 8 and 24 hours, respectively (\( P < 0.05 \); Fig. 4A; Supporting Fig. 13), thus confirming a profound impact of the PI3K-AKT pathway on the stability of the MDM4 protein in human HCC cells. To assess whether active AKT signaling may protect MDM4 from proteasomal degradation, we pharmacologically inhibited the proteasome activity using MG132 while treating HepG2, Hep3B, or HuH7 cells with LY294002. Interestingly, MG132 treatment rescued the decrease of the MDM4 protein level observed following LY294002 single treatment (Fig. 4B). This protective effect was observed in all cell lines analyzed, indicating an underlying p53- and MDM2-independent molecular mechanism.

Since ubiquitination is a crucial step in flagging proteins for proteasomal degradation and the ubiquitin-specific protease 2a (USP2a), which acts as a deubiquitinase, was recently reported to be induced by AKT in human HCC,\(^\text{17}\) and to protect MDM4 from proteolysis,\(^\text{24}\) we explored a possible protective effect of USP2a on the MDM4 protein levels. We not only detected decreased MDM4 protein levels after siRNA-mediated silencing of USP2a in the Focus cell line (expressing high levels of USP2a),\(^\text{17}\) but we were also able to coinmunoprecipitate USP2a and MDM4 indicating their interaction, which was impaired following USP2a knockdown (Fig. 4C). Also, since a direct interaction between MDM4 and AKT in various tumor cell lines was documented in vitro,\(^\text{16}\) we evaluated whether the same occurs in human HCC cell lines. Immunoprecipitation analyses confirmed a physical interaction between AKT and MDM4 in human HCC cells, indicating that MDM4 is a substrate of AKT kinase activity in human HCC. Of note, the interaction between MDM4 and AKT was significantly decreased when the protein lysates were incubated with lambda protein phosphatase (which removes phosphate groups from phosphorylated serine, threonine, and tyrosine residues in proteins) prior to immunoprecipitation (Fig. 4D). The latter observation indicates that MDM4 requires being phosphorylated in order to bind to AKT.

The crosstalk between AKT and MDM4 was further investigated in the human HLE cell line stably overexpressing AKT1.\(^\text{17}\) The latter cell line showed increased levels of AKT, pAKT, and MDM4 when compared with the nontransfected counterpart (Fig. 4E). Of note, suppression of MDM4 by siRNA induced a much stronger growth inhibition in HLE cells stably transfected with AKT1 when compared with untransfected cells (Fig. 4F; \( P < 0.01 \)). These results indicate that the cell growth properties of AKT on HCC cells depend, at least partly, on MDM4.

**Activation of the EEF1A2-AKT-MDM4 Cascade Has Functional Relevance In Vivo and Is Associated With Shorter Patient Survival.** To investigate whether activated AKT signaling stabilizes MDM4 also in vivo, we took advantage of the recently generated...
AKT mouse model of hepatocarcinogenesis, which has been shown to express elevated protein levels of the deubiquitinase USP2a.\textsuperscript{17} Increased protein levels of activated/phosphorylated AKT, USP2a, and MDM4 were detected by western blotting in 12- and 28-week-old AKT mice compared to wild-type littermates (Fig. 5A,B; each $P < 0.001$). Also, a strong correlation between AKT phosphorylation and USP2a expression was observed (Spearman’s rho = 0.88, $P < 0.001$), pAKT and MDM4 protein expression (Spearman’s rho = 0.93, $P < 0.001$), and USP2a and MDM4 protein expression (Spearman’s rho = 0.91, $P < 0.001$), while the mRNA levels of MDM4 were not altered in AKT mice compared to the wild-type controls (3.3 ± 0.6, respectively, 2.9 ± 0.7 versus 3.2 ± 0.5; each $P > 0.05$; Fig. 5C). Immunohistological analyses revealed an up-regulation of MDM4 in pAKT-expressing neoplastic lesions in AKT mice (Fig. 5D). Of note, treatment of AKT mice with the mTORC1 inhibitor rapamycin resulted in down-regulation of p-AKT, USP2a, and MDM4, as assessed by western blot analysis (Fig. 5E).

Finally, to investigate whether the EEF1A2-PI3K-AKT-mTOR-MDM4 cascade is relevant in human liver tumors, we analyzed the expression of the main signaling components in a collection of human HCCs (n = 48). Using western blotting, we observed a significant increase of EEF1A2 protein expression in human HCCs compared to normal liver (NL) and peritumorous, nonneoplastic liver tissue (PT; 66 ± 2 [NL] versus 82 ± 2 [PT] versus 285 ± 16 [HCC]; $P < 0.001$). This up-regulation was paralleled by an up-regulation of pAKT (46 ± 4 [NL] versus 83 ± 1 [PT] versus 254 ± 15 [HCC]; $P < 0.001$; Fig. 6A/B), USP2a (36 ± 8 [NL] versus 44 ± 2 [PT] versus 190 ± 10 [HCC]; $P < 0.001$; Fig. 6A/B), and MDM4 (69 ± 5 [NL] versus 72 ± 2 [PT] versus 226 ± 13 [HCC]; NL/PT versus HCC: $P < 0.001$; Fig. 6A/B), while p21 protein levels were significantly reduced in human HCCs (255 ± 10 [NL] versus 242 ± 4 [PT] versus 110 ± 8 [HCC]; NL/PT versus HCC: $P < 0.001$; Fig. 6A/B). Survival analysis revealed that high levels of EEF1A2, pAKT, USP2a, and MDM4 associate with a shorter survival of HCC patients after liver resection (each $P < 0.001$; Fig. 6C). In univariate analyses, tumor size was the only clinico-pathological parameter that showed an association with patient survival in the investigated cohort. Indeed, patients with tumors larger than 3 cm showed a median survival time of 15 months, compared to 51 months for patients diagnosed with smaller tumors (hazard ratio [HR] = 2.20, $P = 0.04$). In multivariate analysis, high levels of EEF1A2, pAKT, USP2, and MDM4 were clearly associated with a shorter patient survival (Table 1). Importantly, correlation analyses revealed a strong positive association between EEF1A2 and pAKT (rho: 0.87, $P < 0.001$), EEF1A2 and USP2a (rho: 0.76, $P < 0.001$), EEF1A2 and MDM4 (rho: 0.88, $P < 0.001$), pAKT and USP2a (rho: 0.74, $P < 0.001$), pAKT and MDM4 (rho: 0.94, $P < 0.001$), USP2a and MDM4 (rho: 0.80, $P < 0.001$), while a strong negative association was recorded between EEF1A2 and p21 (rho: −0.75, $P < 0.001$), pAKT and p21 (rho: −0.75, $P < 0.001$), USP2a and p21 (rho: −0.66, $P < 0.001$), and MDM4 and p21 (rho: −0.79, $P < 0.001$). These data were further substantiated by immunohistological analysis of an independent HCC cohort (n = 76; Supporting Materials), which revealed a positive association of EEF1A2 and pAKT immunostaining (rho: 0.27, $P < 0.05$), EEF1A2 expression and nuclear MDM4 expression (rho: 0.41, $P < 0.001$), nuclear MDM4 and p53 (rho: 0.23, $P < 0.05$), and p53 and p21 immunostains (rho: 0.76, $P < 0.001$), while expression of MDM4 and p21 revealed a significant inverse association (rho: −0.79, $P < 0.001$; Fig. 6D).

Thus, our data clearly show that the activation of the EEF1A2-AKT-MDM4 cascade has not only functional relevance in vivo, but its activation level has a strong impact on the survival time of HCC patients.

**Discussion**

MDM4 is one of the main negative regulators of p53 that blocks its transcriptional activity upon binding to its N-terminal transactivation domain.\textsuperscript{11} Recently, MDM4 has been identified as a candidate oncogene in human liver cancer due to its frequent up-regulation in HCC specimens.\textsuperscript{13} MDM4 inhibition resulted in growth restraint of HCC cell lines both in vitro and in vivo. However, the finding that targeting MDM4 in p53-wildtype HuH6 xenografts only resulted in a moderate reduction of tumor growth compared to p53-deficient cells was unexpected. Besides less efficient engrafting of p53-wildtype cells, we observed a significant reduction of shMDM4-expressing cells as determined by reduced green fluorescent protein (GFP) levels in shMDM4- compared to shNC-treated cells and preinjection controls, indicating that the MDM4 knockdown resulted in selection of shMDM4-negative cells (Supporting Fig. 14). As this finding may affect the translational potential of MDM4 targeting therapies and data on p53-independent functions of MDM4 are limited,\textsuperscript{25} future studies are needed to dissect p53-dependent and -independent functions of MDM4.
Although amplification of the MDM4 locus is an important mechanism leading to MDM4 up-regulation, elevated protein levels of MDM4 are also present in HCC samples without MDM4 amplification. In accordance with the latter finding, emerging evidence indicates that additional molecular mechanisms, such as phosphorylation or ubiquitination, can modulate the activity and the protein turnover of MDM4 as well. Here we demonstrate for the first time that activation of the PI3K-AKT-mTOR pathway promotes the up-regulation of MDM4 in human HCCs. Pharmacological targeting of the AKT (LY294002) and the mTOR (rapamycin) pathway alone or in combination (PI103) not only blocked the downstream signaling cascade, but also resulted in a marked down-regulation of the MDM4 protein expression. The strongest effect was observed when PI3K, mTORC1, and mTORC2 activities were...
silenced simultaneously. The relevance of the AKT-mTOR axis for the protumorigenic activity of MDM4 was further demonstrated in a mouse model overexpressing a constitutively active form of AKT in the liver. In this model, we found that active AKT alone is sufficient to induce MDM4 protein levels in vivo. Furthermore, the treatment of AKT mice with rapamycin prevented MDM4 overexpression, likely due to reduced MDM4 biosynthesis. Accordingly, rapamycin treatment resulted in reduced activity of the eukaryotic translation initiation factor 4E in colon carcinoma cells, which impaired the translation activity and resulted in decreased MDM2 protein levels. A similar mechanism may be involved in the mTOR-mediated regulation of MDM4 in human and murine HCC. Since the MDM4 transcription was neither significantly altered following inhibition of PI3K-AKT-mTOR signaling in vitro nor in the AKT mice, a post-transcriptional mechanism is presumably responsible for the down-regulation of the MDM4 protein expression. Indeed, coadministration of the proteasomal inhibitor MG132 rescued the reduced MDM4 protein levels after LY294002 treatment, supporting a post-translational mechanism. In particular, the half-life of the MDM4 protein was significantly decreased after silencing of the PI3K-AKT pathway in vitro, suggesting an important role of the AKT kinase in protecting MDM4 from proteasomal degradation. We were able to identify two independent mechanisms that increase the stability of the MDM4 protein, namely, the protection of MDM4 from degradation and its phosphorylation by AKT. Indeed, we showed that USP2a physically interacts with MDM4 in human HCC cells and that down-regulation of USP2a reduces the MDM4 protein levels, suggesting that the USP2a-mediated deubiquitination of MDM4 inhibits its proteolysis. In addition, we were able to demonstrate a direct interaction between phosphorylated MDM4 and AKT in vitro, supporting the idea that MDM4 might be phosphorylated by AKT in human HCC cells, as it has been reported for several other tumor types. A schematic model of the EEF1A2-mediated posttranslational stabilization of MDM4 is shown in Fig. 7. Interestingly, recent studies indicated that the phosphorylation of serine 367 by AKT results in the stabilization of MDM4, while phosphorylation of this residue by CHK2 promotes its degradation, implying an important role of the molecular/cellular context in
Since there was no correlation between MDM4 phosphorylation at serine 367 and MDM4 protein levels both in human and AKT mouse tissues (Calvisi et al., data not shown), other, so far undefined serine residues may regulate MDM4 stability in HCC.

Using array-based comparative genomic hybridization and the definition of minimal overlapping regions, we previously identified EEF1A2 as a candidate oncogene in human hepatocarcinogenesis. Recent data indicated that EEF1A2 overexpression is a hallmark of prostate and ovarian cancer and plays an
important role in mammary carcinogenesis.\textsuperscript{28–30} In particular, EEF1A2 has been reported to induce the activation of AKT proto-oncogene in breast cancer and rat cell lines.\textsuperscript{23} Here we demonstrate that the siRNA-mediated silencing of EEF1A2 results in reduced levels of pAKT and pRPS6K. Also, we found that EEF1A2 overexpression was strongly associated with up-regulation of pAKT and MDM4 protein levels in human HCC samples. These data imply that the protumorigenic up-regulation of EEF1A2 in human HCCs activates AKT and mTOR signaling, which in turn promote the functional inactivation of p53 by way of stabilization of the MDM4 protein. Importantly, a strong activation of the EEF1A2/P3K/AKT/mTOR-MDM4 axis was associated with shorter survival of HCC patients. Since PI3K, AKT, and mTOR inhibitors that are well tolerated in tumor patients (including HCC) have been developed,\textsuperscript{31–33} human HCC patients, whose tumors show activation of AKT/mTOR signaling, should benefit most from targeting these pathways. In these patients suppression of the AKT/mTOR cascade may result in both the inhibition of the protumorigenic effects driven by AKT/mTOR as well as the induction of tumor-suppressive effects following p53 reactivation. In order to substantiate this intriguing hypothesis and to analyze the p53-dependency of targeting this pathway, HCC patients treated with inhibitors of PI3K-AKT-mTOR signaling should be tested for the p53 mutation and surrogate markers of AKT/mTOR pathway activation, such as phosphorylation of AKT and RPS6K in tumor tissue.

Biopsy-based HCC diagnosis prior to respective future clinical trials, as routinely performed in other human cancers, may support predictive testing and rational inclusion of patients likely to benefit more from PI3K-AKT-mTOR inhibition.

In summary, by using in vitro and in vivo approaches we provide evidence that the EEF1A2/P3K/AKT/mTOR cascade supports the protumorigenic up-regulation of MDM4 in human HCC through a post-transcriptional mechanism involving AKT-mediated phosphorylation of MDM4 and USP2a deubiquitination. The degree of activation of the EEF1A2/P3K/AKT/mTOR/MDM4 axis has impact on the survival probability of HCC patients in vivo and may thus represent a promising molecular target.

\textbf{Acknowledgment:} We thank Verena Kautz, Marianne Hartmann, Susanne Bößer, Veronika Geissler, Sara Messard, and Eva Eiteneuer for excellent technical assistance. This work was supported by the tissue bank of the National Center for Tumor Diseases Heidelberg.

\textbf{References}

1. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000;408:307-310.
2. May P, May E. Twenty years of p53 research: structural and functional aspects of the p53 protein. Oncogene 1999;18:7621-7636.
3. Kubicka S, Trautwein C, Schrem H, Tillmann H, Manns M. Low incidence of p53 mutations in European hepatocellular carcinomas with heterogeneous mutation as a rare event. J Hepatol 1995;23:412-419.
4. Guichard C, Amaddeo G, Imbeaud S, Ladeiro Y, Pelletier L, Maad IB, et al. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet 2012;44:694-698.
5. Hussain SP, Harris CC. Molecular epidemiology of human cancer. Recent Results Cancer Res 1998;154:22-36.
6. Seemann S, Maurici D, Olivier M, Caron de Fromentel C, Hainaut P. The tumor suppressor gene TP53: implications for cancer management and therapy. Crit Rev Clin Lab Sci 2004;41:551-585.
7. Momand J, Zambetti GP. Mdm-2: “big brother” of p53. J Cell Biochem 1997;64:343-352.
8. Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 1992;69:1237-1245.
9. Shvarts A, Bazuine M, Dekker P, Ramos YF, Steegenga WT, Merckx G, et al. Isolation and identification of the human homolog of a new p53-binding protein, Mdmx. Genomics 1997;43:5349-5357.
10. Shvarts A, Steegenga WT, Riteco N, van Laar T, Dekker P, Bazuine M, et al. MDMX: a novel p53-binding protein with some functional properties of MDM2. EMBO J 1996;15:5349-5357.
11. Marine JC, Jochemsen AG. Mdmx as an essential regulator of p53 activity. Biochem Biophys Res Commun 2005;331:750-760.
12. Riemenschneider MJ, Buschges R, Wolter M, Reifenberger J, Bostrom J, Kraus JA, et al. Amplification and overexpression of the MDM4 (MDMX) gene from 1q32 in a subset of malignant gliomas without TP53 mutation or MDM2 amplification. Cancer Res 1999;59:609-616.
13. Schlaeger C, Longerich T, Schiller C, Bewerunge P, Mehrabi A, Toedt G, et al. Etiology-dependent molecular mechanisms in human hepatocarcinogenesis. HEPATOLOGY 2008;47:511-520.
14. Pereg Y, Shkedy D, de Graaf P, Meulmeester E, Edelson-Averbukh M, Salek M, et al. Phosphorylation of Hdmx mediates its Hdm2- and ATM-dependent degradation in response to DNA damage. Proc Natl Acad Sci U S A 2005;102:5056-5061.

15. Chen L, Gilkes DM, Pan Y, Lane WS, Chen J. ATM and Chk2-dependent phosphorylation of MDMX contribute to p53 activation after DNA damage. EMBO J 2005;24:3411-3422.

16. Lopez-Pajares V, Kim MM, Yuan ZM. Phosphorylation of MDMX mediated by Akt leads to stabilization and induces 14-3-3 binding. J Biol Chem 2008;283:13707-13713.

17. Calvisi DF, Wang C, Ho C, Ladu S, Lee SA, Mattu S, et al. Increased lipogenesis, induced by Akt-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma. Gastroenterology 2011;140:1071-1083.

18. Villanueva A, Chiang DY, Newell P, Peix J, Thung S, Alsinet C, et al. Pivotal role of mTOR signaling in hepatocellular carcinoma. Gastroenterology 2008;135:1972-1983, 1983 e1971-1911.

19. Ho C, Wang C, Mattu S, Destefanis G, Ladu S, Delogu S, et al. Akt (v-akt murine thymoma viral oncogene homolog 1) and N-Ras (neuroblastoma ras viral oncogene homolog) coactivation in the mouse liver promotes rapid carcinogenesis by way of mTOR (mammalian target of rapamycin complex 1), FOXM1 (forkhead box M1)/SKP2, and c-Myc pathways. Hepatology 2012;55:833-845.

20. Calvisi DF, Donninger H, Vos MD, Birrer MJ, Gordon L, Leaner V, et al. NORE1A tumor suppressor candidate modules p21CIP1 via p53. Cancer Res 2009;69:4629-4637.

21. Pellegrino R, Calvisi DF, Ladu S, Ehemann V, Stanisca T, Evert M, et al. Oncogenic and tumor suppressive roles of polo-like kinases in human hepatocellular carcinoma. Hepatology 2010;51:857-868.

22. Reed D, Shen Y, Shelat AA, Arnold LA, Ferreira AM, Zhu F, et al. Identification and characterization of the first small molecule inhibitor of MDMX. J Biol Chem 2010;285:10786-10796.

23. Amir A, Noei F, Jeganathan S, Kulkarni G, Pinke DE, Lee JM. eEF1A2 activates Akt and stimulates Akt-dependent actin remodeling, invasion and migration. Oncogene 2007;26:3027-3040.

24. Allende-Vega N, Sparks A, Lane DP, Saville MK. MdmX is a substrate for the deubiquitinating enzyme USP2a. Oncogene 2010;29:432-441.

25. Matijaevic Z, Steinman HA, Hoover K, Jones SN. MdmX promotes bipolar mitosis to suppress transformation and tumorigenesis in p53-deficient cells and mice. Mol Cell Biol 2008;28:1265-1273.

26. Kao CL, Hsu HS, Chen HW, Cheng TH. Rapamycin increases the p53/MDM2 protein ratio and p53-dependent apoptosis by translational inhibition of mdm2 in cancer cells. Cancer Lett 2009;286:250-259.

27. LeBron C, Chen L, Gilkes DM, Chen J. Regulation of MDMX nuclear import and degradation by Chk2 and 14-3-3. EMBO J 2006;25:1196-1206.

28. Scaglia B, Papas B, Bonin S, Grassi M, Zennaro C, Farra R, et al. Dissecting the expression of EEF1A1/2 genes in human prostate cancer cells: the potential of EEF1A2 as a hallmark for prostate transformation and progression. Br J Cancer 2012;106:166-173.

29. Pinke DE, Lee JM. The lipid kinase PI4KIIIbeta and the eEF1A2 oncogene co-operate to disrupt three-dimensional in vitro acinar morphogenesis. Exp Cell Res 2011;317:2503-2511.

30. Pinke DE, Kalloger SE, Franchetti T, Huntsman DG, Lee JM. The prognostic significance of elongation factor eEF1A2 in ovarian cancer. Gynecol Oncol 2008;108:561-568.

31. Bendell JC, Rodon J, Burris HA, de Jonge M, Verweij J, Birle D, et al. Phase I, dose-escalation study of BKM120, an oral pan-Class I PI3K inhibitor, in patients with advanced solid tumors. J Clin Oncol 2012;30:282-290.

32. Yap TA, Yan L, Patnaik A, Fearen I, Olmos D, Papadopoulos K, et al. First-in-man clinical trial of the oral pan-AKT inhibitor MK-2206 in patients with advanced solid tumors. J Clin Oncol 2011;29:4688-4695.

33. Zhu AX, Abrams TA, Miksad R, Blaszczovsky LS, Meyerhardt JA, Zheng H, et al. Phase 1/2 study of everolimus in advanced hepatocellular carcinoma. Cancer 2011;117:5094-5102.