Xho I and Not I sites. The resulting mutant receptor gene sequence was confirmed. However, the engineered stop codon TGA was removed in this construct, and the extra sequence AAATLDHNQPYHICRGFTC-FKKPPTPPPEPET was added. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; T, Thr; and Y, Tyr.

19. FDC-P1 cells were maintained in RPMI 1640 Hepes supplemented with 10% fetal bovine serum (FBS), 50 μM 2-mertcaptoethanol, streptomycin sulfate (10 μg/ml), penicillin G (10 units/ml), 112 nM Thr^{175} → Gly–hGH, and 50 μM 5-chloro-2-trichloromethyl benzimidazole (**E8**). In preparation for assays, cells were incubated in 1% FBS (fetal bovine serum) media for 16 to 18 hours. For the JAK2 phosphorylation assays, small volumes of Thr^{175} → Gly–hGH or stock solutions of ligand in DMSO, or both, were added to 3 × 10^6 fasting cells in 0.5 ml of 1% FBS media to the desired concentrations. The mixtures were incubated for 10 to 30 min at 37°C. Cells were deactivated and lysed, and JAK2 was immunoprecipitated and electrophoresed essentially as described by Pearce *et al.* (*17*). After blotting on nitrocellulose membranes (Bio-Rad), phosphorylated JAK2 was probed with antibody to phosphotyrosine (4G10; Upstate Biotechnology) and detected with the ECL Western blotting analysis system (Amersham Lifescience). For cell proliferation assays, starved cells were aliquoted in 96-well plates with 1.5 × 10^4 cells in 100 μl of media per well; triplicate wells were incubated at 37°C for 48 hours containing various concentrations of Thr^{175} → Gly–hGH, compound **E8**, or control ligands. Growth was measured with Celltiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega) and normalized to IL-3 response at 5 units/ml.

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Proliferation, But Not Growth, Blocked by Conditional Deletion of 40*S* Ribosomal Protein S6

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Because ribosome biogenesis plays an essential role in cell proliferation, control mechanisms may have evolved to recognize lesions in this critical anabolic process. To test this possibility, we conditionally deleted the gene encoding 40*S* ribosomal protein S6 in the liver of adult mice. Unexpectedly, livers from fasted animals deficient in S6 grew in response to nutrients even though biogenesis of 40*S* ribosomes was abolished. However, liver cells failed to proliferate or induce cyclin E expression after partial hepatectomy, despite formation of active cyclin D–CDK4 complexes. These results imply that abrogation of 40*S* ribosome biogenesis may induce a checkpoint control that prevents cell cycle progression.

A proliferating eukaryotic cell expends 80% of its energy on the biosynthesis of protein synthetic components, most notably ribosomes. This energy is used not only to generate ribosomal components, but also to transport, process, and assemble them into mature ribosomal subunits (*1*). A change in the ratio of ribosomes to mRNA can modify the pattern of proteins synthesized (*2*), ultimately leading to deleterious effects on the cell. Thus, regulatory mechanisms may have evolved to sense the need and state of the translational machinery. Although such mechanisms have been described in bacteria (*3*, *4*), none have been reported in higher eukaryotes. To address this issue, we conditionally deleted the S6 gene, 1 of 33 40*S* ribosomal protein genes (*5*), in the liver of adult mice. The effect of deleting the S6 gene was then examined in two experimental paradigms thought to require biosynthesis of nascent ribosomes: (i) nutrient-induced growth after a period of fasting, and (ii) mitogen-stimulated proliferation after partial hepatectomy (*6*).

To delete S6, we used the interferon-α (IFN-α)–inducible Cre/loxP system (*7*). The mouse S6 gene was cloned (*8*), inserted into a loxP-targeting vector (Fig. 1A), and introduced into embryonic stem (ES) cells by homologous recombination (*9*). Heterozygous mice were generated and crossed to obtain homozygous floxed S6 (fS6) offspring (Fig. 1B), which were phenotypically normal. These mice were then crossed to transgenic mice expressing CRE under the control of an IFN-α–inducible, Mx promoter (*7*). Southern blot analysis of adult tissues from fS6/CRE^+ mice after intraperitoneal injection of IFN-α (*10*) revealed either the 4.6-kb fS6 band or the expected 9.1-kb band (Fig. 1C), representing the S6 gene fragment after CRE-induced recombination (Fig. 1A). The deletion was complete in liver, whereas the effect was variable in other tissues, with no detectable deletion of the gene in either testis or brain (Fig. 1C). No deletion of the fS6 gene was observed in untreated fS6/CRE^+ mice (*11*). Livers of IFN-α–treated fS6/CRE^+ mice contained no detectable S6 mRNA, but mRNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and other ribosomal proteins was present in similar amounts as in fS6/CRE^- mice (Fig. 1D) (*11*). Hepatic histology was normal in liver cells in both fS6/CRE^+ and fS6/CRE^- mice, although differences in fS6/CRE^+ mice were apparent by 30 days after IFN-α treatment (*11*). Thus, the S6 gene can be efficiently deleted in the liver with no immediate deleterious effects on the mouse.

Fasting triggers an almost 50% reduction in liver weight, total protein, and ribosome content (*12*). Upon refeeding, ribosome biogenesis is activated, and the liver regrows to its normal size, with no effect on cell number (*13*). Therefore, homozygous fS6/CRE^- and fS6/CRE^+ mice were treated with IFN-α, then 2 days later deprived of food for 48 hours, before being refed for 24 hours. Liver mass of fS6/CRE^- and fS6/CRE^+ mice decreased during the 48-hour fasting period, with total weight and protein content declining by greater than 40% in both cases (Fig. 2A) (*14*). After refeeding, liver mass and total protein content recovered to the same extent and at the same rate in fS6/CRE^+ as in fS6/CRE^- mice (Fig. 2A) (*14*). To examine the effect of the S6 deficiency on global translation, we analyzed ribosomes from livers of fS6/CRE^+ mice on sucrose gradients (*15*). The proportion of ribosomes in actively translating polysomes was indistinguishable in normally feeding fS6/CRE^+ and fS6/CRE^- mice, with the mean polysome size decreasing to the same extent in both cases during fasting (Fig. 2B). Upon refeeding, the mean polysome size in the livers of fS6/CRE^- mice recovered to that of normally feeding animals, whereas in livers from refed fS6/CRE^+ mice, 40*S* subunits were absent and 60*S* subunits increased (Fig. 2B). Despite the absence of 40*S* subunits, the total number of ribosomes in polysomes increased, although the mean size and total amount of polysomes was less than in fS6/CRE^- mice (Fig. 2B). Thus, deletion of S6 alters 40*S* ribosome biogenesis, but the remaining ribo-

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somes are sufficient for liver mass to recover.

To measure 40*S* ribosome biogenesis, we analyzed either nascent ribosomal proteins or ribosomal RNA (rRNA) in the livers of IFN-α–treated mice that were fasted and then refed for 3 hours in the presence of ^{35}S-methionine or inorganic phosphate-32 (^{32}Pi). Staining of ribosomal proteins (Fig. 2C) or rRNA (Fig. 2D) from livers of fS6/CRE$^+$ and fS6/CRE$^-$ mice showed no difference in their steady-state levels. In both fS6/CRE$^+$ and fS6/CRE$^-$ mice, increased phosphorylation of S6 was detected, as measured by its decreased mobility in both dimensions of electrophoresis (insets, Fig. 2C) (*16*). However, no newly synthesized ^{35}S-labeled 40*S* ribosomal proteins (Fig. 2C) nor ^{32}P-labeled 18*S* rRNA (Fig. 2D) were detected in livers of fS6/CRE$^+$ mice. In addition, an rRNA species of 34*S* accumulated in the livers of fS6/CRE$^+$ mice, most likely representing the 34*S* precursor of 18*S* rRNA (*17*). There was no difference in the production of 60*S* proteins (Fig. 2C) or 28*S* rRNA (Fig. 2D) in livers of fS6/CRE$^+$ or fS6/CRE$^-$ mice. Thus, conditional deletion of the S6 gene in liver abrogates 40*S* ribosome biogenesis at a defined step of maturation.

After partial hepatectomy, the remaining liver cells proliferate to replace the lost liver mass (*6*). To examine liver cell proliferation, we treated fS6/CRE$^+$ and fS6/CRE$^-$ mice with IFN-α and 2 days later subjected them to partial hepatectomy. The livers of fS6/CRE$^+$ mice failed to regenerate after 10 days, whereas the liver mass of fS6/CRE$^-$ mice was totally recovered (Fig. 3A). Hematoxylin and eosin staining of histological sections from liver of fS6/CRE$^-$ mice 40 hours after hepatectomy revealed lobular disarrays and mitotic figures typical of regenerating liver (*18*) (Fig. 3B). In contrast, liver sections from fS6/CRE$^+$ mice were similar to those of IFN-α–treated, nonhepatectomized mice and showed no sign of regenerative or pathological responses (Fig. 3B). In agreement with these results, livers of mice deficient for fumarylacetoacetate hydrolase gene, a model system for liver transplantation studies (*19*), were repopulated by fS6/CRE$^-$, but not fS6/CRE$^+$ liver cells (*20*). To determine whether fS6/CRE$^+$ liver cells progressed to S phase after partial hepatectomy, we monitored DNA synthesis by either [^{3}H]thymidine or bromodeoxyuridine (BrdU) incorporation. Two peaks of [^{3}H]thymidine incorporation were observed in liver from fS6/CRE$^-$ mice, at 40 and 60 hours after partial hepatectomy (Fig. 3C). In contrast, no [^{3}H]thymidine incorporation (Fig. 3C) or BrdU label (Fig. 3D) was detected in livers of fS6/CRE$^+$ mice. Thus, in the absence of nascent 40*S* ribosome biogenesis, the remnant liver cells fail to enter S phase.

A key step required for passage through the G1/S boundary is the induction of cyclin D and activation of cyclin D–CDK4 and cyclin D–CDK6 complexes (*21*). Liver cells from IFN-α–treated fS6/CRE$^+$ and fS6/CRE$^-$ mice expressed similar amounts of cyclin D1 protein at 40 and 60 hours after hepatectomy (Fig. 4A) (*22*). The expression of p21^{CIP1}, which acts as a positive and negative modulator of CDK function (*21*), paralleled that of cyclin D1 in fS6/CRE$^+$ and fS6/CRE$^-$ liver cells (Fig. 4A). In addition, p27^{KIP}(*22*), a negative effector of cyclin-CDK function, decreased in both fS6/CRE$^+$ and fS6/CRE$^-$ liver cells (Fig. 4A). Consistent with these observations, cyclin D1–associated and CDK4 kinase activities increased in fS6/CRE$^+$ and fS6/CRE$^-$ cells (Fig. 4B). These findings indicate that liver cells of fS6/CRE$^+$ and fS6/CRE$^-$ mice had been stimulated

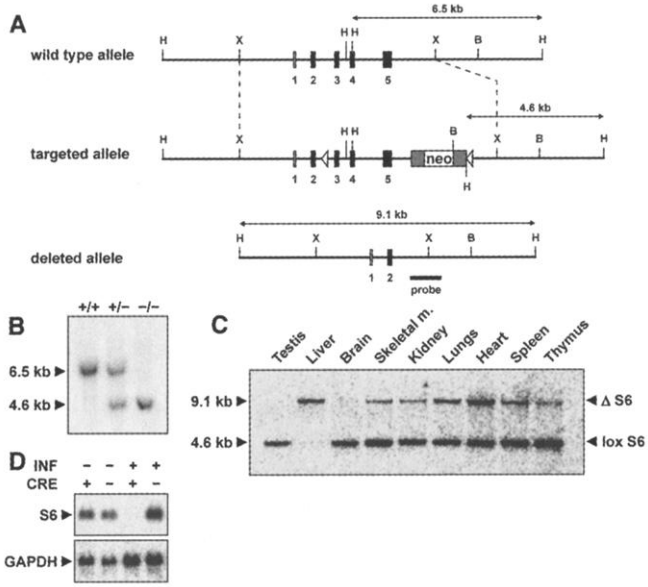

Fig. 1. Targeted disruption of the S6 locus. (**A**) The wild-type S6 gene, targeted allele, and deleted allele are shown. Bam HI (B), Hind III (H), and Xba I (X) sites are shown, as well as the neomycin cassette (neo). Exons are denoted by filled boxes, and loxP sequences, by triangles. The DNA probe used for Southern blot analysis (*30*) and Hind III–restricted fragments are indicated. (**B**) Southern blot analysis of genomic DNA from wild-type mice and fS6 mice. (**C**) Southern blot analysis of specific tissues from homozygous fS6/CRE$^+$ mice after IFN-α treatment. (**D**) Northern blot analysis (*30*) of S6 and control GAPDH mRNA before and after IFN-α treatment were carried out with full-length cDNAs to each mRNA.

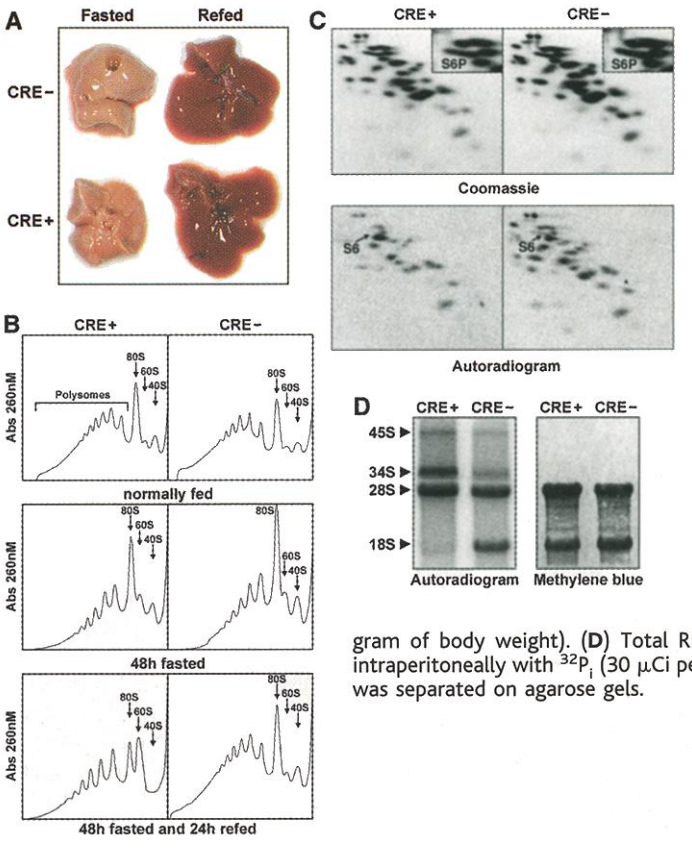

Fig. 2. Effects of S6 deletion on liver growth and ribosome biogenesis. (**A**) Livers from fS6/CRE$^+$ and fS6/CRE$^-$ mice fasted for 48 hours or fasted and refed for 24 hours. The color change is due to the fasting regime. (**B**) Polysome profiles from livers of mice that were fed normally, fasted, or fasted and refed for the indicated times. The abscissa represents the effluent volume of the sucrose gradient. (**C**) Two-dimensional polyacrylamide gel electrophoresis of ribosomal proteins (*16*) from mice injected intraperitoneally with ^{35}S-methionine (10 μCi per gram of body weight). (**D**) Total RNA from mice injected intraperitoneally with ^{32}P$_i$ (30 μCi per gram of body weight) was separated on agarose gels.

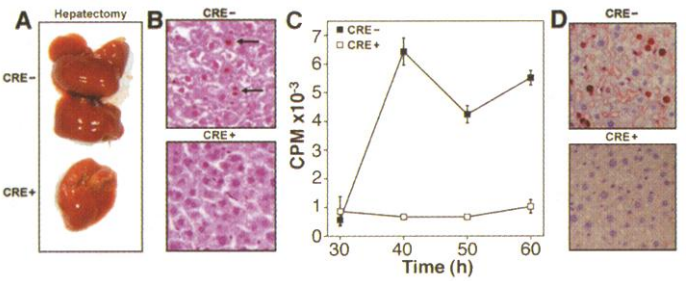

Fig. 3. Effect of S6 deletion on liver regeneration and cell proliferation. (**A**) Livers from fS6/CRE$^+$ and fS6/CRE$^-$ mice 10 days after partial hepatectomy. (**B**) Histological sections (*31*) of livers from fS6/CRE$^+$ and fS6/CRE$^-$ mice 40 hours after partial hepatectomy. Mitotic figures are indicated by arrows. (**C** and **D**) Incorporation of [^{3}H]thymidine or BrdU (*31*), respectively, into DNA.

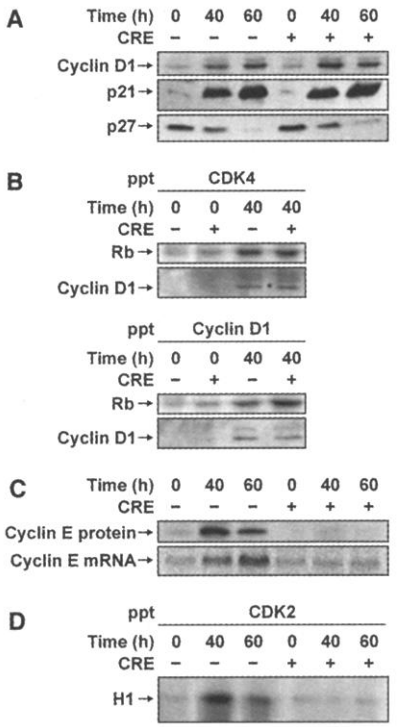

Fig. 4. Analysis of cell cycle components in S6-deficient mice. Liver samples from IFN-α–treated fS6/CRE$^-$ and fS6/CRE$^+$ mice were analyzed at the indicated time points after partial hepatectomy. (**A**) Analysis of cyclin D1, p21^{CIP1}, and p27^{KIP} protein levels by Western blot. (**B**) Cyclin D1–associated and CDK4 kinase activities were measured with Rb as a substrate (*32*) (upper panel); the level of immunoprecipitated cyclin D1 is shown in the lower panel. (**C**) Analysis of cyclin E protein and mRNA levels by Western and Northern blot, respectively. (**D**) CDK2-associated kinase activity was measured with histone H1 as a substrate (*32*).

to proliferate and progress to late G_1. Progression beyond this point and entry into S phase requires increased expression of cyclin E (*23*, *24*). After partial hepatectomy, such an increase in cyclin E protein and mRNA expression was observed in the livers of fS6/CRE$^-$ mice, but not in fS6/CRE$^+$ mice (Fig. 4C). Equivalent results were obtained for cyclin A (*11*). Consistent with these findings, activation of the cyclin E– or cyclin A–dependent kinase, CDK2, was observed in fS6/CRE$^-$ but not fS6/CRE$^+$ liver cells (Fig. 4D). Thus, S6-deficient liver cells can initiate the early events required for entry into S phase, but fail to induce those required to progress beyond this point.

These studies show a distinction between the ability of liver cells to grow and their ability to proliferate (*25*–*27*). The lesion in proliferation seems not to result from a lack of translational capacity, because there is no difference in the abundance or rate of accumulation of p21^{CIP1} or cyclin D1 protein in S6-deficient versus wild-type liver cells (Fig. 4). Furthermore, S6-deficient liver cells have the ability to synthesize proteins and grow (Fig. 2) (*11*). Instead, the failure to progress through the cell cycle appears to be due to a block in expression of cyclin E mRNA. Expression of cyclin E is essential in the proliferative response (*23*), and inhibition of cyclin E function is the major route by which DNA-damaging agents, through a p53-mediated checkpoint control, inhibit cell cycle progression (*28*). Although a direct effect of S6 on cell division cannot be excluded, failure to express cyclin E may result from activation of a checkpoint (*29*), induced by a defect in ribosome biogenesis to avoid improper execution of the genetic program.

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9. The first loxP sequence was introduced into the second S6 intron. The second loxP site along with the neomycin cassette was inserted into 3′-flanking sequence. The targeting vector contained 3 kb of homologous DNA upstream of the 5′ loxP site and 1.1 kb of homologous DNA downstream of the second loxP site. The linearized vector was electroporated into E14 cells derived from 129/OlaH mice, maintained on subconfluent embryonic fibroblasts. G418-resistant colonies were selected and expanded. The correctly targeted ES cells were aggregated with F_1 (C57BL/6 × BALB/c) morulae. The out-coming blastocyst was transferred to the uteri of pseudopregnant female F_1 (C57BL/6 × BALB/c) recipients. To establish the S6 mutant allele in the germ line, we mated the resulting chimeras with C57BL/6 females [M. R. Capecchi, *Science* **244**, 1288 (1989)].
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15. Batches (500 mg) of liver were suspended in 5 ml of buffer and homogenized with a Polytron (Kinematica, Switzerland). Extracts were then centrifuged at 2300*g* for 10 min at 0°C, and 200 μl of supernatant was analyzed on 17.1 to 51% sucrose gradients [H. B. J. Jefferies *et al.*, *EMBO J.* **12**, 3693 (1997)].
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