Met acts on Mdm2 via mTOR to signal cell survival during development

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Coordination of cell death and survival is crucial during embryogenesis and adulthood, and alteration of this balance can result in degeneration or cancer. Growth factor receptors such as Met can activate phosphatidyl-inositol-3′ kinase (PI3K), a major intracellular mediator of growth and survival. PI3K can then antagonize p53-triggered cell death, but the underlying mechanisms are not fully understood. We used genetic and pharmacological approaches to uncover Met-triggered signaling pathways that regulate hepatocyte survival during embryogenesis. Here, we show that PI3K acts via mTOR (Frap1) to regulate p53 activity both in vitro and in vivo. mTOR inhibits p53 by promoting the translation of Mdm2, a negative regulator of p53. We also demonstrate that the PI3K effector Akt is required for Met-triggered Mdm2 upregulation, in addition to being necessary for the nuclear translocation of Mdm2. Inhibition of either mTOR or Mdm2 is sufficient to block cell survival induced by Hgf-Met in vitro. Moreover, in vivo inhibition of mTOR downregulates Mdm2 protein levels and induces p53-dependent apoptosis. Our studies identify a novel mechanism for Met-triggered cell survival during embryogenesis, involving translational regulation of Mdm2 by mTOR. Moreover, they reinforce mTOR as a potential drug target in cancer.

KEY WORDS: Met receptor, Hepatocyte growth factor, Receptor tyrosine kinase (RTK), Signaling in vivo, Cell survival, Mdm2, mTOR (Frap1), Mouse

INTRODUCTION

Cell survival is controlled by crosstalk between pro- and anti-apoptotic signals. During development, the tight regulation of cell survival is crucial, and deregulation can lead to pathologies such as degeneration and oncogenesis (Hanahan and Weinberg, 2000; Mattson, 2000; Meier et al., 2000). Growth factors and their receptor tyrosine kinases (RTKs) ensure accurate functioning of the signaling circuitry controlling the survival and death decisions of cells during organogenesis (Schlessinger, 2000; Simon, 2000). So far, only one growth factor has been shown to regulate the survival of embryonic hepatocytes in vivo: hepatocyte growth factor (Hgf) (Bertotti and Comoglio, 2003; Birchmeier et al., 2003). Indeed, loss-of-function of either *hgf* or of its corresponding RTK *met* leads to hepatocyte apoptosis in developing livers (Bladt et al., 1995; Schmidt et al., 1995).

Activated Met transmits signals by recruiting a variety of SH2-domain-containing proteins via the multifunctional docking site located in its C-terminal tail (Ponzetto et al., 1994; Weidner et al., 1996; Bertotti and Comoglio, 2003). In vivo mutation of this anchor (Met[d]) (Maina et al., 1996; Maina et al., 1997; Maina et al., 1998; Helmbacher et al., 2003) recapitulates all of the phenotypes found in *met* or *hgf* knockout embryos (Bladt et al., 1995; Schmidt et al., 1995). Signaling by Met during embryogenesis has been studied further by generating *met*-specificity-switch mutant mice. This genetic analysis has been instrumental in demonstrating that, superimposed on genetic threshold signaling levels, distinct pathways are required to achieve specific biological functions triggered by Met in vivo (Maina et al., 2001; Segarra et al., 2006; Moumen et al., 2007). These genetic studies also revealed that in vivo survival of hepatocytes requires a more complex intracellular signaling

network downstream of Met (Maina et al., 2001; Moumen et al., 2007). It is interesting to notice that loss-of-function mutations of different intracellular signals, such as Raf (also known as Raf1), Jun, N-Myc (also known as Mycn – Mouse Genome Informatics), NFκB and GSK3β, cause the death of embryonic hepatocytes (Beg et al., 1995; Fruman et al., 2000; Hoeflich et al., 2000; Li et al., 1999a; Li et al., 1999b; Rudolph et al., 2000; Taub, 2004). Thus, in embryonic hepatocytes, the balance of cell survival and death appears to be established by distinct modulators that are each necessary, but not individually sufficient.

Survival and death decisions are often controlled by antagonistic signaling networks. The clearest example is provided by the antagonistic functions of p53 and PI3K, which trigger cell death and survival, respectively (Trotman and Pandolfi, 2003; Vivanco and Sawyers, 2002). Crosstalk between the p53 and PI3K pathways occurs at multiple levels via specific downstream effectors. For example, p53 inhibits cell survival by inducing transcription of the phosphatase *pten* (Stambolic et al., 2001), which antagonizes PI3K signaling (Vivanco and Sawyers, 2002). Pten, in turn, can modulate p53 protein levels and function (Freeman et al., 2003). In addition, negative regulation of PI3K signaling by Pten causes decreased Akt phosphorylation and activity, thereby decreasing its pro-survival properties (Vivanco and Sawyers, 2002).

How PI3K acts to prevent cell death induced by p53 is less well understood. Cell culture studies have shown that activation of PI3K upon growth factor stimulation leads to Akt-dependent phosphorylation of the E3 ubiquitin ligase Mdm2, thus promoting both the nuclear entry (Mayo and Donner, 2001; Zhou et al., 2001) and the stabilization of Mdm2 (Feng et al., 2004). This, in turn, favors the nuclear export, ubiquitylation and degradation of p53, thereby reducing its pro-apoptotic activity. However, it remains to be determined whether PI3K can influence the Mdm2-p53 pathway through other signaling mechanisms. Moreover, it is important that the role of such pathways be demonstrated in vivo.

By studying cell survival mechanisms induced by Met, we are searching for novel PI3K-dependent mechanisms that might lead to the inhibition of p53 activity and to the regulation of cell

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survival in vivo. Here, we demonstrate that Met regulates translation and translocation, but not protein stability, of Mdm2. The Met-triggered signaling pathway acting on Mdm2 requires the PI3K effector Akt for both the translation and the translocation of Mdm2. In addition, we demonstrate that mTOR (also known as Frap1 – Mouse Genome Informatics) signaling is also required for the translation of Mdm2 and to antagonize p53-dependent apoptosis. The relevance of these mechanisms is demonstrated both in vitro and in vivo, in the context of the Met-triggered survival of hepatocytes in developing livers.

MATERIALS AND METHODS
Antibodies and reagents
Antibodies used were anti-tubulin (Sigma), anti-ERKs, anti-phospho-T_{389}-p70^{s6k}, anti-phospho-T_{421}/S_{424}-p70^{s6k}, anti-$S_{235/236}$-S6 ribosomal protein, anti-S_{65}-4EBP1, anti-S_{473}-Akt, anti-S_{18}-p53, anti-$S_{21/9}$-GSK3α/β (all Cell Signaling), anti-Mdm2 (Oncogene), rhodamine-conjugated goat anti-digoxigenin (Roche), Cy-5-conjugated goat anti-mouse and peroxidase-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories). Hgf (R&D Systems) was used at a concentration of 50 ng/ml. The pharmacological inhibitors used were PD98059 (MEK inhibitor), SB203580 (p38 inhibitor), L-JNKI 1 (JNK inhibitor), SU6656 (Src family member inhibitor), LY 294002 (PI3K inhibitor), pifithrin-α (p53 inhibitor), rapamycin (mTOR inhibitor) and Nutlin-3 (Mdm2 inhibitor) (all purchased from Calbiochem). A-443654 (Akt inhibitor) was kindly provided by V. L. Giranda (ABBOTT Laboratories, Illinois, USA). The concentrations of each inhibitor are indicated in the figure legends. No toxic effects were observed at the concentrations used. Actinomycin D (Sigma) was used at 0.2 µg/ml and Cycloheximide (Sigma) at 20 µg/ml.

Mice
The generation and genotyping of *met*$^{2P/2P}$ knock-in signaling mutants have been described previously (Maina et al., 2001). To partially rescue placental development, heterozygous males on a mixed C57BI/6×129/sv background were intercrossed with heterozygous females on the outbreed strain CD1 (Maina et al., 2001). Pifithrin-α (2.2 mg/kg) or rapamycin (3 mg/kg) were administered with intra-peritoneal injection into pregnant females at E10.5 and E11.5. Nutlin-3 (200 mg/kg) was injected sub-cutaneously into pregnant females at E10.5 and E11.5. Mice were kept at the Developmental Biology Institute of Marseille-Luminy (IBDML) animal facilities and all experiments were performed in accordance with institutional guidelines.

Hepatocyte cultures
Embryonic hepatocyte cultures were performed as previously described (Maina et al., 2001; Moumen et al., 2007). Briefly, E15.5 dissected livers were digested with collagenase, debris was removed by filtration and cells were collected by centrifugation. Hepatocytes were plated on collagen-treated dishes in hepatocyte attachment media (HAM; Gibco BRL), supplemented with 10% fetal calf serum, 10 µg/ml insulin and 50 ng/ml EGF, and the medium was replaced after 4 hours of attachment. To assay Mdm2 translocation, cells were cultured in the presence or absence of Hgf after an overnight starvation. Knockdown experiments using *Akt* (Cell Signaling) and *Mdm2* (Santa Cruz) RNA interference were performed according to the manufacturer's procedures (lipofectamine; Invitrogen). The *akt* siRNA oligos used in these studies are known to affect both Akt1 and Akt2 isoforms (Kakazu et al., 2004). A control non-silencing RNA interference was used (Gaggar et al., 2003). Briefly, siRNA was transfected into primary embryonic hepatocytes, cells were left in media plus 0.1% FBS for 6 hours, and survival or biochemical assays were performed 48 hours after transfection.

Immunofluorescence staining
Dissociated embryonic hepatocytes were seeded on collagen-coated 12 mm diameter wells in the presence or absence of Hgf for 24 hours. Cultures were fixed with cold MetOH, washed three times in PBS, and incubated in PBS with 0.3% Tween (PBST) for 30 minutes and then in blocking solution (10% goat serum in PBST). Cells were incubated overnight at room temperature with primary antibodies diluted in blocking solution, were washed with PBST and were then incubated for 1 hour at room temperature with the

corresponding secondary antibodies diluted in blocking solution. After washing with PBST, slides were mounted using DABCO mounting solution containing DAPI and examined by fluorescent microscopy.

Western blot analysis
Western blots were performed as previously described (Maina et al., 1996; Maina et al., 2001). For Mdm2 analysis, total extracts were prepared using boiling Total Protein Extraction Buffer (TPEB: 1 part of 10% SDS, 1 part of 1 M Tris-HCl, pH 6.8, 2 parts of water), then sonication and protein determination was performed.

Metabolic labeling
After 24 hours starvation in media without serum, embryonic hepatocytes were cultured in the presence or absence of Hgf and rapamycin in medium containing 100 µCi/ml of [^{35}S]-methionine/cysteine (PerkinElmer Life Sciences). After 30 minutes, cells were washed twice with cold PBS and lyzate in EBM buffer (20 mM Tris-HCl pH 7.5; 150 mM NaCl; 5 mM EDTA; 5 mM EGTA; 10% glycerol; 1% Triton) with proteases inhibitors, then sonication and protein determination was performed. Pre-cleared lysates were immunoprecipitated for 2 hours with anti-Mdm2 antibodies. Immunoprecipitated proteins were resolved by SDS-PAGE. Gels were dried and labeled proteins were visualized after exposure to X-MR films (Kodak).

Mdm2 half-life determination
After overnight starvation, cells were cultured in the presence of Hgf for 24 hours (high levels of Mdm2), followed by treatment with 20 µg/ml of cycloheximide for the indicated times. Alternatively, starved cells were cultured in media without Hgf to maintain basal levels of Mdm2, and Mdm2 half-life was determined using cycloheximide, as described above. Cell extracts were analyzed by western blots.

In situ detection of apoptosis
E12.5 embryos were dissected in PBS and fixed overnight in 4% paraformaldehyde in PBS. TUNEL staining was performed on 12 µm frozen sections using ApopTag reagents (Chemicon), according to the manufacturer's instructions, with rhodamine-conjugated anti-digoxigenin antibodies. To evaluate the extent of cell death, TUNEL-positive cells in liver sections were counted at 200× magnification on randomly chosen fields of 600 µm^2. Numbers of apoptotic cells corresponding to between three and five different fields were used to evaluate the average number of cells that underwent apoptosis per embryo. For rapamycin or Nutlin-3 injections, only wild-type and homozygous littermate embryos were used. Statistical comparisons were made using the paired Student's *t*-test.

Immunohistochemistry
Analysis of p53 phosphorylation on the Ser$_{18}$ residue was performed on 12 µm frozen sections as follows. Sections were dehumidified at 37°C for 1 hour and fixed in 4% PFA for 5 minutes at room temperature. After PBS washes, tissues were permeabilized with acetone at –20°C for 10 minutes. Endogenous peroxidase was quenched with 3% H_2O_2 in PBS for 10 minutes. Unspecific binding was blocked with 10 % goat serum, and primary antibodies (1:50 dilution) were applied overnight at 4°C. Secondary antibodies were then applied for 45 minutes at room temperature and immunoreaction was revealed using the peroxidase substrate kit DAB (Vector), according to the manufacturer's instructions.

RESULTS
Met-triggered activation of the PI3K pathway is required for Mdm2 translocation into the nucleus
Signaling by some RTKs regulates Mdm2 activity by promoting its nuclear translocation (Mayo and Donner, 2001; Zhou et al., 2001). We first assayed whether Mdm2 translocation into the nucleus requires intact PI3K-Akt signaling downstream of Met. Whereas Mdm2 was restricted to the cytoplasm of un-stimulated wild-type embryonic hepatocytes isolated from E15.5 mouse liver, Mdm2 predominantly translocated to the nucleus upon Hgf stimulation (Fig. 1A). Nuclear accumulation of Mdm2 was abolished in the presence of LY 294002 (Fig. 1B), a PI3K inhibitor,

indicating that Met-triggered Mdm2 translocation is dependent on PI3K activation. We next analyzed whether Mdm2 nuclear translocation occurs in $met^{2P/2P}$ signaling-mutant hepatocytes, which show efficient activation of PI3K, but reduced Akt phosphorylation, upon Hgf stimulation (Maina et al., 2001; Segarra et al., 2006). In $met^{2P/2P}$ mutant cells, the remaining Akt activity does not contribute to Met-triggered cortical neuron migration (Segarra et al., 2006). We found that Mdm2 translocation was impaired in embryonic hepatocytes derived from $met^{2P/2P}$ signaling mutants (Fig. 1A,B). These studies show genetically that an intact PI3K pathway is essential for Mdm2 translocation downstream of activated RTKs such as Met.

In vivo inactivation of p53 restores hepatocyte survival in $met^{2P/2P}$ mutant embryos

Nuclear translocation allows Mdm2 to bind to p53 and promote its degradation. The early embryonic lethality of $mdm2$ mutants has prevented genetic analysis of the general biological relevance of Mdm2 for cell survival (Jones et al., 1995; Montes de Oca Luna et al., 1995). We have previously reported that $met^{2P/2P}$ signaling mutants show exacerbated death of embryonic hepatocytes during liver development (Maina et al., 2001). To analyze the contribution of p53 to this increased death, we used pharmacological and genetic tools to reduce p53 activity in $met^{2P/2P}$ mutant embryos. The chemical compound pifithrin-α, which blocks p53-dependent transcription and apoptosis (Komarov et al., 1999; Pani et al., 2002), was administered shortly

after the start of liver organogenesis to pregnant $met^{2P/+}$ females crossed with $met^{2P/+}$ males (E10.5 and E11.5), and hepatocyte survival was assayed on liver sections of E12.5 embryos by TUNEL staining. Remarkably, pifithrin-α injections completely prevented cell death in $met^{2P/2P}$ livers (Fig. 1C,D). Although p53 can also regulate cell cycle progression (Bargonetti and Manfredi, 2002), pifithrin-α injection did not perturb the cell cycle of embryonic hepatocytes in vivo (see Fig. S1 in the supplementary material), thus confirming that p53 is dispensable for embryonic hepatocyte proliferation in developing livers (Dumble et al., 2001). These results were confirmed genetically, because levels of cell death observed in liver sections from E12.5 double-homozygous $met^{2P/2P}; p53^{-/-}$ embryos were similar to those from wild-type livers (Fig. 1C,D). These data indicate that, when Met survival signaling is compromised, p53-dependent mechanisms trigger cell death. Thus, under normal conditions, Met signaling inhibits p53 activity in embryonic livers.

Met-triggered PI3K activation is required for Mdm2 translation via an Akt- and mTOR-dependent pathway

In addition to its effects on translocation, we also found that Hgf caused a time-dependent increase in the levels of Mdm2 protein (Fig. 2A) in a cell-type-specific manner (see Fig. S2A in the supplementary material). To assess whether the Hgf-induced increase in Mdm2 levels reflects an enhanced rate of $Mdm2$ translation, embryonic hepatocytes were pulse-labeled with [35]S-
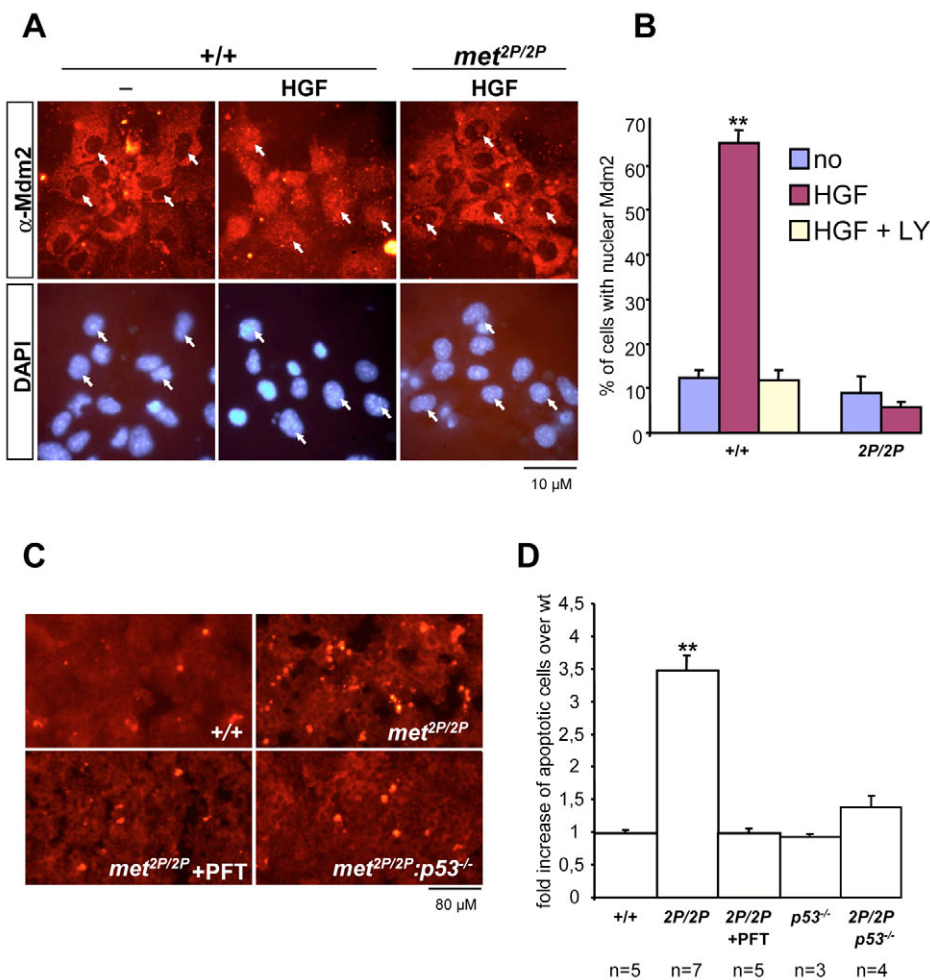
Fig. 1. An intact PI3K pathway is required for Hgf-induced Mdm2 translocation. (A) Upon Hgf stimulation, wild-type (+/+) Met induces Mdm2 translocation from the cytoplasm into the nucleus of cultured primary embryonic hepatocytes. By contrast, Met[2P], which has intact PI3K activity but reduced Akt signaling ($met^{2P/2P}$), does not trigger Mdm2 translocation. (B) Quantitative analysis of Mdm2 translocation in wild-type (+/+) and mutant (2P/2P) embryonic hepatocytes. Mdm2 translocation in wild-type hepatocytes is abolished in the presence of the PI3K inhibitor LY 294002 (10 μM). (C) In vivo inactivation of p53 restores hepatocyte survival in $met^{2P/2P}$ mutant embryos. TUNEL staining of liver cryosections from E12.5 wild-type (+/+), $met^{2P/2P}$ treated ($met^{2P/2P}$+PFT) or not ($met^{2P/2P}$) with the p53 inhibitor pifithrin-α and double-homozygous $met^{2P/2P}; p53^{-/-}$ embryos. (D) Quantitative analysis of TUNEL-positive cells in E12.5 liver sections of the indicated genotypes. The proportion of apoptotic cells is represented as fold increase compared with wild-type livers. The numbers of embryos analyzed in these studies are indicated (n). Arrows indicate nuclei. **$P < 0.001$.

A

B

C
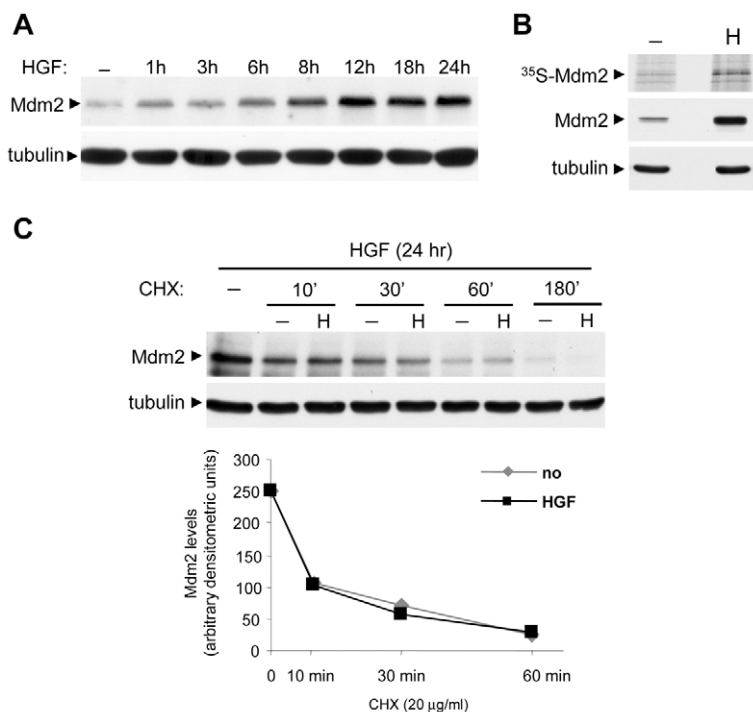
Fig. 2. Met triggers translation of Mdm2 in cultured embryonic hepatocytes without affecting protein stability. (**A**) Time-course analysis of Mdm2 protein levels upon Hgf stimulation. Total extracts from wild-type embryonic hepatocytes were blotted and probed with α-Mdm2 (top) and α-tubulin (bottom). (**B**) Levels of newly synthesized Mdm2 in α-Mdm2 immunoprecipitates (top panel) of ^{35}S-methionine/cysteine labeled embryonic hepatocytes treated or not with Hgf (H). The middle and lower panels show levels of Mdm2 and tubulin, respectively, on total lysates. (**C**) Rate of decay of Mdm2 protein in embryonic hepatocytes with high Mdm2 levels (Hgf stimulation for 24 hours; top panel) as determined by cycloheximide (CHX) treatment. Hgf treatment (H) does not change Mdm2 protein stability. CHX does not affect the half-life of tubulin. The decrease in Mdm2 protein levels was quantified after normalization with tubulin (bottom panel).

methionine/cysteine in the presence or absence of Hgf, and levels of newly synthesized Mdm2 were detected by immunoprecipitation. Newly synthesized Mdm2 was detected in Hgf-treated embryonic hepatocytes, but not in untreated cells (Fig. 2B). As Mdm2 protein has a short half-life (Trotta et al., 2003), we tested whether Hgf also increased Mdm2 protein stability by blocking nascent translation using cycloheximide. We addressed this issue by following the rate of decay of Mdm2 in primary hepatocytes that had high levels of Mdm2 (after 24 hours of Hgf stimulation). Hgf did not significantly change the half-life of Mdm2 following cycloheximide treatment (Fig. 2C). Moreover, Hgf did not affect the rate of decay of basal Mdm2 levels in primary hepatocytes (starved cells; see Fig. S2B in the supplementary material). Thus, a Met-triggered increase in Mdm2 protein level reflects its effects upon Mdm2 translation rather than on the stability of the protein.

Although increased Mdm2 levels have been linked to the activation of MAPK pathways (Phelps et al., 2005; Ries et al., 2000), inhibition of neither MAPKs nor Src family members significantly interfered with the Met-triggered increase of Mdm2 levels (see Fig. S2C in the supplementary material). By contrast, Mdm2 upregulation was blocked by the PI3K inhibitor LY 294002 in a dose-dependent manner (Fig. 3A). Inhibition of PI3K by LY 294002 was verified by following phosphorylation of its downstream target Akt on amino acid residue S_{473} (Fig. 3A). To assay the requirement of the PI3K effector Akt for Met-triggered Mdm2 translation, we used the oligo RNA interference approach. Transfection of embryonic hepatocytes with *Akt* siRNA oligos decreased Akt protein levels and impaired Met-triggered upregulation of Mdm2 protein (Fig. 3B). These results were confirmed further using A-443654, which is known to specifically inhibit Akt activity in cultured cells (Luo et al., 2005). Inhibition of Akt by A-443654 was confirmed following GSK3α phosphorylation on residue S_{21} (Fig. 3C). Met-triggered Mdm2 upregulation was blocked by the Akt inhibitor A-443654 in a dose-dependent manner (Fig. 3C). Thus, PI3K and its downstream effector Akt are both required for Hgf-mediated Mdm2 translation.

Another well-established PI3K effector is mTOR, the serine/threonine kinase that specifically phosphorylates p70^{s6k} kinase and 4E-BP1 (Bjornsti and Houghton, 2004a; Hay and Sonenberg, 2004; Huang and Houghton, 2003; Inoki et al., 2005). Western blot analysis of p70^{s6k} phosphorylation is commonly used to monitor mTOR activity (Brown et al., 1995). We found that Hgf stimulation led to phosphorylation of p70^{s6k} on amino acid residues T_{389} and T_{421}/S_{424} (Fig. 4A,B). This occurs through PI3K and mTOR, because either LY 294002 or rapamycin, an inhibitor of mTOR (Huang and Houghton, 2003), were sufficient to block p70^{s6k} phosphorylation (Fig. 3C and data not shown). We observed a slight reduction in p70^{s6k} phosphorylation in un-stimulated cells, suggesting that basal levels are also controlled by these pathways. In addition, Hgf induced the phosphorylation of a p70^{s6k} effector, S6 ribosomal protein, on residues $S_{235/236}$, and of 4E-BP1 on residue S_{65} (Fig. 4B). These results indicate that the mTOR pathway is properly activated by Hgf in embryonic hepatocytes.

Inhibition of mTOR with rapamycin completely blocked the Hgf-induced Mdm2 protein increase in embryonic hepatocytes (Fig. 4C). By performing ^{35}S-pulse labeling, we found that rapamycin inhibits the translation of Mdm2, whereas translation rates of other proteins, such as p53, were not inhibited (Fig. 4D). These results demonstrate that mTOR activation is specifically required to enhance Mdm2 translation. Thus, activation of PI3K and of its effectors Akt and mTOR is required to enhance Met-induced Mdm2 translation.

Inhibition of Mdm2- or mTOR-activity prevents Met-triggered cell survival in cultured embryonic hepatocytes
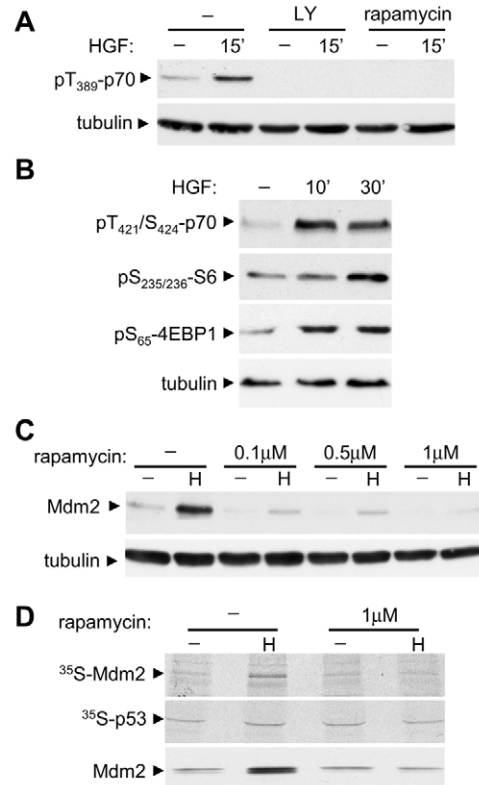
Rapamycin can amplify drug-induced cell death in vitro, and has anti-tumor effects on both *Pten*-deficient and *Akt*-promoted tumors (Bjornsti and Houghton, 2004b; Majumder et al., 2004; Wendel et al., 2004). However, the underlying mechanism is still unclear. Our demonstration that mTOR controls Mdm2 protein levels raised the possibility that rapamycin might alter cell survival responses in a p53-dependent manner. Therefore, we tested whether this signaling
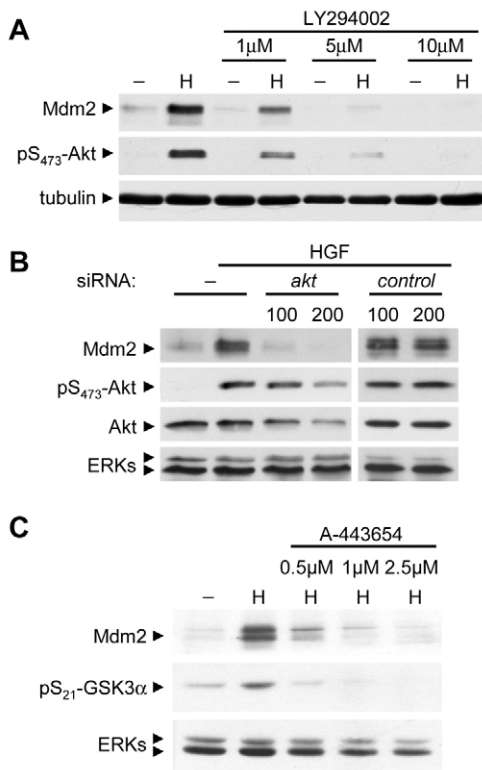
Fig. 3. PI3K and its downstream signal Akt are required for Met-triggered Mdm2 translation in embryonic hepatocytes. (**A**) Hgf-mediated Mdm2 induction and Akt phosphorylation in wild-type hepatocytes is inhibited by LY 294002 in a dose-dependent manner. (**B**) Western blot analysis showing that *Akt* siRNA causes a reduction in Mdm2 protein levels in a concentration-dependent manner (100 and 200 pmol). Levels of phospho-S_{473} Akt and Akt, but not ERKs, were reduced in the presence of Akt siRNA. Control siRNA did not alter protein levels. (**C**) Hgf-induced Mdm2 translation is prevented by the Akt inhibitor A-443654 in a dose-dependent manner. Inhibition of Akt by A-443654 was confirmed following GSK3α phosphorylation.

Fig. 4. The mTOR pathway is activated upon Hgf stimulation and is required for Met-triggered Mdm2 translation in embryonic hepatocytes. (**A**) Hgf activates mTOR-p70^{s6k} signaling. p70^{s6k} is phosphorylated on T_{389} upon Hgf stimulation. Because this phosphorylation is abolished in the presence of LY 294002 (5 μM) or rapamycin (rapa: 1 μM), it must require intact PI3K and mTOR signaling. (**B**) Hgf stimulation induces phosphorylation of the following mTOR downstream signals: p70^{s6k} on residues T_{421}/S_{424}, S6 ribosomal protein on $S_{235/236}$ and 4EBP1 on S_{65}. (**C**) Rapamycin prevents the upregulation of Mdm2 by Hgf in embryonic hepatocyte cultures. (**D**) Newly synthesized Mdm2 and p53 proteins were analyzed by the immunoprecipitation of ^{35}S-methionine/cysteine-labeled protein extracts from embryonic hepatocytes treated or not with Hgf and/or rapamycin. Rapamycin inhibits the translation of Mdm2, but not of p53. The bottom panels show levels of Mdm2 on total lysates.

mechanism is required for the survival of embryonic hepatocytes in vitro. Inhibition of Mdm2-p53 interactions stabilizes p53, leading to its activation (Vassilev et al., 2004). Nutlin-3, a new selective Mdm2 inhibitor, binds to the p53-binding pocket in Mdm2, thus preventing its interaction with p53 (Thompson et al., 2004; Vassilev et al., 2004). This leads to p53-dependent apoptosis and growth inhibition of cultured cells and tumors.

Treatment of primary embryonic hepatocytes with tumor necrosis factor α (TNFα) and actinomycin D increased phosphorylation and p53 protein levels, thus affecting Mdm2 expression (see Fig. S3A,B in the supplementary material) and inducing cell death (Fig. 5A,B). We found that addition of Hgf promoted survival in these culture conditions (Fig. 5A,B). Therefore, this experimental set-up was used to analyze the effects of Nutlin-3 on embryonic hepatocyte survival. We found that the uncoupling of Mdm2 from p53 using Nutlin-3 abolished the survival response induced by Hgf in primary embryonic hepatocytes (Fig. 5A,B) in a dose-dependent manner (data not shown). Similarly, downregulation of Mdm2 protein levels

using RNA interference (Fig. 5C) suppressed Hgf-induced cell survival (Fig. 5A,B). Thus, intact Mdm2 signaling regulates the survival of cultured embryonic hepatocytes. Interestingly, cell survival induced by Hgf was also prevented by rapamycin (Fig. 5A,B), showing that mTOR inhibition recapitulates the effects of blocking Mdm2. Altogether, these results demonstrate that the inhibition of p53 by intact mTOR signaling is required for Met-triggered embryonic hepatocyte survival in vitro.

Rapamycin induces p53-dependent cell death by affecting Mdm2 protein levels in vivo

The findings above, which argue that mTOR acts through Mdm2 to prevent p53-dependent apoptosis, led us to investigate the physiological relevance of Mdm2 for cell survival in developing livers. This problem cannot be addressed genetically because of the early lethality of *mdm2*-knockout embryos (Jones et al., 1995; Montes de Oca Luna et al., 1995) and because of minor changes in *Mdm2* mRNA levels in livers of *mdm2* hypomorphic mice
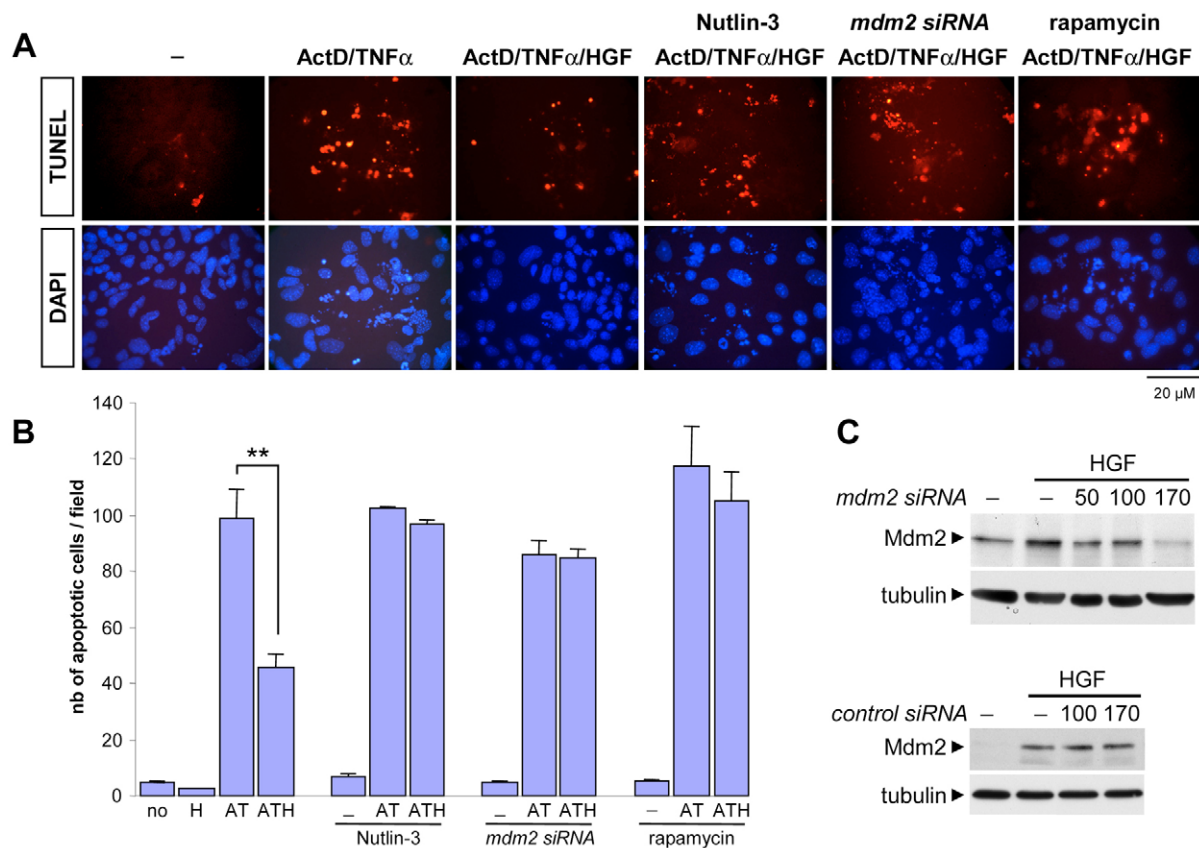
Fig. 5. Inhibition of Mdm2 and mTOR prevents Met-triggered cell survival in vitro. (**A**) Death of primary hepatocytes was triggered by actinomycin D (ActD) plus TNFα. Hgf-mediated cell survival was prevented by blocking Mdm2 function using the Mdm2 inhibitor Nutlin-3 or *Mdm2* siRNA. The Hgf survival effects were also impaired by the mTOR inhibitor rapamycin (1 μM). (**B**) Quantitative analysis of cell death in primary embryonic hepatocyte cultures treated or not with actinomycin D plus TNFα (AT), Hgf (H), actinomycin D plus TNFα plus Hgf (ATH), Nutlin3, *Mdm2* RNA interference and rapamycin. Data are representative of three independent experiments performed in duplicate. **P<0.001; Student's *t*-tests. (**C**) Western blot analysis showing that *Mdm2* siRNA causes a reduction in Mdm2 protein levels in a concentration-dependent manner (50, 100 and 170 pmol). A non-silencing siRNA was used as control at concentration of 100 and 170 pmol. Control siRNA did not affect Mdm2 protein levels.

(Mendrysa et al., 2003). Therefore, we addressed this issue pharmacologically using the Mdm2 inhibitor Nutlin-3 in vivo. Nutlin-3 was injected into pregnant *p53*^{+/–} females that had been crossed with *p53*^{+/–} males, and cell survival was assayed on E12.5 liver sections by TUNEL. Inhibition of Mdm2 function significantly increased the number of apoptotic liver cells in wild-type embryos compared with *p53*^{–/–} mutants (which have indistinguishable basal levels of TUNEL-positive cells; Fig. 6A). The average number of apoptotic cells in a 600 μm^2 area was: un-injected wild-type and *p53*^{–/–} embryos, 48.2±7.8; wild-type embryos treated with Nutlin-3, 101.2±11.0; *p53*^{–/–} embryos treated with Nutlin-3, 71.2±6.1; *P*<0.001 (Fig. 6A). Thus, Mdm2 suppresses cell survival induced by Hgf in vitro and impairs p53-dependent cell death in vivo. As with Nutlin-3, rapamycin administration significantly increased the number of apoptotic liver cells in wild-type embryos compared with non-injected wild type and *p53*^{–/–} mutants (Fig. 6A). Strikingly, this increase was significantly reduced in *p53*^{–/–} mutants injected with rapamycin (Fig. 6A). The average number of apoptotic cells in a 600 μm^2 area was: un-injected wild-type and *p53*^{–/–} embryos, 48.2±7.8; wild-type embryos treated with rapamycin, 106.2±7.4; in *p53*^{–/–} embryos treated with rapamycin, 70.4±5.4; *P*<0.001 (Fig. 6A). Although mTOR has been shown to be required for proliferation and cell cycle progression during early mouse embryogenesis

(Gangloff et al., 2004; Murakami et al., 2004), rapamycin injection did not perturb the cell cycle of embryonic hepatocytes in vivo (see Fig. S1 in the supplementary material). Thus, at least part of the cell death caused by the inhibition of the mTOR pathway in vivo is p53-dependent.

The effect of mTOR inhibition in developing livers was addressed further by analyzing Mdm2 protein levels and the phosphorylation of p53 on the amino acid residue S$_{18}$, as a read-out of its activity (Shieh et al., 1997). Strikingly, western blot analysis of embryonic liver extracts showed that rapamycin injection significantly reduced Mdm2 protein levels in vivo (Fig. 6B). This event correlates with an increased phosphorylation of p53 on the S$_{18}$ residue (Fig. 6B,C). The average number of phospho-S$_{18}$-p53-positive cells in a 600 μm^2 area was: un-injected wild-type embryos, 10.3±2.1; wild-type embryos treated with rapamycin, 21.3±2.6; *P*<0.01 (Fig. 6C). Altogether, our results demonstrate that in vivo inhibition of mTOR causes cell death by altering the balance of Mdm2 protein levels and p53 phosphorylation.

DISCUSSION
The antagonistic properties of p53 and PI3K have had a major impact on the understanding of how cell survival is controlled during development, adult life and pathology. Although incompletely understood, crosstalk between these two main actors
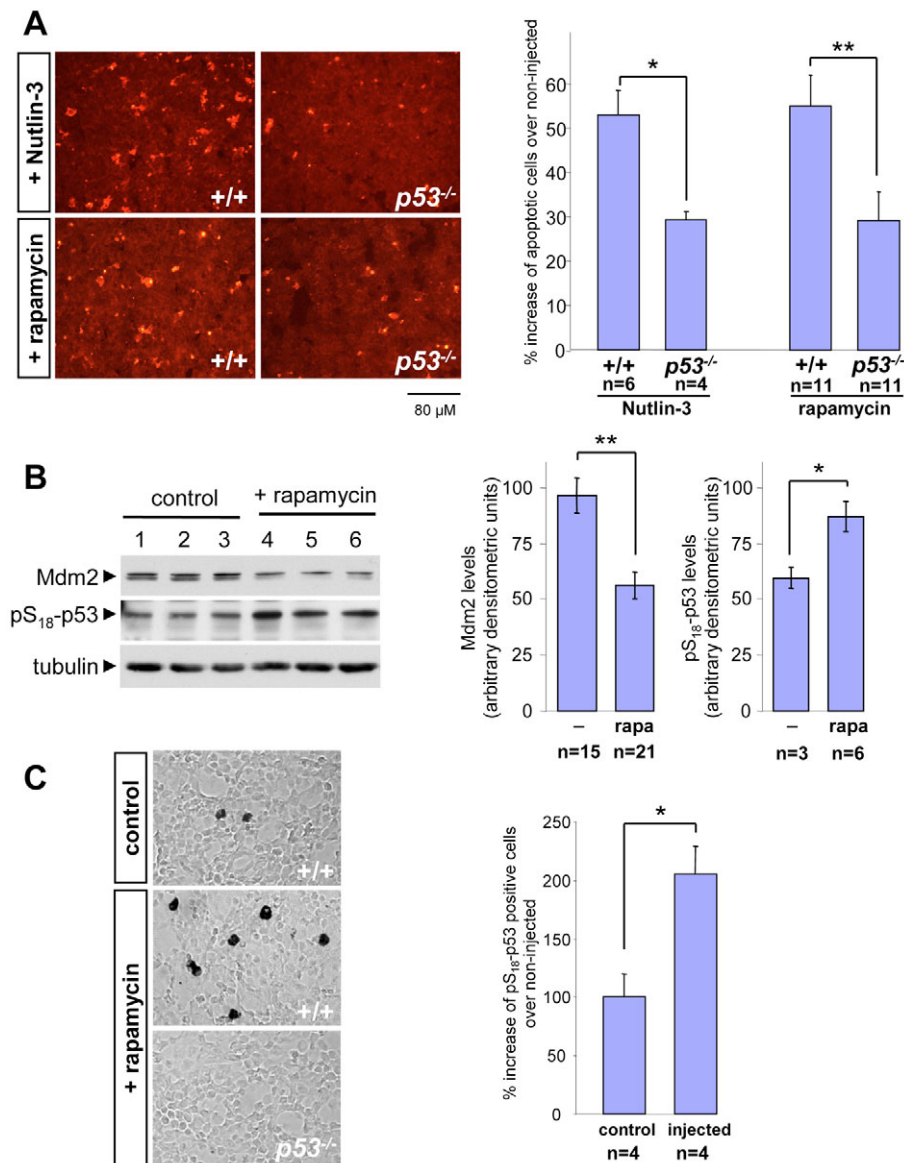
Fig. 6. mTOR inhibition causes p53-dependent cell death by affecting Mdm2 protein levels in developing livers. (**A**) Analysis of Mdm2 (top) and mTOR (bottom) inhibition in E12.5 wild-type (+/+) and $p53^{-/-}$ mutant livers. Panels show TUNEL-positive cells in representative liver sections treated with Nutlin-3 or rapamycin. Cell death for each genotype is shown (right) as a percentage increase in the number of apoptotic cells compared with non-injected mice (*$P<0.01$; **$P<0.001$; Student's t-tests). Basal levels of TUNEL labeling were indistinguishable in non-injected wild-type and $p53^{-/-}$ mutant embryos. Analysis on p53 mutants indicated that death of embryonic hepatocytes is also controlled by unknown p53-independent pathways. (**B**) Western blot analysis of total lysates of E12.5 livers of wild-type embryos treated or not with rapamycin. Rapamycin treatment causes a reduction in the levels of Mdm2 (top), which correlates with an increased phosphorylation of p53 on the S_{18} residue (pS_{18}-p53; middle), whereas tubulin levels are unchanged. Levels of Mdm2 protein and p53 phosphorylation were quantified (right) after normalization with tubulin. *$P<0.01$; **$P<0.001$; Student's t-tests. (**C**) Immunohistochemical analysis of p53 phosphorylation on the S_{18} residue of untreated (control), wild-type and $p53^{-/-}$ mice injected with rapamycin. Quantitative analysis (right) is reported as the percentage increase of cells positive for p53 phosphorylation in rapamycin-injected wild-type mice over the number of positive cells in non-injected mice. *$P<0.01$; Student's t-tests. The numbers of embryos analyzed in these studies are indicated (n).

has been thought to involve transcriptional and post-transcriptional mechanisms. Here, we provide evidence that, during embryogenesis, cell survival signaling by PI3K involves translational control of the p53 negative regulator Mdm2. By studying Hgf-mediated cell survival in developing livers, we found that Met-triggered PI3K activation leads to the modulation of Mdm2 at two distinct levels (Fig. 7). Firstly, PI3K enhances Mdm2 protein levels via Akt and mTOR signals; and, secondly, PI3K requires Akt to trigger the subsequent nuclear translocation of Mdm2 (Fig. 7). Pharmacological inhibition of mTOR causes a reduction of Mdm2 protein levels and leads to p53-dependent cell death in vivo. Consistent with this, disruption of Mdm2 function in mouse embryos by Nutlin-3 causes apoptosis of hepatocytes. The ability of PI3K to regulate Mdm2 at multiple levels further emphasizes its importance as an antagonist of p53-dependent apoptosis (Fig. 7).

Hgf-Met is the only ligand-receptor couple for which there is a genetically proven role in regulating in vivo survival of embryonic hepatocytes and regeneration in adult liver (Bladt et al., 1995; Borowiak et al., 2004; Huh et al., 2004; Maina et al., 1996; Schmidt et al., 1995). The Hgf-Met system therefore represents a unique opportunity to determine how intracellular signals are orchestrated to promote hepatocyte survival during embryogenesis. Hepatocyte apoptosis in *met*-specificity-switch mutants has shown that cell survival in developing livers requires a complex signaling network triggered by Met (Maina et al., 2001). Here, we show that activation of PI3K by Met leads to the inhibition of p53-dependent death in the developing liver. The fact that p53 is dispensable during normal liver development (Lin et al., 2002) suggests that its role is to be a 'guardian' in circumstances where the normal survival signaling is perturbed. This 'guardian' role has so far prevented studies on the temporal and spatial susceptibility of embryonic cells to p53 action. Recently, the use of embryos lacking *Mdm2* in the central nervous system revealed that Mdm2-p53 interplay is required for neuronal survival (Xiong et al., 2006). It will be interesting to know whether the PI3K-Akt-mTOR pathway regulates Mdm2 in embryonic cell types other than hepatocytes.

Gene ablation of several members of the PI3K signaling pathway has underlined its pivotal role in hepatocyte survival during liver development. Mice lacking all isoforms of PI3K p85α showed hepatocyte death leading to extensive liver necrosis (Fruman et al., 2000). Genetic mutation of the PI3K-Akt effector GSK3β results in
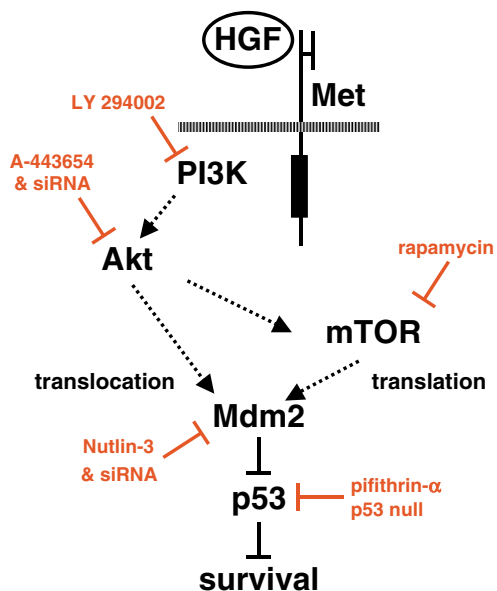
Fig. 7. A novel pathway involving mTOR and Mdm2 signals cell survival. Upon Hgf stimulation, Met triggers the nuclear translocation of Mdm2 by activating the PI3K-Akt pathway. In addition, Met enhances Mdm2 translation by acting on Akt-mTOR via PI3K. The relevance of this mechanism for Met-triggered cell survival has been genetically and pharmacologically demonstrated. Blocking a single step of this pathway alters the cell survival/death balance in embryonic cells.

excessive TNFα toxicity, reduced NF-κB activation and hepatocyte apoptosis (Hoeflich et al., 2000). Disruption of NF-κB signaling, which can also be downstream of PI3K, leads to embryonic lethality associated with massive liver degeneration by hepatocyte death (Beg et al., 1995; Li et al., 1999a; Li et al., 1999b; Rudolph et al., 2000). In addition, we have recently found that Met-triggered PI3K-Akt activation is also required to prevent FLIP$_L$ degradation and cell death in embryonic hepatocytes exposed to Fas (Moumen et al., 2007). Thus, by modulating its effectors, the PI3K-Akt pathway appears to be a key regulator of cell survival in developing livers. In addition to signaling to NF-κB and GSK3β, PI3K-Akt acts on Mdm2 and FLIP to prevent death triggered by p53 and Fas, respectively.

In addition to modulating several downstream effectors directly involved in cell proliferation and survival, it has emerged, only recently, that PI3K can also control protein synthesis. In particular, PI3K stimulation leads to the activation of mTOR, which, in turn, can dramatically change the translation rates of a small proportion of mRNAs, mainly acting through the intermediates 4E-BP1 and the p70^{s6k}-S6 ribosomal protein (Hay and Sonenberg, 2004; Holland et al., 2004). Although it is well established that mTOR can control cell metabolism by modulating the translation of ribosomal-binding proteins, it is still unclear whether other growth and survival genes are also translational targets of mTOR. By identifying Mdm2 as a novel target, our data emphasize the importance of looking carefully at other molecules, which may be also be translationally controlled by mTOR.

Upregulation of Met signaling in combination with the downregulation of p53 activity is often found in human liver carcinomas (Kiss et al., 1997), suggesting that aberrant Met signaling confers a neoplastic phenotype to cells only when it is not restrained by p53 activity. Thus, our findings not only contribute to a better understanding of the molecular mechanisms involved in normal liver development, but they may cast light on the mechanisms by which cells acquire a growth advantage, invasive properties and resistance to anoikis during metastasis in a variety of Met-associated cancers. Therefore, it will be important to examine whether the inhibition of either mTOR or Mdm2 alone or in combination with the inhibition of RTKs, such as Met, will also reduce the frequency and metastasis of Met-related tumors. Our studies also confirm the importance of identifying which intermediates in the PI3K and p53 pathways are altered in neoplastic cells as a rational approach to combined molecular therapies.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/7/1443/DC1

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DEVELOPMENT