structure within a volume along the ray path whose Fresnel width (22) for 1-Hz P'_{DF} rays in the inner core is about 150 km. Lateral variations at much smaller scales will be smeared by the physical propagation of the waves. My observations exhibit considerable coherence on length scales up to 100 km, but variability at longer scales (Fig. 2A). The steepest lateral gradient that is admissible by the ASN data at 100-km length scales is 0.1% per degree (Fig. 3A; azimuths 310° to 317°). The rotation rate inferred from the COL observations and this gradient is 0.05° per year.

My preferred estimate is that the inner core currently rotates 0.2 to 0.3° per year faster than the mantle, but rates as low as 0.05° per year cannot be ruled out by the available data. The preferred values are 3 to 15 times smaller than, but in the same direction as, previous estimates (5, 6). This lower rotation rate raises Buffett's (8) estimate of the upper limit for inner core viscosity from 3×10^{16} to 10^{17} Pa·s. The low inferred differential rotation rate is as much as 10 times slower than predicted from some dynamo calculations (3) and can be used to constrain parameters for future dynamo models.

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14. Differential times were picked by first aligning the P'_{BC} and P'_{DF} phases and visually determining how many cycles of the seismograms were coherent. This varied from one cycle to five, and typically was two or three. Differential times were determined, using the longest time windows for which the two phases were coherent. For each seismogram, differential times were also picked, using small windows around the first, second, third, and fourth prominent peaks or troughs in the seismograms. The standard deviations among these different picks for each seismogram is less than 0.1 s, which is a realistic estimate of the picking uncertainties. Because the P'_{DF} phase passes through the strongly attenuating inner core, it is often deficient in short-period energy relative to P'_{BC}. This difference is expected to cause differential times to be frequency dependent, and possibly produce a bias in the differential times that is sensitive to the chosen time windows. For the observations analyzed here, this bias is insignificant. The mean difference among picks from the first and second peaks is 0.02 s, from second to third is −0.06 s, and from third to fourth is −0.01 s.
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23. The idea of jointly analyzing COL and ASN data came from a discussion with T. Dahlen during an Incorporated Research Institutions for Seismology (IRIS) field trip. I thank X. Song for sending me his hand-digitized seismograms at COL for earthquakes before 1982; B. Buffett, T. McSweeney, R. Merrill, M. Brown, J. Castle, and J. Winchester for discussion; and B. Buffett, R. Engdahl, X. Song, and S. Tanaka for sending preprints. I also thank IRIS, the ASN, C. Rowe, and D. Christensen for providing high-quality waveform data. Supported by NSF.

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Regulation of Distinct Stages of Skeletal Muscle Differentiation by Mitogen-Activated Protein Kinases

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The signal transduction pathway or pathways linking extracellular signals to myogenesis are poorly defined. Upon mitogen withdrawal from C2C12 myoblasts, the mitogen-activated protein kinase (MAPK) p42Erk2 is inactivated concomitant with up-regulation of muscle-specific genes. Overexpression of MAPK phosphatase-1 (MKP-1) inhibited p42Erk2 activity and was sufficient to relieve the inhibitory effects of mitogens on muscle-specific gene expression. Later during myogenesis, endogenous expression of MKP-1 decreased. MKP-1 overexpression during differentiation prevented myotube formation despite appropriate expression of myosin heavy chain. This indicates that muscle-specific gene expression is necessary but not sufficient to commit differentiated myocytes to myotubes and suggests a function for the MAPKs during the early and late stages of skeletal muscle differentiation.

C2C12 myoblasts proliferate in response to mitogens and upon mitogen withdrawal differentiate into multinucleated myotubes (*1*). Although much progress has been made in defining the mechanisms governing myogenesis at the transcriptional level (*1*, *2*), the signal transduction pathways involved in myogenesis are poorly defined. Differentiation of C2C12 myoblasts is inhibited by serum, growth factors (*3*), oncogenic tyrosine kinases (*4*), and oncogenic forms of the small guanine nucleotide-binding protein Ras (*5*). These observations implicate the Ras pathway in relaying to the nucleus extracellular signals that repress myogenesis. One of the effector pathways of Ras leads to activation of MAPKs, which have been implicated directly in regulating proliferation and differentiation by phosphorylation of transcription factors in a variety of cellular systems (*6*, *7*). However, the function of the MAPKs during skeletal muscle differentiation has not been elucidated.

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We induced C2C12 myoblasts to differentiate by transfer from growth medium (GM) to differentiation medium (DM) (*8*). Within 24 hours in DM, p42Erk2 activity (*9*) was reduced by 50%, whereas the activity of the related MAPK, c-Jun NH_2-terminal kinase (JNK), remained unaffected (Fig. 1A). Concomitantly, expression of the skeletal muscle-specific basic helix-loop-helix transcription factors MyoD and myogenin and the cell cycle inhibitor p21 (*10*) were increased, marking the onset of myogenesis and cell cycle withdrawal (Fig. 1B). By day 3, multinucleated myotubes had formed (*11*), correlating with expression of the terminal differentiation marker myosin heavy chain (MHC) (Fig. 1B). Between days 2 and 3 of differentiation, p42Erk2 activity remained low, whereas JNK activity increased by 1.8-fold (Fig. 1A). The amount of p42Erk2 and JNK proteins remained constant throughout differentiation (*11*).

These results suggest that inactivation of p42Erk2 might be required for C2C12 myoblasts to initiate myogenesis. If so, ectopic expression of a dual-specificity protein phosphatase with substrate specificity toward the MAPKs should facilitate myogenesis in the presence of mitogens. We sought to overex-

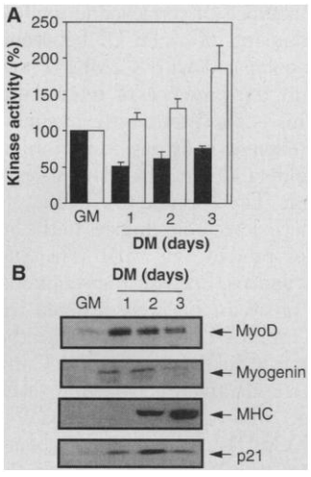

Fig. 1. Activity of p42Erk2 and JNK during C2C12 differentiation (**A**) C2C12 myoblasts were induced to differentiate by transfer from GM to DM. At the times indicated, p42Erk2 (closed bars) and JNK kinase (open bars) activities were assessed. Results are representative of the mean ± SEM from five separate experiments for p42Erk2 and four separate experiments for JNK. (**B**) C2C12 myoblasts were assessed for their progression through differentiation by immunoblot analysis for expression of MyoD, myogenin, MHC, and p21 at the indicated times.

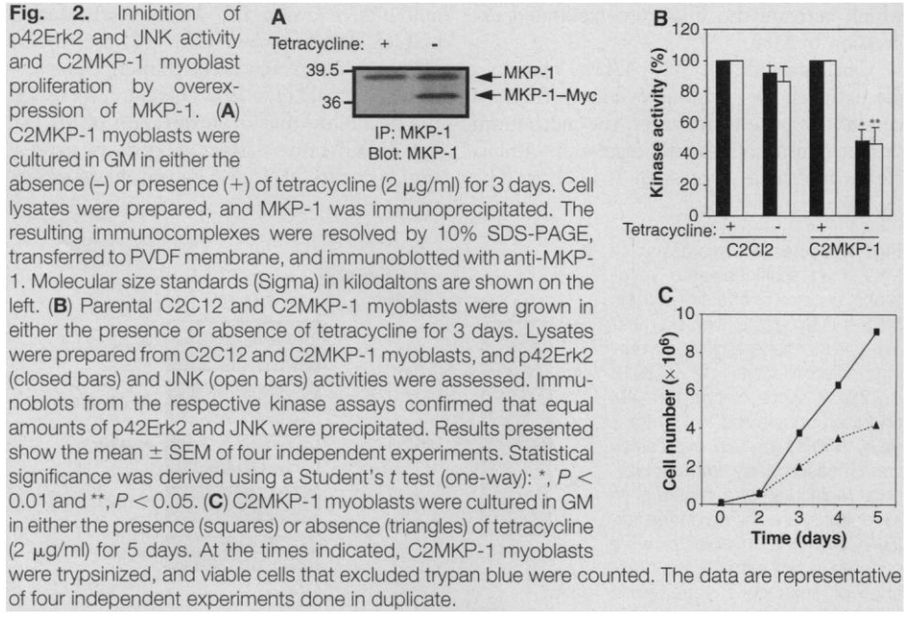

Fig. 2. Inhibition of p42Erk2 and JNK activity and C2MKP-1 myoblast proliferation by overexpression of MKP-1. (**A**) C2MKP-1 myoblasts were cultured in GM in either the absence (–) or presence (+) of tetracycline (2 μg/ml) for 3 days. Cell lysates were prepared, and MKP-1 was immunoprecipitated. The resulting immunocomplexes were resolved by 10% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-MKP-1. Molecular size standards (Sigma) in kilodaltons are shown on the left. (**B**) Parental C2C12 and C2MKP-1 myoblasts were grown in either the presence or absence of tetracycline for 3 days. Lysates were prepared from C2C12 and C2MKP-1 myoblasts, and p42Erk2 (closed bars) and JNK (open bars) activities were assessed. Immunoblots from the respective kinase assays confirmed that equal amounts of p42Erk2 and JNK were precipitated. Results presented show the mean ± SEM of four independent experiments. Statistical significance was derived using a Student's *t* test (one-way): *, $P < 0.01$ and **, $P < 0.05$. (**C**) C2MKP-1 myoblasts were cultured in GM in either the presence (squares) or absence (triangles) of tetracycline (2 μg/ml) for 5 days. At the times indicated, C2MKP-1 myoblasts were trypsinized, and viable cells that excluded trypan blue were counted. The data are representative of four independent experiments done in duplicate.

press the MAPK phosphatase-1 (MKP-1), which inactivates the MAPKs selectively by dephosphorylation (*12*). We generated stable C2C12 lines expressing the first 314 residues of MKP-1 fused at the COOH-terminus to the Myc epitope (MKP-1–Myc) under the control of a tetracycline-repressible promoter (*13*). Ten clones were isolated that inducibly express MKP-1–Myc (*14*). Results presented were obtained from one such clone, C2MKP-1; similar results were obtained from two other clones.

C2MKP-1 myoblasts cultured for 3 days in GM in the absence of tetracycline expressed amounts of MKP-1–Myc similar to those of endogenous MKP-1 (Fig. 2A). Maximal MKP-1–Myc expression was achieved within 3 days after transfer of C2MKP-1 myoblasts to tetracycline-free GM (*11*), resulting in the inactivation of p42Erk2 and JNK (Fig. 2B) and inhibition of C2MKP-1 myoblast proliferation (Fig. 2C). The activities of p42Erk2 and JNK in parental C2C12 myoblasts were unaffected by tetracycline (Fig. 2B). These data indicate that MKP-1–Myc expression is sufficient to interfere with endogenous MAPK activity and myoblast growth. We tested whether increased expression of MKP-1–Myc in C2MKP-1 myoblasts could facilitate muscle-specific gene expression in the presence of mitogens. C2MKP-1 myoblasts cultured in GM containing tetracycline failed to express detectable levels of MyoD, myogenin, or MHC (Fig. 3A). However, when C2MKP-1 myoblasts were cultured in GM in the absence of tetracycline, MyoD and myogenin were detected by day 4, and by day 5, expression of MHC was detected (Fig. 3A). MyoD expression is linked to cell cycle withdrawal through MyoD-induced transcriptional activation of p21 (*15*). We investigated whether expression of p21 was also facilitated by MKP-1–Myc. C2MKP-1 myoblasts cultured in GM in the absence of tetracycline expressed p21 after 3 to 4 days (Fig. 3A). Control C2MKP-1 myoblasts cultured in GM containing tetracycline expressed p21 more slowly; this likely occurs because these cells proliferate more rapidly than C2MKP-1 myoblasts grown in the absence of tetracycline and consequently achieve density-dependent arrest. In contrast, the cell cycle inhibitor p27, which is not regulated at the transcriptional level by MyoD (*16*), remained unaffected (Fig. 3A). These results demonstrate that inhibition of p42Erk2 or JNK, or both, by MKP-1 in C2MKP-1 myoblasts, in the presence of mitogens, is sufficient to initiate the process of muscle-specific gene expression, analogous to that of mitogen withdrawal. Because p42Erk2 but not JNK activity declines during normal C2C12 differentiation induced by mitogen withdrawal, p42Erk2 likely participates in mediating the inhibitory effects of mitogens on myoblast differentiation. This notion was supported by the observation that treatment of C2C12 myoblasts in GM with an inhibitor (PD 098059) of the dual-specificity kinases MEK [or MAPK/extracellular signal-regulated kinase (ERK) kinases] 1 and 2,

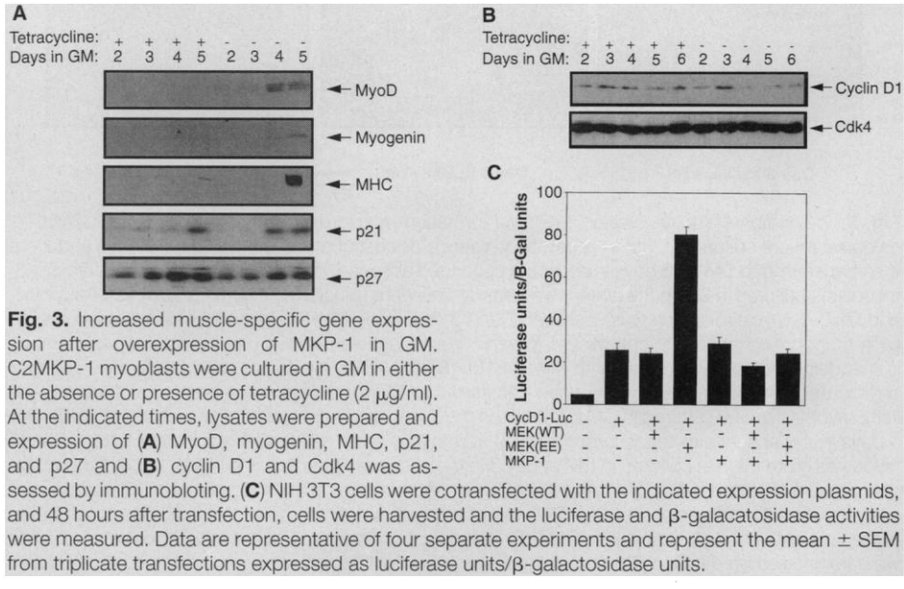

Fig. 3. Increased muscle-specific gene expression after overexpression of MKP-1 in GM. C2MKP-1 myoblasts were cultured in GM in either the absence or presence of tetracycline (2 μg/ml). At the indicated times, lysates were prepared and expression of (**A**) MyoD, myogenin, MHC, p21, and p27 and (**B**) cyclin D1 and Cdk4 was assessed by immunoblotting. (**C**) NIH 3T3 cells were cotransfected with the indicated expression plasmids, and 48 hours after transfection, cells were harvested and the luciferase and β-galacatosidase activities were measured. Data are representative of four separate experiments and represent the mean ± SEM from triplicate transfections expressed as luciferase units/β-galactosidase units.

which activate the Erks, also facilitated expression of MHC (*11*).

Our data indicate that p42Erk2 may couple extracellular signaling events to the process of myogenesis; however, the mechanism or mechanisms of p42Erk2 regulation of muscle-specific gene expression is unclear. The amounts of cyclin D1 decline early during C2C12 differentiation (*17*, *18*), and cyclin D1 negatively regulates the transcriptional activity of MyoD (*17*, *19*). Therefore, we tested the possibility that overexpression of MKP-1 in C2MKP-1 myoblasts could decrease expression of cyclin D1. C2MKP-1 myoblasts grown in the absence of tetracycline expressed reduced amounts of cyclin D1 between days 4 and 6, compared with C2MKP-1 myoblasts grown in the presence of tetracycline (Fig. 3B). This is consistent with maximal MKP-1–Myc expression by day 3 and inhibition of cell proliferation in MKP-1-Myc–expressing myoblasts (Fig. 2, B and C). Tetracycline did not induce detectable changes in the amounts of Cdk4 protein (Fig. 3B). The effects of MKP-1 and p42Erk2 on transcriptional activation of cyclin D1 were assessed with the human cyclin D1 promoter fused to the luciferase gene [CycD1-Luc (*20*, *21*)]. Cotransfection of constitutively active MEK [MEK(EE)] but not wild-type MEK [MEK(WT)] increased CycD1-Luc activity by about threefold (Fig. 3C). When MEK(EE) was cotransfected with an amount of MKP-1 cDNA that did not affect basal CycD1-Luc activity, the MEK(EE)-inducible component of CycD1-Luc activity was inhibited (Fig. 3C). Similar effects of p42Erk2 on cyclin D1 transcription have been described in CCL39 cells (*22*). These data support the notion that early during myogenesis, inactivation of p42Erk2 is coupled to the transcriptional repression of cyclin D1. Therefore, the decision between either proliferation or differentiation in C2C12 myoblasts is coordinated by the amount of p42Erk2 activity. Consistent with this, we observed that MEK(EE) was also sufficient to repress the transcriptional activity of MyoD (*23*).

By overexpressing MKP-1, we demonstrated that the p42Erk2/MAPK pathway may supress the early stages of myogenesis and thus couple extracellular signals to regulate skeletal muscle differentiation. However, expression of endogenous MKP-1 declines in differentiated multinucleated myotubes (Fig. 4A), suggesting that decreased MKP-1 activity might serve to modulate the MAPKs later during myogenesis. To assess the functional significance of MKP-1 down-regulation during skeletal muscle differentiation, we induced C2MKP-1 myoblasts to express MKP-1–Myc during differentiation initiated by mitogen withdrawal. Ectopic expression of MKP-1–Myc before and during C2MKP-1 myoblast differentiation did not affect the expression of MyoD, myogenin, p21, or MHC (Fig. 4B). Despite the appropriate expression of MHC, C2MKP-1 myocytes expressing MKP-1–Myc during differentiation (Fig. 5A) failed to undergo appropriate cell-cell fusion to form multinucleated myotubes (Fig. 5B). In contrast, C2MKP-1 myoblasts grown in the presence of tetracycline differentiated and fused to form multinucleated myotubes (Fig. 5B). Under these conditions, both p42Erk2 and JNK activities were inhibited by ~50% in myocytes expressing MKP-1–Myc in DM (*11*). Readdition of tetracycline to repress expression of MKP-1–Myc

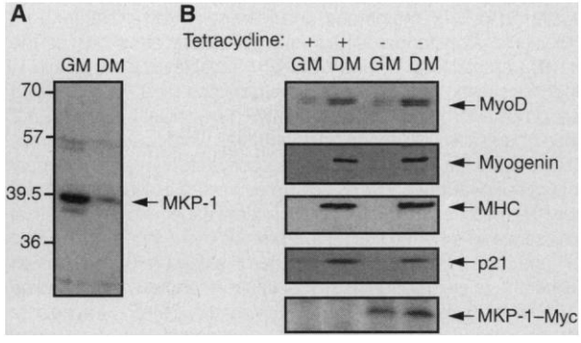

Fig. 4. Decreased expression of MKP-1 in multinucleated myotubes, but lack of effect of ectopic MKP-1–Myc expression on muscle-specific gene regulation during differentiation. (**A**) C2C12 myoblasts were cultured in GM and then transferred to DM for 3 days. MKP-1 protein expression was measured by immunoblotting. Molecular size markers (in kilodaltons) are shown on the left. (**B**) C2MKP-1 myoblasts were cultured in GM either in the presence or absence of tetracycline for 3 days to induce MKP-1–Myc expression. Differentiation was then induced by transferring cultures to DM for 3 days in either the continued absence or presence of tetracycline. Lysates prepared from C2MKP-1 myoblasts at 3 days in GM and DM were assessed by immunoblotting for the expression of MyoD, myogenin, MHC, p21, and MKP-1–Myc.

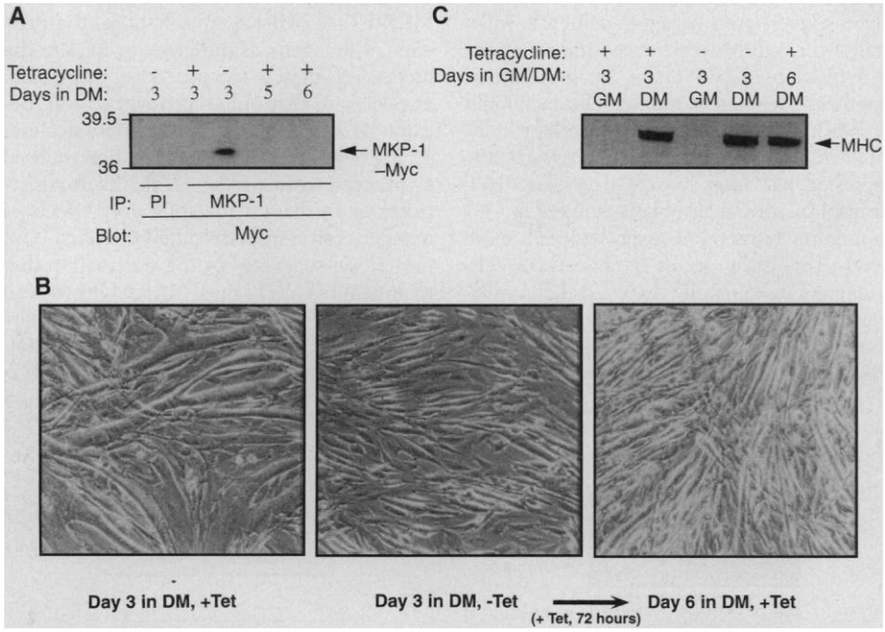

Fig. 5. Prevention of multinucleated myotube formation by overexpression of MKP-1. (**A**) C2MKP-1 myoblasts were cultured in GM in either the presence or absence of tetracycline for 3 days and were then transferred to DM for 3 days in either the continued absence or presence of tetracycline. On day 3, myoblasts cultured in DM in the absence of tetracycline were transferred to DM containing tetracycline, and MKP-1–Myc expression was assessed 2 and 3 days later. MKP-1–Myc expression was assessed by immunoprecipitation with anti-MKP-1 [preimmune (PI) anti-serum was used as control], followed by immunoblot analysis with antibody to Myc (9E10). (**B**) C2MKP-1 myoblasts were cultured in GM for 3 days in the presence of tetracycline, then transferred to DM for 3 days in the presence of tetracycline (left); myoblasts were cultured in GM for 3 days in the absence of tetracycline, then transferred to DM for 3 days in the absence of tetracycline (middle); and myocytes at day 6 after addition of tetracycline to myocytes (from middle) cultured in DM at day 3 in the absence of tetracycline (right). Light photomicrographs were obtained with a Zeiss Axiovert 10 light microscope at 100× magnification. (**C**) Immunoblot analysis of MHC expression in C2MKP-1 myoblasts cultured as in (A) for the indicated times in the presence or absence of tetracycline. C2MKP-1 myoblasts cultured in DM in the absence of tetracycline were transferred on day 3 to DM containing tetracycline; MHC expression was assessed 3 days later.

(Fig. 5A) rescued the fusion defect (Fig. 5B). Thus, inhibition of myotube formation by MKP-1 overexpression is reversible. The formation of multinucleated myotubes upon readdition of tetracycline to differentiated C2MKP-1 myocytes was not accompanied by changes in the amounts of MHC (Fig. 5C), indicating that the formation of multinucleated myotubes upon readdition of tetracycline stemmed from the preexisting population of differentiated myocytes.

These observations indicate that down-regulation of MKP-1 late during myogenesis is functionally required for the process of myotube formation. Thus, expression of the muscle-specific genes is necessary but not sufficient to commit terminally differentiated myocytes irrevocably to the formation of multinucleated myotubes. The uncoupling of muscle-specific gene activation from myotube formation suggests that a distinct pathway involving a member or members of the MAPK family regulates the formation of multinucleated myotubes.

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8. C2C12 myoblasts were cultured in GM containing fetal bovine serum (FBS, 10%) in Dulbecco's modified Eagle's medium (DMEM) with antibiotics and were transferred to DM containing FBS (0.1%) in DMEM plus insulin (5 μg/ml), transferrin (5 μg/ml), and antibiotics.
9. C2C12 myoblasts were washed in ice-cold phosphate-buffered saline and lysed in 1% NP-40, 50 mM Hepes (pH 7.4), and 150 mM NaCl in the presence of 1 mM sodium orthovanadate, 5 mM NaF, 5 mM phenylmethylsulfonyl fluoride, aprotonin (5 μg/ml), leupeptin (5 μg/ml), 1 mM benzamidine, and pepstatin (0.1 μg/ml). Cell lysates were clarified at 20,000*g* for 20 min, and protein concentration was assessed by Bradford assay. Following immunoprecipitation of p42Erk2 (Santa Cruz, Santa Cruz, CA; SC-154) from 250 μg of total cell lysate, p42Erk2 activity was assessed by immunocomplex kinase assay with myelin basic protein as substrate (0.25 mg/ml) with 50 μM adenosine triphosphate (ATP) and 5 μCi of [γ-^{32}P]ATP for 15 min at 30°C. Immunocomplex kinase reactions were stopped by the addition of 0.5 M EDTA, and 20-μl aliquots of the supernatant were spotted onto p81 phosphocellulose paper, washed extensively, and counted by liquid scintillation. JNK activity was assessed by incubating a glutathione-S-transferase (GST) fusion protein of c-Jun (GST–c-Jun, containing amino acids 5 through 89 of c-Jun) with 250 μg of total cell lysate. After washing the beads, the kinase reactions were initiated by the addition of 25 μM ATP and 10 μCi of [γ-^{32}P]ATP for 30 min at 30°C. Reactions were terminated by the addition of sample buffer, and GST–c-Jun was resolved by 10% SDS–polyacrylamide gel electrophoresis (PAGE), excised, and counted by liquid scintillation.
10. Approximately 50 to 100 μg of total cell lysate prepared as described was resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF). Membranes were blocked in nonfat dry milk (5%) and immunoblotted with either anti-MyoD (5.8A), anti-myogenin (F5D), anti-myosin heavy chain (MF20), anti-p21, anti-cyclin D1 (Transduction Laboratories, 1 μg/ml), or anti-p27 (Transduction Laboratories, 1 μg/ml) antibodies. Primary antibodies in nonfat dry milk (5%) were incubated with PVDF membranes for 2 to 4 hours at room temperature or overnight at 4°C. Proteins were visualized with either sheep antibody to mouse immunoglobulin G (IgG) or donkey antibody to rabbit IgG peroxidase-linked secondary antibodies (Amersham) at a dilution of 1:4000 and were developed with Enhanced Chemiluminescence (Amersham).
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21. NIH 3T3 cells were transfected with the human cyclin D1 promoter fused to the luciferase gene (1 μg) and β-galactosidase (1 μg) with either wild-type [pEXV3.MEK(WT)] (4 μg) or constitutively active MEK [pEXV3.MEK(EE)] (4 μg) and pSG5.MKP-1–Myc (4 μg). We prepared lysates 2 days after transfection, and luciferase activity was measured on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI) and β-galactosidase activity was assayed according to the manufacturer's protocol. Results are expressed as relative luciferase units normalized to β-galactosidase activity.
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23. NIH 3T3 cells were cotransfected with plasmids encoding MyoD (0.5 μg), rabbit muscle creatine kinase (MCK) promoter/enhancer luciferase reporter gene (1 μg), and β-galactosidase (1 μg), with either vector control, MEK(WT), or MEK(EE) (3 μg). We harvested cells 2 days after transfection and measured the luciferase and β-galactosidase activities. Expression of MEK(EE) inhibited MyoD transcriptional activity from the MCK luciferase reporter gene by ~50% relative to MEK(WT).

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Recovery of Replication-Competent HIV Despite Prolonged Suppression of Plasma Viremia

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In evaluating current combination drug regimens for treatment of human immunodeficiency virus (HIV) disease, it is important to determine the existence of viral reservoirs. After depletion of CD8 cells from the peripheral blood mononuclear cells (PBMCs) of both patients and normal donors, activation of patient CD4 lymphocytes with immobilized antibodies to CD3 and CD28 enabled the isolation of virus from PBMCs of six patients despite the suppression of their plasma HIV RNA to fewer than 50 copies per milliliter for up to 2 years. Partial sequencing of HIV pol revealed no new drug resistance mutations or discernible evolution, providing evidence for viral latency rather than drug failure.

Treatment with potent antiretroviral regimens can produce sustained suppression of HIV-1 replication, with reduction of HIV RNA in infected individuals to below the limits of detection in blood for 2 years or more (*1*). Although viral DNA remains detectable in PBMCs as well as in lymphoid tissues, several groups have reported that virus cannot