MICROBIOLOGY. For the article “Isolates of Zaire ebolavirus from wild apes reveal genetic lineage and recombinants,” by Tatiana J. Wittmann, Roman Biek, Alexandre Hassanin, Pierre Rouquet, Patricia Reed, Philippe Yaba, Xavier Pourrut, Leslie A. Real, Jean-Paul Gonzalez, and Eric M. Leroy, which appeared in issue 43, October 23, 2007, of Proc Natl Acad Sci USA (104:17123–17127; first published October 17, 2007; 10.1073/pnas.0704076104), the affiliation for Jean-Paul Gonzalez should have appeared as “Institut de Recherche pour le Développement, UR178, Center for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University at Salaya, Phutthamonthon 4, Nakhonpathom 73170, Thailand.” The corrected affiliation line appears below.

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NEUROSCIENCE. For the article “Dissociating the neural mechanisms of memory-based guidance of visual selection,” by David Soto, Glyn W. Humphreys, and Pia Rotshtein, which appeared in issue 43, October 23, 2007, of Proc Natl Acad Sci USA (104:17186–17191; first published October 16, 2007; 10.1073/pnas.0703706104), the authors note that, due to a printer’s error, the third line of the Abstract appeared incorrectly in part. “How WM and implicit priming affects influence visual selection remains poorly understood, however” should read: “How WM and implicit priming influence visual selection remains poorly understood.” Additionally, on page 17186, in the second line of the second paragraph, left column, “This work has led to the development of the influentially biased competition model of visual selection (1), where it is hypothesized that memory acts to bias the competition for selection between different objects in the visual scene” should read: “This work has led to the development of the influentially biased competition model of visual selection (1), where it is hypothesized that memory acts to bias the competition for selection between different objects in the visual scene.” These errors do not affect the conclusions of the article.

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MEDICAL SCIENCES. For the article “The EGF receptor is required for efficient liver regeneration,” by Anuradha Natarajan, Bettina Wagner, and Maria Sibilia, which appeared in issue 43, October 23, 2007, of Proc Natl Acad Sci USA (104:17081–17086; first published October 16, 2007; 10.1073/pnas.0704126104), the authors note that, due to a printer’s error, line 3 of the Acknowledgments appeared incorrectly. The sentence should read as follows: “This project was supported by Austrian National Bank ÖNB-10556 and the European Community Grant Growthstop LSHC-CT-2006-037731.”

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SUSTAINABILITY SCIENCE. For the article “Poverty and Hunger Special Feature: The African Millennium Villages,” by Pedro Sanchez, Cheryl Palm, Jeffrey Sachs, Glenn Denning, Rafael Flor, Rebbie Harawa, Bashir Jama, Tsegazeab Kiflemariam, Bronwen Konecky, Raffaela Kozar, Eliud Lelerai, Alia Malik, Vijay Modi, Patrick Mutuo, Amadou Niang, Herine Okoth, Frank Place, Sonia Ehrlich Sachs, Amir Said, David Siriri, Awash Teklehaimanot, Karen Wang, Justine Wangila, and Colleen Zamba, which appeared in issue 43, October 23, 2007, of Proc Natl Acad Sci USA (104:17777–17780; first published October 17, 2007; 10.1073/pnas.0700423104), due to a printer’s error, the author name Vijay Modi did not appear in the Table of Contents. The online version has been corrected.

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The EGF receptor is required for efficient liver regeneration

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Mice lacking the EGF receptor (EGFR) die between midgestation and postnatal day 20 with various defects in neural and epithelial organs. Here, we generated mice carrying a floxed EGFR allele to inactivate the EGFR in fetal and adult liver. Perinatal deletion of EGFR in hepatocytes resulted in decreased body weight, whereas deletion in the adult liver did not affect body mass. Although liver function was not affected, after partial hepatectomy mice lacking EGFR in the liver showed increased mortality accompanied by increased levels of serum transaminases indicating liver damage. Liver regeneration was showed increased mortality accompanied by increased levels of serum transaminases indicating liver damage. Liver regeneration was impaired in the mutants because of reduced hepatocyte proliferation. Analysis of cell cycle progression in EGFR-deficient livers indicated a defective G1–G2 phase entry with delayed transcriptional activation and reduced protein expression of cyclin D1 followed by reduced cdk2 and cdk1 expression. Impaired liver regeneration was accompanied by compensatory up-regulation of TNFα in the serum and prolonged activation of c-Jun. Moreover, p38α and NF-κB activation was reduced in regenerating mutant livers, indicating an impaired stress response after hepatectomy. Our studies demonstrate that EGFR is a critical regulator of hepatocyte proliferation in the initial phases of liver regeneration.

Conditional EGF receptor knockout mice | cyclin D1 | hepatocyte proliferation | liver function | partial hepatectomy

The EGF receptor (EGFR) is highly expressed in the adult liver and has been proposed to play an important role during liver development, function, and regeneration (1). Together with erbB2/neu, erbB3, and erbB4, the EGFR/erbB1 belongs to the ErbB family of receptor tyrosine kinases which can homo- or heterodimerize on ligand stimulation. The EGFR can be activated by several ligands like EGF, TGFα, amphiregulin, heparin-binding EGF, betacellulin, and epiregulin (2–4). Many of these ligands are potent mitogens for hepatocytes (5), and overexpression of EGFR and its ligands are implicated in hepatocellular carcinoma (6). Mice lacking EGFR die between midgestation and postnatal day 20 (P20) depending on their genetic background, with defects in placenta, brain, bone, skin, and lung (7–11). The early lethality of EGFR−/− mice has prevented a careful analysis of EGFR function in adult liver and during liver regeneration.

The liver has the unique capacity to regulate its growth and mass and is a well characterized biological system for studying cell proliferation and differentiation. Liver mass in rodents increases severalfold in the first 4 postnatal weeks until the liver/body weight ratio approaches adult levels. Although adult hepatocytes are quiescent cells, they retain their ability to proliferate and regenerate damaged hepatic tissue after toxic injury and infections (12). One of the models to investigate liver regeneration is rodents is two-thirds partial hepatectomy (PH), which has been studied extensively at the molecular level (13). On acute-phase response induced by PH, the hepatocytes synchronously exit their quiescent state, re-enter the cell cycle, and undergo one to two rounds of replication before returning to the quiescent state (5). During the initial “priming” phase, hepatocytes undergo a transition from G0 to G1. This highly regulated process is mediated by different cytokines such as TNFα and IL-6 (13, 14). These factors in turn activate transcription factors like activator protein 1 (AP-1), STAT3, and NF-κB, followed by the expression of other genes that encode cell cycle regulators such as cyclin D (12, 15–17). In the second phase, hepatocytes leave G1 and enter S phase, which is accompanied by up-regulation of a number of genes including cyclin E, cyclin A, and their respective kinases (14, 18).

Many growth factors such as TGFα, EGF, heparin-binding EGF, and amphiregulin are highly expressed after PH and are thought to be important in driving liver regeneration (19–21). Mice lacking TGFα develop normally and liver regeneration is not impaired most likely because other EGFR ligands may compensate for its absence (22). Recently, heparin-binding EGF has been shown to regulate hepatocyte DNA replication after 70% PH (23). Similarly, mice lacking amphiregulin also displayed impaired hepatocyte proliferation and delayed induction of cyclin D1 after PH (19). However, it cannot be excluded that the high redundancy among the EGFR ligands might mask additional functions of the EGFR during liver regeneration. Therefore, definitive conclusions about the requirement of EGFR signaling during liver function and regeneration can be reached only after analyzing mice harboring liver-specific deletions of the EGFR.

Here, we have generated mice harboring a loxp-flanked EGFR allele (EGFR<sup>flp</sup>) and have used the Alfp-cre transgenic line to inactivate EGFR in hepatocytes before birth (EGFR<sup>flp</sup>-<sup>cre</sup>) and an inducible Mx-cre line to delete EGFR in the livers of adult mice (EGFR<sup>cre</sup>). Whereas EGFR is dispensable for liver development and function, liver regeneration is impaired in mice lacking the EGFR in the liver. Analysis of cell cycle progression in mutant livers indicates reduced cyclin D expression followed by reduced expression of cdk2 and cdk1. Furthermore, mutant regenerating livers displayed an impaired stress response that is accompanied by compensatory up-regulation of TNFα in the serum and prolonged c-Jun protein expression. Our results identify EGFR as a critical regulator of hepatocyte proliferation by regulating efficient G1–S transition in the initial phases after PH.

**Results**

**Generation of Mice Harboring a Conditional EGFR Allele.** A targeting vector in which the promoter and the first exon of the EGFR gene are flanked by loxP sites was used to generate ES cells harboring a floxed EGFR allele (EGFR<sup>flp</sup>) (Fig. L4). Correct gene targeting at the EGFR locus was confirmed by PCR (data not shown) and Southern blot analysis (Fig. 1B). Homozygous EGFR<sup>flp</sup> mice were fertile and phenotypically indistinguishable from control mice suggesting that the genetic manipulations and the presence of the

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**Abbreviations:** EGF, EGF receptor; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvic transaminase; PH, partial hepatectomy; pIpC, poly(I/C); P<sub>n</sub>, postnatal day n.

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Deletion of EGFR in the Liver. To analyze the function of EGFR during perinatal and early postnatal liver development, EGFR was inactivated tissue specifically in hepatocytes by using Alfp-cre transgenic mice, in which the cre recombinase is under the control of the liver-specific albumin promoter and albumin and Mox2Cre mice, in which the cre recombinase is under the control of the notypical abnormalities in the liver (data not shown). After cre-mediated recombination of the EGFR allele the first exon and part of the promoter region are deleted giving rise to the EGFR allele (Fig. 1A and C). To confirm that cre-mediated removal of the loxP-flanked region of the EGFR generates a null allele, we crossed EGFR mice with Mox2-Cre transgenic mice that express cre in all embryonic cells (24). EGFR/Mox2-Cre (EGFR) mice displayed the same phenotypes as previously described for EGFR knockout mice (8, 10, 25) and Western blot analysis confirmed that no EGFR protein was expressed in EGFR mice [supporting information (SI) Fig. 7].

A genomic EGFR locus (EGFR

B f/+ f/+ f/+ X/HindIII

C Δ/+ Δ/+ X/HindIII

D

E

F

Fig. 1. Generation of EGFR mice. (A) The first line depicts the mouse genomic EGFR locus with the restriction sites and DNA probe used for Southern blot analysis. The targeting construct for homologous recombination in ES cells harbors a neomycin (neo) resistance cassette flanked by frt sites (black circles) for positive and a diphtheria toxin (DT) cassette for negative selection. Cre-mediated recombination at the loxP sites (black triangles) generates the deleted EGFR allele. C, XmaCI; E, EcoRI; H, HindIII; K, KpnI; S, Sall; X, XbaI. (B) Southern blot analysis of genomic DNA of electroporated ES clones showing the presence of the wild-type (+) and the EGFR allele. (C) Southern blot analysis of offspring from crosses with Mox2-cre transgenic mice.

Fig. 2. Analysis of mice lacking EGFR in the liver. (A) Body weight of EGFR and EGFR mice at various times after birth. Results represent the mean ± SEM of six litters from five independent breeding cages. *, P < 0.05; **, P < 0.005. (B) Southern blot analysis of liver DNA of Alfp-cre mice at the indicated postnatal days. (C) Western blot analysis of EGFR and EGFR mice at the indicated times after birth. Tubulin was used as loading control. (D) Southern blot analysis of livers of EGFR mice 3 days after the last plpC injection. (E) Kaplan–Meier plot showing significant reduction in survival of EGFR (P = 0.033) and EGFR (P = 0.019) mice after PH. (F) Liver/body weight ratio in EGFR mice and EGFR mice 7 days after PH or sham surgery. Data indicate mean ± SEM of five mice per group. *, P < 0.05; **, P < 0.005.

We further confirmed these results by employing a second independent transgenic line expressing cre under the control of the IFN-α-inducible Mx promoter, which is active in parenchymal and nonparenchymal liver cells as well as several other organs (27). Adult EGFR Mx-cre (EGFR) mice were injected with the IFN-α inducer poly(I/C) (plpC) and recombination of the floxed allele was observed in the liver and other organs 3 days after the last injection (Fig. 2D and SI Fig. 8A). Despite complete EGFR recombination at the genomic level, absence of the EGFR protein in the liver occurred only ∼3–4 weeks later similarly to what was observed in EGFR mice (SI Fig. 8B). These results indicate that the EGFR protein still persists long after the EGFR allele is deleted. EGFR deletion in adult mice did not lead to growth retardation even when EGFR deletion was induced around day 9 after birth, the earliest time point at which plpC injection did not lead to lethality (data not shown). EGFR mice did not develop any apparent phenotypical alterations or premature death up to 18 months after plpC injection (data not shown). These results demonstrate that additional deletion of the EGFR in nonparenchymal liver cells does not lead to a more severe phenotype than what was observed in EGFR mice.

Analysis of several parameters of liver function, including serum bilirubin, triglycerides, cholesterol, and aspartate aminotransferase/alanine aminotransferase did not reveal differences in EGFR and EGFR mice when compared with control littersmates at 6 and 12 months of age (data not shown). Moreover, the liver-to-body weight ratio was comparable in EGFR mice and EGFR mice and their respec-
tive controls (SI Fig. 8C). These results demonstrate that, apart from the reduction in body size of EGFR<sup>lox/lox</sup> mice, both EGFR<sup>lox/lox</sup> and EGFR<sup>lox/lox</sup> mice did not develop any signs of impaired liver function.

Liver Regeneration Is Impaired in the Absence of EGFR. To investigate whether EGFR is required during liver regeneration, two-thirds PH was performed on both EGFR<sup>lox/lox</sup> and EGFR<sup>lox/lox</sup> mice. Western blot analysis confirmed the absence of EGFR protein before and after PH in both EGFR<sup>lox/lox</sup> and EGFR<sup>lox/lox</sup> mice (SI Fig. 8E). Absence of EGFR resulted in reduced survival of mice lacking EGFR in the liver, which became evident 36 h after PH (Fig. 2E). No mortality was encountered after sham surgery, excluding that surgery-induced stress is responsible for the increased mortality of hepatectomized mice lacking EGFR in the liver (data not shown). Seven days after PH, the liver-to-body weight ratio of EGFR<sup>lox/lox</sup> and EGFR<sup>lox/lox</sup> mice was significantly below that observed for control mice, whereas sham surgery did not affect this ratio (Fig. 2F). However, 15 days after PH, no significant differences in the liver-to-body weight ratio could be detected in both EGFR<sup>lox/lox</sup> and EGFR<sup>lox/lox</sup> mice, indicating that after the initial delay mutant livers can complete regeneration (SI Fig. 8D). Liver morphology of EGFR<sup>lox/lox</sup> and EGFR<sup>lox/lox</sup> mice was comparable to controls before and after PH (SI Fig. 9A–F) (data not shown). These results show that, despite apparent normal liver architecture, liver regeneration is delayed in the absence of EGFR.

Reduced Cell Proliferation in Regenerating Livers of EGFR Mutants. To determine whether EGFR signaling is required for proliferation after PH, the number of S-phase cells was investigated by BrdU incorporation in EGFR<sup>lox/lox</sup> and EGFR<sup>lox/lox</sup> mice. The results represent the mean ± SEM of six to seven independent livers. **, *P < 0.005; *, *P < 0.05.

To exclude that EGFR has an antiapoptotic role during liver regeneration, similar to its function in epithelial tumors and cortical astrocytes (11, 28), apoptosis was measured by TUNEL staining in EGFR<sup>lox/lox</sup> and EGFR<sup>lox/lox</sup> mice after PH. The number of apoptotic cells was overall very low and comparable between EGFR mutants and controls at all time points after PH (SI Fig. 9G–L). Similarly, the expression of several apoptotic and antiapoptotic genes was also unchanged between mutant and controls (data not shown). These data show that reduced proliferation and not increased apoptosis is responsible for impaired liver regeneration in mice lacking EGFR in the liver.

Analysis of Liver Enzyme and Cytokine Production After PH. Increased levels of transaminases like glutamyl oxaloacetic transaminase (GOT) and glutamyl pyruvic transaminase (GPT), which are released in the serum on liver damage, were observed in the serum of EGFR<sup>lox/lox</sup> and EGFR<sup>lox/lox</sup> mice after PH and could partially account for the increased mortality. Other liver parameters like bilirubin, alkaline phosphatase, and cholesterol were not affected and were comparable to controls (Fig. 4A and B) (data not shown). Cytokines like TNFα and IL-6 are known to play a significant role in the early phases of liver regeneration (14, 29). Increased TNFα levels immediately after PH can induce hepatocyte cell cycle entry. However, sustained TNFα after PH can also trigger cell death as observed in Timp3<sup>−/−</sup> mice (30). In mice lacking EGFR in the liver, the expression of several cytokines including IL-6 was not significantly altered and IL-6 levels were also not affected in mutant liver extracts and serum (SI Fig. 10). In the first 8 h after PH, the total levels of TNFα present in liver extracts were also comparable between mutant and controls (Fig. 4C). However, reduced levels of membrane-bound TNFα were detectable by Western blot analysis in EGFR<sup>lox/lox</sup> and EGFR<sup>lox/lox</sup> mice early after PH (Fig. 4D) (data not shown), suggesting that more TNFα is secreted by liver cells lacking the EGFR. Indeed, higher levels of TNFα were observed in the serum of EGFR<sup>lox/lox</sup> and EGFR<sup>lox/lox</sup> mice 24 h after PH (Fig. 4D)
EGFR Is Required for Cell Cycle Progression in Regenerating Hepatocytes. To further investigate the defects in liver regeneration at the molecular level, we examined the expression of several cell cycle regulators after PH. The induction of cyclin D1 mRNA was delayed in the mutants and appeared only 72 h after PH (Fig. 5A and D) (data not shown). Moreover, cyclin D1 protein levels were also significantly reduced and the induction delayed compared with controls. Whereas at 24 h after PH, cyclin D1 protein levels were significantly increased in control livers, no cyclin D1 protein could be detected in mutant livers and cyclin D1 became apparent only 12–24 h later (Fig. 5B and E) (data not shown). Cyclin D1-dependent kinase activity was fully induced 36 h after PH in controls, but only after 48 h in mutants (Fig. 5C). At 72 h, the amount of cdk4 associated with cyclin D1 was still high in EGFR-deficient livers compared with controls. Interestingly, p21 protein expression was reduced in EGFR<sup>ΔIV</sup> and EGFR<sup>ΔIV hops</sup> mice when cyclin D1 protein levels were very low. However, at 48 h, when cyclin D1 started to appear in EGFR mutant livers, the levels of p21 were similar to controls (Fig. 5B) (data not shown). Moreover, cdk2 and cdk1, which are present during the G1–S and G2–M phases, respectively, were expressed at reduced levels in EGFR mutant livers after PH. Expression of other cell cycle inhibitors like p27, p53, and p16 was not significantly affected (Fig. 5B) (data not shown). These results show that EGFR regulates cell cycle progression of regenerating hepatocytes by controlling the expression of cyclin D1 both at the mRNA and protein level.

Signaling Pathways Affected After PH in the Absence of EGFR in the Liver. Growth factors and cytokines mediate hepatocyte proliferation after PH by activating several signaling pathways and transcription factor complexes including AP-1, STAT, and NF-κB (14, 16, 17, 29). We next investigated whether impaired activation of these pathways was responsible for impaired liver regeneration in the early phases after PH. Activation of Erk1/2 after PH was comparable in EGFR mutant and control livers (SI Fig. 11A) (data not shown). Phosphorylation of Akt and GSK3-β was overall very low after PH and not significantly altered in EGFR<sup>ΔIV</sup> and EGFR<sup>ΔIV hops</sup> mice (SI Fig. 11A) (data not shown). Furthermore, STAT3 activation was similar in mutants and controls, which is in accordance with the comparable IL-6 levels observed (data not shown).

To exclude compensatory mechanisms, the expression of other growth factor receptors was investigated. The induction of ErbB3 after PH was similar in control and EGFR mutant livers and PH did not induce the expression of ErbB2 and ErbB4, as previously reported (1) (data not shown). Analysis of EGFR ligand expression by quantitative real-time RT-PCR revealed that the expression of heparin-binding EGF, TGFα, and amphiregulin was slightly reduced in EGFR-deficient livers at 36 h after PH (SI Fig. 12A–D). We also investigated c-Met receptor activation to exclude compensatory mechanisms via this signaling pathway (31, 32). Met protein levels were comparable between mutant and control livers after PH and its phosphorylation was overall very low and not affected by the absence of EGFR (SI Fig. 11B). Moreover, the expression of its ligand hepatocyte growth factor (HGF) was also not affected and comparable to controls both at the mRNA and protein level (SI Fig. 12 E and F). These results exclude that up-regulation of these receptors are compensating for the loss of EGFR.

Immediate-early gene products such as Jun and NF-κB can also control the expression of cyclin D in fibroblasts (33, 34). Surprisingly, the transcriptional activation of c-jun was prolonged in EGFR<sup>ΔIV</sup> and EGFR<sup>ΔIV hops</sup> mice after PH, whereas mRNA expression of other AP-1 family members was comparable between controls and mice lacking EGFR in the liver (SI Fig. 13). Similarly, also Jun protein levels remained high and activated for a longer time after PH in mutant livers (Fig. 6), although JNK activity was unchanged (data not shown). These results suggest that the delayed cyclin D1 activation is not due to impaired Jun expression. Livers lacking EGFR exhibited reduced activation of the stress kinase p38α, which is consistent with sustained Jun activation (Fig. 6), because it was recently shown that c-jun negatively regulates p38α in the liver (35). NF-κB is also activated on stress and can bind to the cyclin D1 promoter (12, 33). Interestingly, the activation of NF-κB was severely impaired in the absence of EGFR in the liver. In control livers, phosphorylation of the p65 subunit of NF-κB occurred already at 4 h after PH and was maximal at 36 h after which it declined. In contrast, in EGFR-deficient livers NF-κB activation was almost absent in the early phases after PH and became apparent only after 48 h (Fig. 6). These results show that mice lacking EGFR in the liver might have an impaired stress response after PH. Because cyclin D1 transcriptional activation occurred.
Discussion
In the present study, we generated mice harboring floxed EGFR alleles to assess the function of EGFR in liver homeostasis and regeneration. Surprisingly, the absence of EGFR in adult liver had little impact on its function in the absence of additional stress. Mice lacking EGFR in the liver survived, were fertile, and had a normal life span. However, perinatal deletion of the EGFR in hepatocytes with the Alfp-crere transgenic line resulted in reduced body weight, suggesting that the EGFR might be important in the first weeks after birth when hepatocyte proliferation occurs. This is supported by our previous findings that in chimeraEGFR−/− ES cell derivatives can efficiently contribute to the livers at embryonic day 14.5, but liver contribution of EGFR−/− cells decreases after birth, suggesting that hepatocytes lacking the EGFR might encounter a proliferative disadvantage when compared with wild-type cells (8).

Two receptor signaling systems mainly involved in driving cell cycle progression during liver regeneration are c-Met and the EGFR with their respective ligands (12, 13). Mice lacking c-Met in the liver display defects in liver regeneration and repair, confirming the importance of c-Met in hepatocyte proliferation and survival (31, 32). Mice lacking the EGFR in the liver also show impaired proliferation and cell cycle progression after PH. Although the activation of Erk and Akt was impaired in c-Met-deficient livers after PH (31, 32), surprisingly these pathways were not affected in EGFR-deficient livers despite the reduced and delayed expression of cyclin D1. There was also no compensatory increase in Met signaling or up-regulation of other erbB family members after PH, which could have provided a possible explanation for the lack of changes in Erk/Akt activation.

After PH, increased mortality was observed in mice lacking EGFR in the liver. The elevated levels of GOT and GPT detected in the serum of mutant mice clearly indicate extended liver damage in these mice. Notably, we also found increased levels of TNFα in the serum of mice lacking EGFR in the liver. Because reduced levels of membrane-bound TNFα were observed in EGFR-deficient livers, it is possible that in the absence of EGFR more soluble TNFα is secreted by liver cells to compensate for the partial cell cycle block. Similar observations have been made for IL-6 in mice lacking c-Met in the liver (31). TNFα and IL-6 can function as a priming agent for hepatocyte replication at the early stages of liver regeneration (36). Furthermore, TNFα increases the sensitivity of hepatocytes to growth factors such as TGFα and can also modulate TGFα expression in regenerating livers (37, 38). The induction of TNFα and IL-6 within the first 8 h after PH was normal in mice lacking EGFR in the liver, thereby excluding that an impaired induction of these cytokines would be responsible for the delayed regeneration.

Sustained levels of TGFα after PH can also trigger cell death as observed in Timp3−/− mice (30). In addition, we have previously shown in astrocytes and in skin tumors that EGFR controls cell survival via the activation of the Akt pathway (11, 28). Despite the high TGFα levels, we did not observe increased apoptosis nor impaired Akt activation in EGFR-deficient livers after PH. We did observe increased c-Jun expression and activation, which might be mediated by the increased TNFα levels and represents a compensatory response to the loss of EGFR and impaired liver regeneration. We speculate that, in EGFR-deficient livers, hepatocyte apoptosis after PH is prevented by the higher c-Jun levels because it has been shown that hepatocytes lacking c-Jun show increased sensitivity to TNFα-induced apoptosis (39). Moreover, mice lacking c-Jun in the liver display increased apoptosis of liver cells after PH, suggesting that Jun might play an antiapoptotic function during liver regeneration (16).

From our results, it seems that EGFR primarily functions to promote hepatocyte proliferation and not hepatocyte survival after PH. Cyclin D was reduced at the mRNA and protein levels, suggesting that G1–S phase progression is impaired in the absence of EGFR. As a consequence, cdk1 and cdk2 expression was also delayed, indicating that mice lacking the EGFR in the liver show an overall delay in cell cycle progression. Our results are in accordance with published data showing that reduced expression of cyclin D1 is associated with impaired liver regeneration (19, 40). Jun has been shown to directly regulate cyclin D1 expression in fibroblasts (34). From our results, it seems unlikely that c-Jun is the key factor positively controlling cyclin D1 expression, because in hepatocytes lacking EGFR c-jun mRNA and protein levels accumulate after PH. The prolonged Jun expression is associated with reduced activity of p38α, which is likely responsible for the reduced p21 levels. These results are in agreement with recent findings that Jun negatively regulates p38α, thereby preventing the accumulation of p21 (35). Recently, Jun-dependent EGFR expression has been demonstrated in cultured keratinocytes and K5-SOS induced tumors (39). Whether Jun is controlling EGFR expression in regenerating hepatocytes remains to be determined.

It is likely that, in the absence of EGFR, there is an impaired stress response after PH. Besides the reduced activity of the stress kinase p38α in both EGFRflh and EGFRAliv livers, we also observed a strong reduction in NF-κB activity in regenerating mutant livers. Our data are in accordance with published results that inhibition of NF-κB activity after PH results in impaired cell cycle progression because of block in G1–S phase entry (41). Furthermore, it has been demonstrated that NF-κB regulates levels and kinetics of cyclin D1 expression during G1 phase by binding to the cyclin D1 promoter (33). These data imply that reduced cell proliferation and reduced levels of cyclin D1 observed in EGFR mutant livers after PH are due to reduced NF-κB activity.

In summary, we have demonstrated that mice lacking EGFR in the liver show impaired liver regeneration with reduced and delayed expression of cyclin D1. In the absence of EGFR in hepatocytes, increased levels of TNFα are released in the serum of mutant mice, which are likely responsible for increased c-Jun expression and could be part of a compensatory response to the delayed liver regeneration. Moreover, p38α and NF-κB activation was reduced in regenerating mutant livers, indicating an impaired stress response after PH. Because NF-κB can directly activate cyclin D1 transcription, we propose that during liver regeneration EGFR signaling controls cyclin D1 expression via NF-κB activation.
Materials and Methods

Generation of EGFR Floxed ES Cells and Mice. A 7-kb genomic XbaI–BsaII EGFR fragment starting 5 kb upstream of the ATG was used to clone the targeting construct. The 5′-loxP site with a diagnostic XmaCI site was introduced into the BamHI site upstream of the EGFR promoter. The frt-flanked neomycin resistance cassette (42) with the 3′-loxP site was cloned into the SexAI site. The diphteria toxin A fragment cassette for negative selection was inserted at the 3′ end of the construct. The linearized targeting plasmid (20 μg) was electroporated into 2 × 10⁷ HM1 ES cells. Clones were analyzed for homologous recombination by two rounds of nested PCR as previously described in refs. 8 and 10. Positive clones were verified by Southern blot analysis by using a HindIII–XmaCI digest, which identifies correct targeting and the presence of the 5′-loxP site. Two correctly targeted EGFR<sup>f/f</sup> ES cells were injected into blastocysts. Germline male chimeras were mated to C57BL/6 females to obtain EGFR<sup>f/f</sup> mice, which were used for further breedings. Removal of the neo cassette from the EGFR<sup>f</sup> allele was obtained by breeding with β-actin-flp mice (43) but took more than five to six generations. Therefore, the following study was conducted with EGFR<sup>f/f</sup> mice still harboring the neo cassette, which did not affect EGFR expression. Genotyping of the mice was performed on tail biopsies by Southern blot analysis or by PCR. Mice were kept in the facilities of the Medical University of Vienna in accordance with institutional policies and federal guidelines.

PH and plpC Injection. All mice subjected to PH were between 8 and 12 weeks old. PH was performed under anesthesia as described previously in ref. 16. The abdominal cavity was entered through a transverse incision just below the sternum, parallel to the rib cage. The large left lateral lobe and median lobes were ligated and removed. The gall bladder was always removed before liver lobe resection to avoid its damage during surgery. Eight-week-old EGFR<sup>f/f</sup> Mx-cre mice and controls were injected three times at 3-day intervals with 400 μg of plpC. PH was performed 3 weeks after the last plpC injection.

Histology, Immunohistochemistry, and TUNEL Assay. Mice were injected i.p. with BrdU at 100 μg/g of body weight 1 h before killing. Histology (Haltag, Burlingame, CA) was performed by using the ABC staining kit (Vector Laboratories, Burlingame, CA). An in situ cell-death detection kit (Roche, Indianapolis, IN) was used for TUNEL staining.

RNase Protection Assay and Real-Time PCR Analysis. Total RNA from frozen tissues was isolated by using TRizol reagent (Invitrogen, Carlsbad, CA). Ten micrograms of RNA were used for each RNase protection reaction using the RibonQuant multiprobe RNase protection assay systems mCyC-1, mFos/Jun, and mCy2B (BD Pharmingen, San Diego, CA). Quantitative real-time RT-PCR was performed with Light Cycler (Roche) as described previously in refs. 11 and 28, employing ALAS gene as loading control.

Western Blot Analysis, Kinase Assay, and ELISA. Western Blot analysis was performed according to standard procedures (11). Kinase assays were performed as described previously in ref. 16. ELISAs for TNFα, IL-6, and hepatocyte growth factor were performed with commercial kits (DY410, DY406, and DY2207; R&D Systems, Minneapolis, MN). Antibodies were as follows: cyclin D1, cyclin E, cyclin B, pan-ERK, Met (Santa Cruz Biotechnology, Santa Cruz, CA); EGFR (Upstate Biotechnology, Lake Placid, NY); p-c-Jun, p-p38α, p38α, p-NF-κB, NF-κB, p-Met, p-GSK-3β, GSK-3β, p53 (Cell Signaling Technology, Beverly, MA); c-Jun, p27, cdk1, cdk2, cdk4 (BD Transduction, San Jose, CA); p21, TNF (BD Pharmingen); p-ERK (New England Biolabs, Beverly, MA) and tubulin (Sigma, St. Louis, MO).

Statistical Analysis. Each experiment was performed at least three times. The statistical significance of the data was determined by applying the two-tailed Student t test or the log rank test. The difference was considered statistically significant at P < 0.05.

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