ing 8000 understory leaves on transects through the study plot and recording length of each larva present. Larval lengths were converted to biomass using length-mass regressions.
20. No banded fledglings from the 64-ha plot in New Hampshire have returned to the plot as breeding adults, nor have any been resighted on winter quarters. Thus, the relations between annual fecundity and recruitment documented here represent warbler population dynamics occurring at a larger, regional scale.
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31. We used Cormack-Jolly-Seber models (*29*) and the *MARK* computer program (*30*) to estimate warbler survival and recapture probabilities. Survival from $year_i$ to $year_{i+1}$ was modeled as constant, as dependent on year, and as a linear function of mean monthly SOI in $year_i$. Recapture probability was modeled as either constant or as dependent on year. We did not model age- or sex-related differences in these parameters because of small sample sizes from Jamaica. Identical but separate models were run for the Jamaica and New Hampshire populations. Model details can be found at *Science* Online at www.sciencemag.org/features/data/1049756/shl.
32. Funded by grants from the NSF. We are grateful for the advice and assistance of M. Ayres, D. Bolger, K. Cottingham, P. Doran, M. Johnson, W. Lowe, P. Marra, M. McPeek, S. Morrison, L. Nagy, J. Nichols, P. Roberts, K. Sillett, S. C. Sillett, and A. Strong. We thank the Jamaica Natural Resources Conservation Authority, the Williams and Sutton families of Jamaica, and the Hubbard Brook Experimental Forest of the U.S. Department of Agriculture's Northeastern Research Station for their support and cooperation.

23 February 2000; accepted 26 April 2000

Designing Small-Molecule Switches for Protein-Protein Interactions

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Mutations introduced into human growth hormone (hGH) ($Thr^{175} \rightarrow Gly$–hGH) and the extracellular domain of the hGH receptor ($Trp^{104} \rightarrow Gly$–hGHbp) created a cavity at the protein-protein interface that resulted in binding affinity being reduced by a factor of 10^6. A small library of indole analogs was screened for small molecules that bind the cavity created by the mutations and restore binding affinity. The ligand 5-chloro-2-trichloromethylimidazole was found to increase the affinity of the mutant hormone for its receptor more than 1000-fold. Cell proliferation and JAK2 phosphorylation assays showed that the mutant hGH activates growth hormone signaling in the presence of added ligand. This approach may allow other protein-protein and protein–nucleic acid interactions to be switched on or off by the addition or depletion of exogenous small molecules.

A large number of cellular processes involve specific protein-protein or protein–nucleic acid interactions, including signal transduction, transcription, cellular trafficking, and mitosis. Many of these interactions are regulated either by posttranslational modification (e.g., phosphorylation, acylation, and methylation) or by binding of ligands such as guanosine triphosphate, cyclic adenosine monophosphate, and hormones. There are few general strategies for the generation of synthetic molecules that directly modulate these interactions. Here we report a two-step approach for regulating biomolecular interactions. First, a cavity is introduced at a protein-protein or protein–nucleic acid interface that results in a loss of binding energy. A library of small molecules is then screened for ligands that bind the cavity and restore the protein-protein or protein–nucleic acid interface. Using this strategy, we generated a ligand-gated growth factor–growth factor receptor interaction between human growth hormone (hGH) and its receptor (*1*, *2*).

The initial event in signaling through the hGH receptor is the binding of hGH to site 1 of the extracellular domain of the receptor (hGHbp) to form a high-affinity complex (dissociation constant K_d = 0.3 nM). A high-resolution x-ray crystal structure (*3*) of this complex reveals an interface of about 1300 Å^2 that involves 31 side chains on the hormone and 33 residues on the receptor (*4*). Alanine scanning mutagenesis revealed that Trp^{104} and Trp^{169} of hGHbp are critical binding determinants (*4*). The binding affinity of the $Trp^{104} \rightarrow Ala$ mutant of hGHbp is reduced by more than 2500-fold relative to wild-type hGHbp. The side chains of these residues pack with surrounding nonpolar side chains from both hGH and hGHbp to form a hydrophobic core that, together with a network of hydrogen bonds and five interprotein salt bridges, makes up the protein-protein interface (*5*). It has been shown that interface remodeling that repacks the 150 Å^3 cavity introduced by the $Trp^{104} \rightarrow Ala$ mutation can restore a substantial fraction of the binding energy (*6*). Similar results have been found with mutations to the hydrophobic cores of other proteins (*7*). In addition, a mutant of T4 lysozyme was stabilized by incorporation of exogenous benzene into a hydrophobic cavity in the protein interior (*8*). Benzene also shifted the dimer-trimer equilibrium of a GCN4 leucine zipper $Asn^{16} \rightarrow Ala$ mutant by binding to a hydrophobic cavity in the trimer (*9*).

These results suggested that it might be possible to selectively repack a hydrophobic cavity created at the hGH-hGHbp interface with an exogenous small molecule. Such a molecule might be expected to act as a molecular switch—addition of the ligand should result in binding of a low-affinity hGH mutant to the receptor and activation of the signaling pathway. To test this notion, we substituted Trp^{104} in hGHbp and Thr^{175} in hGH with glycine. The $Thr^{175} \rightarrow Gly$ mutant was expected to further reduce the interaction between the two proteins (the binding affinity of the $Thr^{175} \rightarrow Ala$ mutant is reduced 25-fold) and make the cavity at the interface larger to accommodate indole analogs that might complement the defect.

To screen for small molecules that complement the hGH-hGHbp interface defect, we panned phage-displayed hGH against immobilized hGHbp in the presence of added ligand. This assay allows one to further optimize cavity shape by screening a phage-displayed hGH library for mutants that improve the ligand-"hole" complementarity. hGH was displayed on the NH_2-terminus of pIII protein of filamentous phage as previously described (*10*). The Trp^{104}-hGHbp mutant was immobilized on streptavidin-coated magnetic beads through a unique biotinylated cysteine generated by mutation of surface Ser^{201} (*11*). This residue is distant from binding site 1 but blocks the second hGH-hGHbp interface (site 2) when linked to a solid support (*4*). A library of roughly 200 indole derivatives and derivatives of structurally related 5- and 6-membered fused aromatic heterocycles including benzimidazoles, quinolines, isoquinolines, benzothiazoles, and purines was screened for ligands that complement the binding epitope mutations (*12*).

To carry out the screen, we divided the small molecule library into 17 groups with about 10 compounds per group; the compounds were dissolved in dimethyl sulfoxide (DMSO)

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and diluted to a final concentration of 100 μM. Phages were panned against immobilized receptor in the presence of ligand, and after extensive washing, the number of recovered phage was quantitated (*13*). The relative phage recovery ratio is shown in Fig. 1A; only group E consistently yielded a substantially higher recovery of phage in the presence versus absence of added ligands (*14*). To identify the highest affinity ligand in group E, we iterated the screening process with single compounds in the binding buffer. Only 5-chloro-2-trichloromethyl benzimidazole (**E8**) increased the recovery of the hGH mutant phage (Fig. 1B); all other compounds had a negative affect on phage recovery. Molecules with similar structures to **E8** were then selected from the original library and assayed individually; several other positive compounds were identified (Figs. 1C and 2). A comparison of these structures with those of compounds that do not affect phage binding reveals that the benzimidazole ring is an essential feature for binding to the cavity at the protein-protein interface. Small hydrophobic groups such as chloro or methyl groups are preferred at the 5-position, whereas the 2-position is more tolerant of larger substituents. Substituents as small as a methyl group at the 6-position abolish binding.

Surface plasmon resonance was used to quantitate the binding affinity between the mutant hormone and its receptor in both the presence and the absence of compounds identified from the phage panning experiments (*15*). The binding affinity of the Thr^{175} → Gly–hGH mutant to the Trp^{104} → Gly–hGHbp mutant in the absence of added ligand was too low to accurately measure (K_d > 1 mM; Fig. 3B). All of the compounds that improved phage recovery substantially increased the binding affinity between the mutant hormone and receptor (Fig. 3A and Table 1); the highest increase in binding affinity was observed for **E8**. The binding affinity of the mutant hormone for the mutant receptor increased greater than 1000-fold in the presence of 100 μM **E8** to a K_d value of 260 nM. Although the phage recovery ratio does not correspond quantitatively to the measured affinity enhancement by the ligands, compounds that increased the phage recovery ratio always improved the binding affinity (phage recovery under nonequilibrium washing conditions also likely depends on other parameters such as association and dissociation rates).

To determine whether Thr^{175} → Gly–hGH acts as an agonist in the presence of **E8**, we carried out JAK2 phosphorylation and cell proliferation assays established for growth hormone signal transduction with the interleukin-3 (IL-3)–dependent promyeloid cell line, FDC-P1 (*16*, *17*). A construct pAC30 (*18*) containing the full-length mutant hormone receptor (Trp^{104} → Gly, Ser^{201} → Cys–hGHR) was used to transfect FDC-P1 cells. A stable cell line AC30C8 was established by proliferating

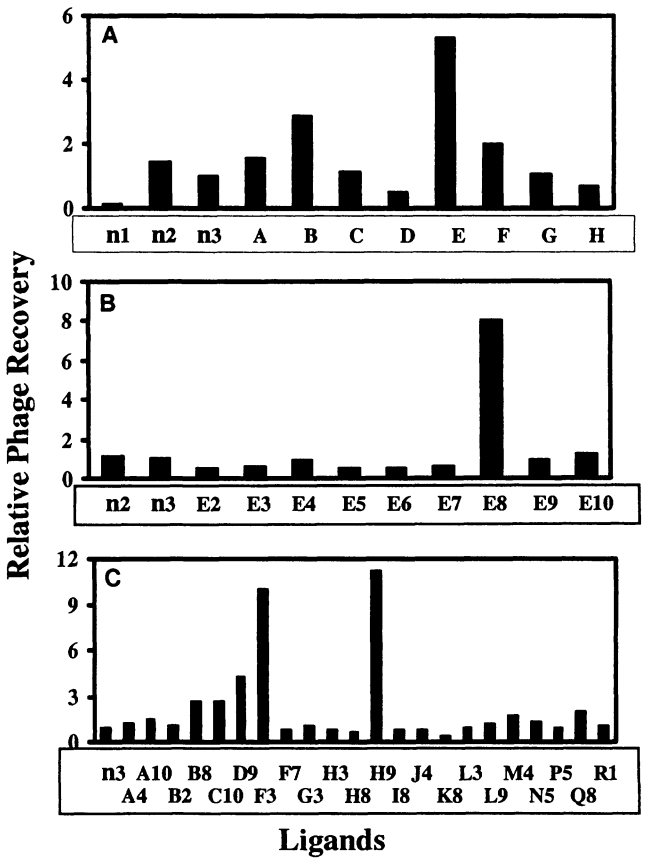


Fig. 1. Phage recovery ratios in the presence of the small molecule mixtures used for screening. n1, negative control without mutant receptor, ligands, and DMSO; n2, negative control without ligands and DMSO; n3, negative control without ligands but with 1% DMSO. The phage recovery ratio was normalized to that for the n3 experiment (relative phage recovery = 1). (**A**) Relative phage recovery in the presence of compound groups A to H. (**B**) Relative phage recovery in the presence of individual compounds of group E (E2 to E10). (**C**) Relative phage recovery in the presence of selected compounds that closely resemble E8 for Thr^{175} → Gly–hGH mutant phage.

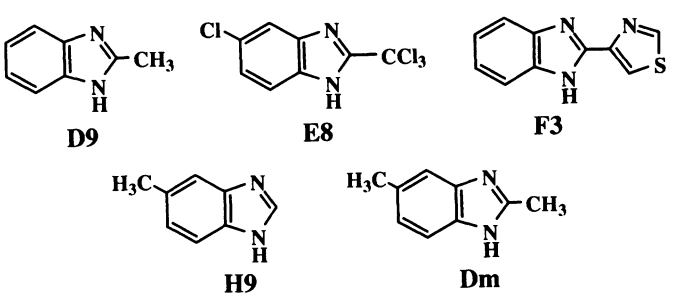


Fig. 2. Structures of ligands that complement the defective interface between Thr^{175} → Gly–hGH and Trp^{104} → Gly–hGHbp.

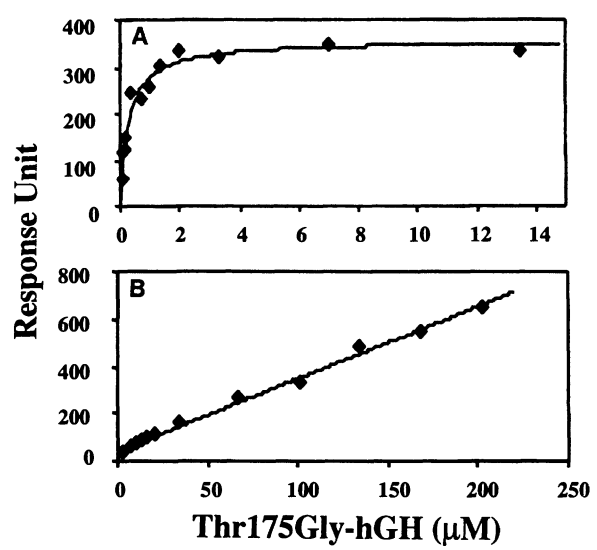
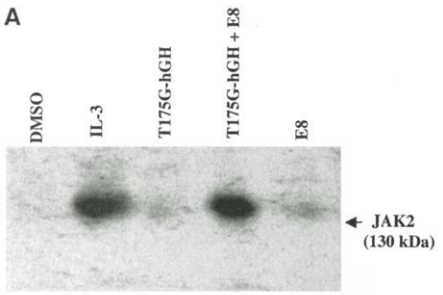


Fig. 3. Effect of 5-chloro-2-trichloromethyl benzimidazole (**E8**) on binding affinity of Thr^{175} → Gly–hGH to Trp^{104} → Gly–hGHbp. (**A**) Sigmoidal association curve of mutant hormone and receptor in the presence of 100 μM **E8**. (**B**) Surface plasmon resonance response in the absence of **E8** compound.



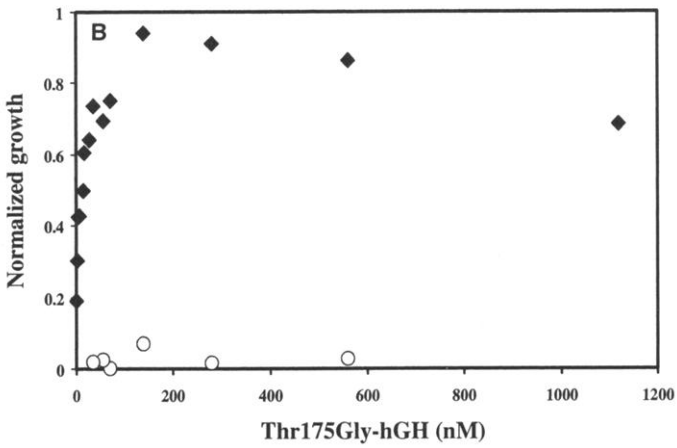


Fig. 4. Mitogenic signaling through the mutant hGH-hGHR complex in FDC-P1 cells. (**A**) Tyrosine phosphorylation of JAK2 in the presence of **E8**. In the positive control, concentration of IL-3 was 10 units/ml. The $Thr^{175} \rightarrow$ Gly–hGH (T175G-hGH) concentration was 112 nM, and the ligand concentration was 50 μM when included. DMSO (0.2% v/v) was also included in all reactions. (**B**) Cell proliferation of transfected FDC-P1 cells for $Thr^{175} \rightarrow$ Gly–hGH in the presence (◆) versus absence (○) of 50 μM ligand **E8**. Cell growth was normalized to the IL-3 response at 5 units/ml.

the transfected cells in the presence of a 112 nM concentration of $Thr^{175} \rightarrow$ Gly–hGH and a 50 μM concentration of **E8** (the compounds in Fig. 2 show no cytotoxicity at concentrations ≤50 μM). Stably transfected cells were then assayed for mitogenesis in the presence and absence of the mutant hormone with and without ligand **E8**. The phosphorylation of JAK2 (Fig. 4A), an early event in the mitogenic intracellular pathway for the hGH receptor, shows that robust mitogenic signaling is stimulated only in the presence of the mutant hormone and 50 μM **E8** (*19*). Dose-dependent proliferation of FDC-P1 cells (Fig. 4B) further confirms that **E8** gates mitogenic signaling by the mutant hGH-hGHR complex (median effective concentration EC_{50} = 10 nM) (*20*, *21*). Moreover, small molecules that showed no affinity in either the phage or surface plasmon resonance assays did not stimulate mitogenesis. These results demonstrate that small molecules can be identified that not only mediate binding of mutant hormone but also switch on the signal transduction pathway.

The success of this approach for modulating macromolecular interactions with a limited set of protein mutations and relatively small library of ligands suggests that it may be relatively easy to generalize this strategy to other systems. These include other growth factors, cytokines, and chemokines; DNA binding molecules such as transcription factors and repressors; and possibly enzyme-substrate complexes. The same strategy could be used to complement an internal cavity in a protein during translation and folding. Ultimately, this approach may allow conditional activation or inactivation of cellular events mediated by macromolecular interactions in vivo (*23*).

Table 1. Dissociation constants (K_d) between wild-type (wt) and mutant hGH and its receptor at binding site 1 and the effects of small ligands on binding affinity.

hGH	hGHbp	Ligands	K_d
wt	wt	None	0.3 nM*
wt	W104A	None	>750 nM*
T175G	W104G	None	>1 mM
T175G	W104G	100 μM **D9**	27 μM
T175G	W104G	100 μM **E8**	0.26 μM
T175G	W104G	100 μM **F3**	2.2 μM
T175G	W104G	100 μM **H9**	52 μM
T175G	W104G	100 μM **Dm**	53 μM

*Data were taken from (*4*).

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11. The human growth hormone gene was amplified from a human pituitary cDNA library (Stratagene) by polymerase chain reaction (PCR) and inserted into the vector pFAB-5c (*22*) between Sfi I and Not I sites to display hGH on M13 phage surface as a fusion protein to gIIIp. The extracellular portion of hGH receptor (residues 1 to 191 of the mature protein) was assembled from exons 3, 4, 5, and 6 and part of exons 2 and 7 amplified from human genomic DNA (Clontech) by PCR with primers overlapping with adjacent exon sequence. The hGHbp gene was subsequently fused to *st*II signal sequence and *pho*A promoter (*22*) and cloned into pUC118 between Eco RI and Bam HI sites in the presence of 3 mM sodium phosphate in the culture medium. Sculptor in vitro Mutagenesis System (Amersham Lifescience) was used to introduce site-directed mutations. To express and purify the wild type and mutant hormone, we digested the corresponding constructs with Eag I and religated them to link a COOH-terminal histidine tag for purification with Ni^{2+}–nitrilotriacetic acid column.
12. Structures of the compounds are available on *Science* Online at www.sciencemag.org/feature/data/1050853.shl or upon request from the authors.
13. In a typical panning experiment, hGH mutant phages (10^{10} to 10^{11} colony-forming units in 50 μl) were mixed with 5 μl of biotinylated $Trp^{104} \rightarrow$ Gly–hGHbp (1.5 mg/ml), 150 μl of binding buffer [1 × phosphate-buffered saline (PBS) (pH 7.4) and 1 mM EDTA], and 2 μl of a 10 mM stock solution of ligand in DMSO. The mixture was then incubated at room temperature for 30 min and centrifuged at 18,000*g* for 10 min. The supernatant was subsequently incubated at room temperature for 30 min with 50 μl of Streptavidin Magnetic Particles (Boehringer Mannheim), which were preblocked with bovine serum albumin (BSA). The captured phages were washed (5 × 1 ml) with binding buffer supplemented with 0.1% Tween 20, 0.1% BSA, and 1% of 10 mM ligand stock solution in DMSO. Finally, the phage particle suspension (10 μl out of 100 μl in binding buffer) was used directly to superinfect 1 ml of midlogarithmic XL1-Blue culture (absorbance at 600 nm = 0.5 to 1.0) and titered on LB ampicillin (100 μg/ml) agar plates.
14. The $Thr^{175} \rightarrow$ Gly, $Thr^{67} \rightarrow$ Gly and $Thr^{175} \rightarrow$ Gly, $Lys^{168} \rightarrow$ Ala double mutants were also panned, but the highest phage recovery ratios in the presence of the ligand library were obtained with the $Thr^{175} \rightarrow$ Gly mutant.
15. The mutant receptor protein ($Trp^{104} \rightarrow$ Gly, $Ser^{201} \rightarrow$ Cys–hGHbp) was modified with *N*-[6-(biotinamido)hexyl]-3′-(2′pyridyldithio)propionamide and captured with streptavidin on a sensor chip, while increasing concentrations of hGH mutants and a 100 μM concentration of the compound were injected at a flow rate of 10 μl/min. PBS buffer was used to mimic physiological ionic strength, and the equilibrium response unit was read after 4.5 min of injection. The receptor matrix was regenerated by washing (2 × 1 min) with 2.5 M $MgCl_2$ solution in PBS buffer. Control experiments showed that this was sufficient to remove all bound hormone. The resonance responses and the corresponding concentrations of the mutant hormone were then fitted with a saturation curve according to Eq. 1 to calculate the dissociation constants:

$$R = R_{max}*K_a*[hGH]/\{1 + K_d*[hGH]\}, K_d = 1/K_a \quad (1)$$

where R and R_{max} are the real and maximum surface plasmon response, respectively, and K_a is the association constant.
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18. The cytoplasmic portion of the receptor gene was amplified from a human liver cDNA library (Stratagene) by PCR. Silent mutations (*16*, *17*) were introduced in the transmembrane portion by PCR primers. The cytoplasmic and extracellular ($Trp^{104} \rightarrow$ Gly, $Ser^{201} \rightarrow$ Cys–hGHbp) portions were then fused through PCR reaction to obtain the full-length receptor gene. The gene was inserted into modified pEGFP-N1 (Clontech), whose replication origin was replaced by the low copy number replication origin from plasmid pACYC177 (New England Biolabs) between the

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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28 March 2000; accepted 20 April 2000

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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