A New Description of Cellular Quiescence

Hilary A. Coller[1☯¤*], Liyun Sang[1,2☯], James M. Roberts[1,3]

1 Department of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington, United States of America, **2** Molecular & Cellular Biology Program, University of Washington, Seattle, Washington, United States of America, **3** Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Center, Seattle, Washington, United States of America

Cellular quiescence, defined as reversible growth/proliferation arrest, is thought to represent a homogenous state induced by diverse anti-mitogenic signals. We used transcriptional profiling to characterize human diploid fibroblasts that exited the cell cycle after exposure to three independent signals—mitogen withdrawal, contact inhibition, and loss of adhesion. We show here that each signal caused regulation of a unique set of genes known to be important for cessation of growth and division. Therefore, contrary to expectation, cells enter different quiescent states that are determined by the initiating signal. However, underlying this diversity we discovered a set of genes whose specific expression in non-dividing cells was signal-independent, and therefore representative of quiescence per se, rather than the signal that induced it. This fibroblast "quiescence program" contained genes that enforced the non-dividing state, and ensured the reversibility of the cell cycle arrest. We further demonstrate that one mechanism by which the reversibility of quiescence is insured is the suppression of terminal differentiation. Expression of the quiescence program was not simply a downstream consequence of exit from the cell cycle, because key parts, including those involved in suppressing differentiation, were not recapitulated during the cell cycle arrest caused by direct inhibition of cyclin-dependent kinases. These studies form a basis for understanding the normal biology of cellular quiescence.

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Introduction

Quiescence is the counterpart to proliferation: a reversible, nondividing state. Although it is a common state for many somatic cells, including stem cells, we know remarkably little about the regulation of cellular quiescence. For instance, is there just one kind of quiescent state, or do cells enter qualitatively different states depending upon the particular stimulus that initiated withdrawal from the cell cycle? Is cellular quiescence static, or is it a dynamic, continually changing physiological state? Is quiescence essentially a passive state (i.e., characterized by the absence of factors needed for cell division), or is it actively maintained?

Answering these questions has been difficult, because a quiescent cell is more notable for what it doesn't do (e.g., synthesize DNA, generate mitotic spindles) than for having readily identifiable traits of its own. One solution may be to use patterns of gene expression as a representation of the unique physiology of nondividing cells. Most of the earlier studies on fibroblasts have shown that there are substantial differences in the patterns of expressed genes in quiescent versus proliferating cells [1–6]. However, these previous studies did not distinguish gene-expression changes that were involved in quiescence, per se, from secondary responses induced by changes in the cellular environment. To circumvent this problem we used DNA microarrays to ask whether the same gene-expression changes occur when cells become quiescent in response to three independent growth-arrest signals, and for various periods of time. We found that fibroblast quiescence is a remarkably more dynamic and diverse collection of states than had been expected, but that it can also be described by a program of gene expression that is characteristic of multiple, independent quiescent states. This program of gene expression reveals the basic biological attributes of cellular quiescence.

Results

General Approach

Primary human fibroblasts were exposed to mitogen deprivation, growth at high cell density ("contact inhibition"), and disruption of cell-substratum adhesions ("loss of adhesion") (Figure 1). Each of these three arrest protocols caused greater than a 95% decrease in the fraction of cells undergoing DNA synthesis (Figure 1). DNA microarrays were used to compare patterns of gene expression in growing cells and cells arrested for varying periods of time. By comparing three independent quiescence pathways, we were able to ask whether there were genes whose expression was associated with quiescence, per se, rather than with the particular mechanism of cell-cycle arrest. Our goal was to determine whether there is a novel set of genes that is associated with movement into and out of the cell cycle (G1 versus G0 [quiescent] cells) as opposed to the more commonly studied set of genes that is associated with progression through the

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Abbreviations: BMP, bone morphogenetic protein; BrdU, bromodeoxyuridine; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; ER, estrogen receptor; GO, gene ontology; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor

* To whom correspondence should be addressed. E-mail: hcoller@molbio.princeton.edu

☯ These authors contributed equally to this work.

¤ Current address: Department of Molecular Biology, Princeton University, Princeton, New Jersey, United States of America

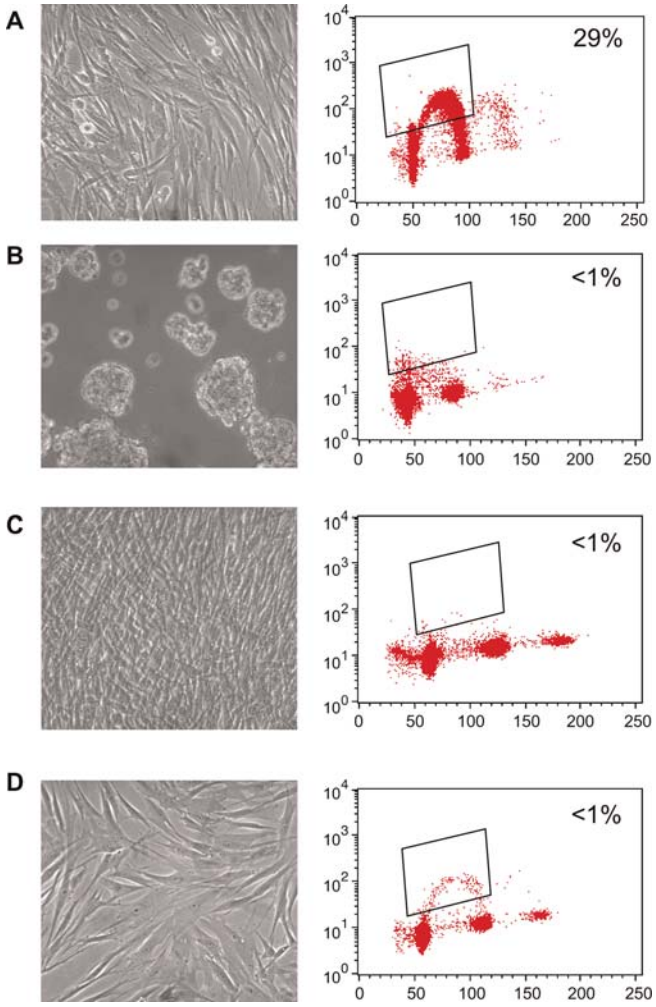

Figure 1. Model System: Growing and Arrested Cells

Photographs of growing cells (A), and cells arrested overnight by loss of adhesion (B), contact inhibition (C), or mitogen withdrawal (D) are shown. Growing cells and cells that were arrested for 4 d by one of the three different arrest signals were incubated with bromodeoxyuridine (BrdU). Cell nuclei were labeled with an anti-BrdU antibody and propidium iodide, and analyzed by FACS. DNA content was plotted along the *x*-axis, and BrdU intensity was plotted along the *y*-axis. In growing cells, 28% of the nuclei incorporated BrdU during a 6-h incubation, while less than 1% of the arrested cells incorporated BrdU during the same time period. At 14 h <10% of the cells were in S phase. 2N (G1) cells in all conditions were purified by sorting.

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cell cycle (see for instance [7]). We therefore excluded from our analysis the many genes whose expression is dependent upon the position of the cell within the cell cycle (e.g., S or M phase specific genes) by using a flow cytometer to collect only those with a 2N (G1) DNA content. Flow cytometry-based sorting was applied to all samples.

To get an overview of the relationships among the different quiescent states, we measured the "genome-wide transcriptional distance" between a pair of samples, which was calculated by summing the differences in expression levels between the samples for all genes on the microarray [8]. A tree representing the relationships among cells arrested by different methods based on this measure is shown in Figure 2A. Note that independent, duplicate samples of cells arrested by each of the three methods are next to each other, demonstrating that cells arrested by different methods execute reproducible and signal-specific gene-expression changes.

We also used two methods to identify specific genes regulated in quiescent cells. In the first, we ranked the genes based on their similarity to a stereotyped example of expression changes, and used permutation tests to determine the extent of correlation expected by chance. In the second, we identified genes regulated 2-fold when cells became quiescent. The expected number of false positives was determined by permutation testing. In addition to determining the number of such genes, we visualized these genes in the relevant samples with "heat maps" [9] in which low expression of a gene in a given sample is indicated by green while high expression is indicated by red.

Quiescence Initiation

Cells were exposed to arrest signals for 14 h, at which time the fraction undergoing DNA synthesis had decreased 70%–85%. The relatedness tree (Figure 2A) showed that the transcriptional profile of each quiescent cell population was reproducibly distinct from proliferating G1 cells. Thus, quiescence is not a prolonged pause in G1 but rather is characterized by distinct gene expression changes. Indeed, a significant number of genes (between 10 and 60) were uniquely upregulated or downregulated in cells exposed to each arrest signal for 14 h (Figure 2B and 2C). Some of the genes regulated by only one or two arrest signals relate to two central features of quiescent cells—decreased proliferation and decreased biosynthetic activity. The former include genes involved in mitogenic signal transduction and in pathways that directly regulate the cell cycle, and the latter include genes involved in lipid, nucleotide, and carbohydrate metabolism (Table 1). We concluded that each signal induced a unique quiescent state, because these fundamental features of quiescence (cessation of growth and division) are realized, at least in part, by different signal-dependent changes in gene expression. These included upregulated as well as downregulated genes, showing that quiescence is an active state.

There was a smaller but statistically significant set of genes that was regulated by all three arrest signals. The upregulated genes included three transcription factors, *ZNF124*, *HES1*, and *RB1*, and downregulated genes included the transcription factor *GABPB2*, the mitotic regulator *MAD2L1*, and *VEGFC*. The fact that four pleiotropic transcription factors were regulated during entry into quiescence suggested that the cells might induce a generalized program of quiescence gene expression that would be manifest at later times.

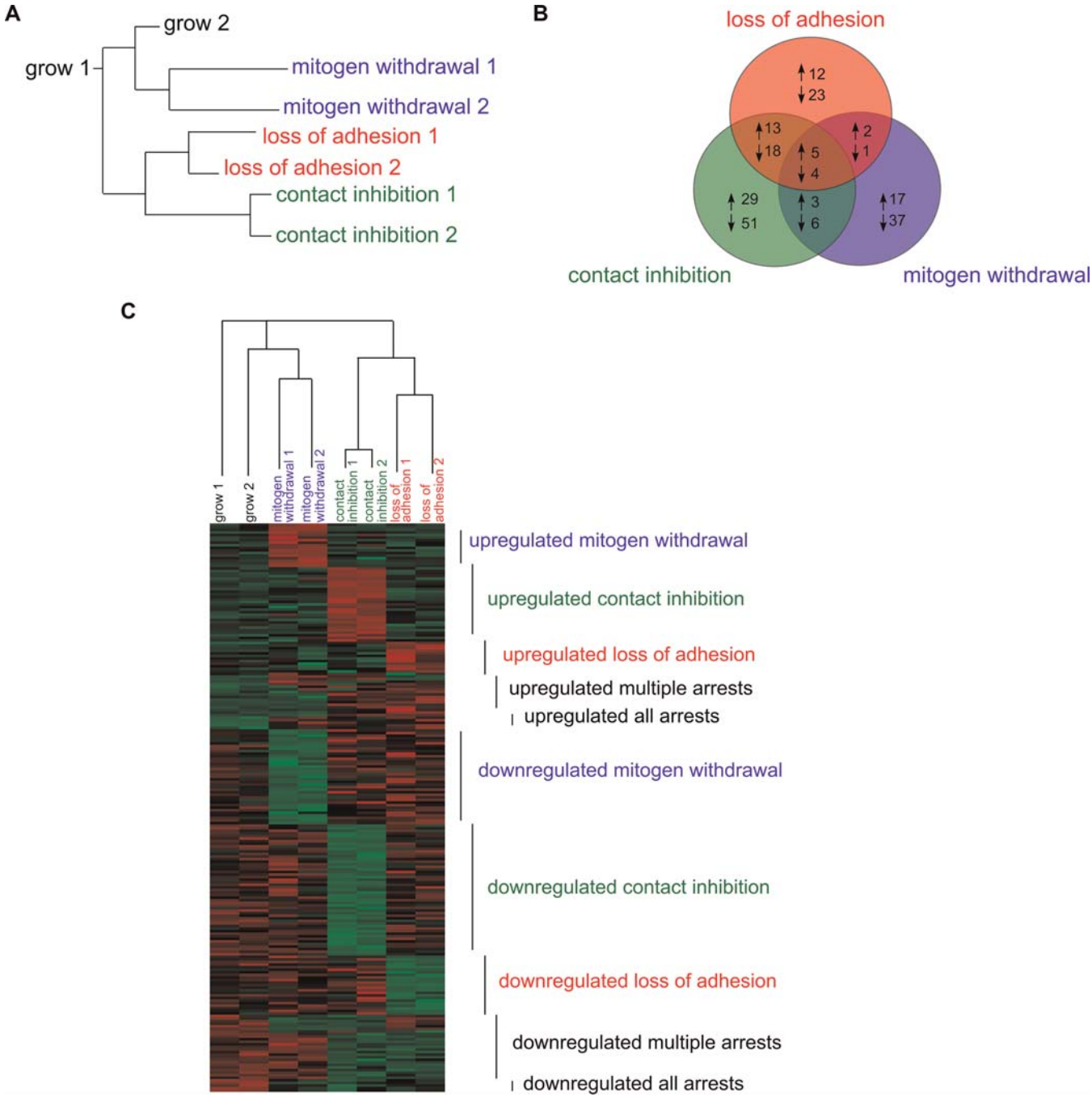
Quiescence Maintenance

We next asked whether the immediate response to arrest signals, which was characterized largely by signal-specific

Figure 2. Quiescence Initiation

(A) Neighbor-joining tree of quiescence initiation. Samples of growing cells and cells arrested by a single signal for 14 h were flow-sorted. Transcriptional distances (Affymetrix Genechip suite 4.0.) were determined for each pairwise comparison among samples as described in Materials and Methods. A matrix of transcriptional distances was used to generate a tree depicting the relationship among the growing and quiescent cells using neighbor-joining methods.

(B) Venn diagrams depicting the number of genes regulated by one, two, or three arrest signals during quiescence initiation. The number of genes that were regulated by a single signal, by two out of three signals, or by all three signals was determined based on either consistent 2-fold



regulation or ''template-matching.'' The number of genes expected to be downregulated by a single signal by chance is 7.8, and the number observed was statistically significant for all three arrest methods ($p < 0.001$). The number of genes expected to be upregulated by a single signal by chance is 5.1. The signature for genes upregulated by mitogen withdrawal or contact inhibition alone is significant at $p < 0.001$, while the signature for genes upregulated by loss of adhesion is significant at $p = 0.004$. One gene was expected to be downregulated by two out of three signals by chance. For mitogen withdrawal combined with contact inhibition, and loss of adhesion combined with contact inhibition, the observed number of downregulated genes was statistically significantly higher than expectation ($p < 0.001$). For mitogen withdrawal combined with loss of adhesion, the result was not statistically significant. The expected number of genes upregulated by two arrest signals is 0.75. For genes upregulated by mitogen withdrawal combined with contact inhibition, and by loss of adhesion combined with contact inhibition, the number of regulated genes is significant ($p < 0.001$). For mitogen withdrawal combined with loss of adhesion, the result was not statistically significant. The number of genes expected to be downregulated by all three signals was 0.85, and the observation of four was significant at $p = 0.009$. The number of genes expected to be upregulated by all three arrest signals is 1.2, and the observation of five is significant at $p = 0.006$.

(C) ''Heat maps'' of genes regulated during quiescence initiation. Samples of growing cells and cells arrested by a single signal for 14 h were flow sorted and analyzed with microarrays. Genes regulated by one, two, or all three signals were identified both by consistent 2-fold change and by ''template-matching'' as described in Materials and Methods. Genes are represented by rows; columns indicate samples. The average difference values for each gene were normalized. The relative expression levels for a gene among the samples are indicated by green for a low value and by red for a high value. The figure was generated with Java Treeview [54]. The dendrogram above the figure depicts the topology of a neighbor-joining tree generated from the transcriptional distances between each sample. The complete tree is shown in (A).

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Table 1. Genes Regulated by Specific Arrest Signals

Time	Gene Name	GO Terms	Mitogen Withdrawal	Contact Inhibition	Loss of Adhesion
14 h					
	UGCG: UDP-glucose ceramide glucosyltransferase	Lipid metabolism	▲		
	GDF5: growth differentiation factor 5	Growth, cell communication, signal transduction	▲		
	IGFBP3: IGF binding protein 3	Regulation of growth, signal transduction, cell communication	▲		
	HMGCL: 3-hydroxymethyl-3-methylglutaryl-Co A lyase	Amino acid metabolism		▲	
	ALDH2: aldehyde dehydrogenase 2 family (mitochondrial)	Carbohydrate metabolism		▲	
	JARID2: Jumonji, AT rich interactive domain 2	Regulation of transcription		▲	
	PPP2R5A: protein phosphatase 2, regulatory subunit B	Cell communication, signal transduction		▲	
	PASK: PAS domain containing serine/threonine kinase	Cell communication, signal transduction			▲
	SLC20A2: solute carrier (phosphate transporter)	Establishment of localization, transport	▲		▲
	RGS2: regulator of G-protein signalling 2, 24kDa	Cell cycle, cell communication, signal transduction	▼		
	CDK10: cyclin-dependent kinase (CDC2-like) 10	No swiss prot identifiers	▼		
	MAP3K5: mitogen-activated protein kinase kinase kinase 5	Cell communication, signal transduction	▼		
	ITPKA: inositol 1,4,5-trisphosphate 3-kinase A	Cell communication, signal transduction	▼		
	PL6: placental protein 6	Cell communication, signal transduction	▼		
	NR4A1: nuclear receptor subfamily 4, group A, member 1	Cell communication, signal transduction, regulation of transcription	▼		
	NR4A2: nuclear receptor subfamily 4, group A, member 2	Cell communication, signal transduction, regulation of transcription	▼		
	CUL2: cullin 2	Cell cycle, negative regulation of cell proliferation		▼	
	CENPA: centromere protein A, 17kDa	Chromosome organization and biogenesis		▼	
	LMNB1: lamin B1	No swiss prot identifiers		▼	
	LBR: lamin B receptor	No swiss prot identifiers		▼	
	FER: fer (fps/fes related) tyrosine kinase	No swiss prot identifiers		▼	
	DHX9: DEAH (Asp-Glu-Ala-His) box polypeptide 9	No swiss prot identifiers		▼	
	FASN: fatty acid synthase	No swiss prot identifiers		▼	
	PRPS1: phosphoribosyl pyrophosphate synthetase 1	Nucleotide metabolism		▼	
	UMPS: uridine monophosphate synthetase	Nucleotide metabolism		▼	
	FNTB: farnesyltransferase, CAAX box, beta	Protein metabolism		▼	
	MRPL12: mitochondrial ribosomal protein L12	Protein metabolism		▼	
	KPNA3: karyopherin alpha 3 (importin alpha 4)	Establishment of localization, protein transport		▼	
	PCNA: proliferating cell nuclear antigen	Regulation of cell cycle, cell proliferation, regulation of DNA replication		▼	
	METTL1: methyltransferase-like 1	RNA metabolism		▼	
	SFPQ: splicing factor proline/glutamine rich	RNA metabolism		▼	
	SFRS2: splicing factor, arginine/serine-rich 2	RNA metabolism		▼	
	SF3A1: splicing factor 3a, subunit 1, 120kDa	RNA metabolism		▼	
	DDX39: DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	RNA metabolism		▼	
	BOP1: block of proliferation 1	RNA metabolism, ribosomal biogenesis		▼	
	INPP5A: inositol polyphosphate-5-phosphatase	Cell communication		▼	
	ARG2: arginase, type II	Amino acid metabolism			▼
	CUL1: cullin 1	Cell cycle, regulation of cell proliferation			▼
	PRKCM: protein kinase C, mu	Cell proliferation, cell communication, signal transduction			▼
	KIAA0092: translokin	Cell communication, signal transduction, intracellular transport			▼
	PCYT2: phosphate cytidylyltransferase 2, ethanolamine	Lipid metabolism			▼
	PRKAR1B: protein kinase, cAMP-dependent, regulatory	Nothing listed			▼
	HIST2H4: histone 2, H4	No swiss prot identifiers			▼
	MAP2K5: mitogen activated protein kinase kinase 5	Protein metabolism			▼
	FGF4: fibroblast growth factor 4	Reglation of cell cycle, cell proliferation, signal transduction			▼
	CCNE1: cyclin E1	Regulation of cell cycle, cell division			▼
	CREBL1: cAMP responsive element binding protein-like 1	Cell communication, signal transduction, regulation of transcription			▼
	ADORA2B: adenosine A2b receptor	Cell communication, signal transduction, response to external stimulus			▼
	NP: nucleoside phosphorylase	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolism	▼	▼	

Table 1. Continued

Time	Gene Name	GO Terms	Mitogen Withdrawal	Contact Inhibition	Loss of Adhesion
	MRPL19: mitochondrial ribosomal protein L19	Protein metabolism	▼	▼	
	MPHOSPH6: M-phase phosphoprotein 6	Regulation of cell cycle	▼	▼	
	ETFDH: electron-transferring-flavoprotein dehydrogenase	Generation of precursor metabolites and energy	▼		▼
	ASL: argininosuccinate lyase	Amino acid metabolism		▼	▼
	SLC1A4: solute carrier (amino acid transporter)	Establishment of localization, amino acid transport		▼	▼
	UMPK: uridine monophosphate kinase	Biosynthesis		▼	▼
	LPIN1: lipin 1	Cell differentiation		▼	▼
	MVD: mevalonate (diphospho) decarboxylase	Lipid metabolism		▼	▼
	IDI1: isopentenyl-diphosphate delta isomerase	Lipid metabolism		▼	▼
	SCAP: SREBP cleavage-activating protein	Lipid metabolism		▼	▼
	FGF5: Fibroblast growth factor 5	Regulation of cell cycle, cell proliferation, cell communication		▼	▼
	SLC12A4: solute carrier (ion transporter)	Regulation of cell cycle		▼	▼
	SNRPC: small nuclear ribonucleoprotein polypeptide C	RNA metabolism		▼	▼
4 d					
	ASNS: asparagine synthetase	Amino acid metabolism	▲		
	ATP6V1A: ATPase, H+ transporting, lysosomal	Generation of precursor metabolites and energy	▲		
	UGCG: UDP-glucose ceramide glucosyltransferase	Lipid metabolism	▲		
	ARHGEF12: Rho guanine exchange factor (GEF) 12	No swiss prot identifiers	▲		
	CALML3: calmodulin-like 3	No swiss prot identifiers	▲		
	DDX3X: DEAD (Asp-Glu-Ala-Asp) box polypeptide 3	No swiss prot identifiers	▲		
	NT5C2: 5′-nucleotidase, cytosolic II	No swiss prot identifiers	▲		
	CACNA1E: calcium channel, voltage-dependent	No swiss prot identifiers	▲		
	CCNG2: cyclin G2	Regulation of cell cycle, cell division	▲		
	TGFB2: transforming growth factor, beta 2	Regulation of cell cycle, cell proliferation, cell growth	▲		
	TGFB3: transforming growth factor, beta 3	Regulation of cell cycle, cell proliferation, cell growth, cell communication	▲		
	GNAI1: Guanine binding protein, alpha inhibiting activity	Cell communication, signal transduction	▲		
	GNAQ: guanine binding protein, q polypeptide	Cell communication, signal transduction	▲		
	E2F4: E2F transcription factor 4, p107/p130-binding	Regulation of cell cycle, regulation of transcription		▲	
	DUSP4: dual specificity phosphatase 4	Regulation of cell cycle, cell communication, signal transduction			▲
	DUSP5: dual specificity phosphatase 5	Protein metabolism			▲
	HARS: histidyl-tRNA synthetase	Amino acid metabolism	▼		
	GK: Glycerol kinase	Nothing listed	▼		
	POLD2: polymerase (DNA directed) regulatory subunit	DNA replication	▼		
	KCNAB1: potassium voltage-gated channel	Establishment of localization, ion transport	▼		
	TOMM34: translocase of outer mitochondrial membrane	Establishment of protein localization, intracellular transport	▼		
	SEC23B: Sec23 homolog B	Protein localization, intracellular transport	▼		
	HADH2: hydroxyacyl-Coenzyme A dehydrogenase	Lipid metabolism	▼		
	POLR2I: polymerase (RNA) II (DNA directed)	No swiss prot identifiers	▼		
	PDXK: pyridoxal (pyridoxine, vitamin B6) kinase	No swiss prot identifiers	▼		
	PMPCA: peptidase (mitochondrial processing) alpha	Protein metabolism, protein catabolism	▼		
	DDX39: DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	RNA metabolism	▼		
	SLC20A1: solute carrier (phosphate transporter)	Cell communication, signal transduction, establishment of localization	▼		
	EIF3S9: eukaryotic translation initiation factor 3	Protein metabolism	▼		
	TNFRSF17: tumor necrosis factor receptor superfamily	Cell proliferation, signal transduction		▼	
	GUCY1A2: guanylate cyclase 1, soluble, alpha 2	Cell communication, signal transduction		▼	
	IDH1: isocitrate dehydrogenase 1 (NADP+), soluble	Carbohydrate metabolism			▼
	SLC25A1: solute carrier (citrate transporter)	Carbohydrate metabolism			▼
	KCNMA1: potassium large conductance channel	Cell communication, establishment of localization, ion transport			▼
	LPIN1: lipin 1	Cell differentiation			▼
	FGF5: fibroblast growth factor 5	Regulation of cell cycle, cell proliferation, cell communication			▼
	MAT2A: methionine adenosyltransferase II, alpha	Cellular metabolism			▼
	SLC25A4: solute carrier (adenine translocator)	Generation of precursor metabolites and energy			▼
	ATP1B1: ATPase, Na+/K+ transporting	Establishment of localization, ion transport			▼
	LDLR: low density lipoprotein receptor	Lipid metabolism			▼
	FDPS: farnesyl diphosphate synthase	Lipid metabolism			▼
	SREBF1: sterol regulatory element binding tx factor 1	Regulation of transcription, lipid metabolism			▼
	SREBF2: sterol regulatory element binding tx factor 2	Regulation of transcription, lipid metabolism			▼
	RRAS2: Related RAS viral (r-ras) oncogene homolog 2	No swiss prot identifiers			▼

Table 1. Continued

Time	Gene Name	GO Terms	Mitogen Withdrawal	Contact Inhibition	Loss of Adhesion
	FASN: fatty acid synthase	No swiss prot identifiers			▼
	GTF2F2: general transcription factor IIF, polypeptide 2	Regulation of transcription			▼
	UBTF: upstream binding transcription factor	Regulation of transcription			▼
	ADCY7: adenylate cyclase 7	Cell communication, signal transduction			▼
	EDG2: endothelial differentiation	Cell communication, signal transduction			▼
	UMPK: uridine monophosphate kinase	Metabolism	▼	▼	
	PFKFB3: 6-phosphofructo-2-kinase	Carbohydrate metabolism	▼		▼
	FABP5: fatty acid binding protein 5	Lipid metabolism	▼		▼
	PAICS: phosphoribosylaminoimidazole carboxylase	Nucleotide metabolism	▼		▼
	MCM5: MCM5 minichromosome maintenance deficient 5	Regulation of cell cycle, DNA replication, regulation of transcription		▼	▼
	MVD: mevalonate (diphospho) decarboxylase	Lipid metabolism		▼	▼
	TTK: TTK protein kinase	No swiss prot identifiers		▼	▼

A subset of genes regulated by one or two arrest signals after 14 h or 4 d are listed along with the direction of change and the arrest conditions in which they are regulated.
Functional categories represent Gene Ontology terms previously assigned to the particular gene. To assign these categories, the Affymetrix-provided Swissprot identifier (http://www.affymetrix.com/support/index.affx) was determined. Using GO::Termfinder modules [55] to read in the latest (June 2005) versions of the GO biological process aspect and the GOA-provided Annotations [56], we identified all unique ancestors within the GO tree. A few informative biological process terms are listed.
▲, upregulated; ▼, downregulated.
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changes, foreshadowed mostly signal-specific expression changes at a later time point. Alternatively, did these short-term largely divergent states ultimately coalesce into a common quiescent state? After 4 d, there were many more expression changes than after an overnight arrest, and the transcriptional distance to each state increased on average 3.3-fold (Figure 3A). Moreover, each arrest state maintained its unique character. There continued to be strong signatures for genes that were regulated specifically by a single arrest signal (Figure 3B and 3C), and many of these genes were regulators of cell growth and cell division (Table 1).

We wanted to ascertain whether these quiescence state-specific expression changes reflect bonafide differences in the gene expression pattern in response to different signals, or whether they might reflect only a difference in the kinetics of the induction of a similar response induced by different signals. We discovered that much of the signal-specific signature observed after 14 h persisted through 4 d (Figure S1). This makes it unlikely that differences reflect simply timeshifting of a common signature. We also discovered a surprisingly strong signature for genes upregulated by mitogen withdrawal and downregulated by loss of adhesion, or vice versa, at both quiescence initiation and quiescence maintenance (Figure S2). For these reasons, we concluded that the gene expression changes observed most likely reflect qualitatively different quiescent states and do not represent the same gene expression changes induced by different signals with different kinetics.

Superimposed on this signal-specific pattern, however, was also a convergence of gene expression changes. After 4 d of arrest, 35 genes were downregulated and 96 were upregulated by all three signals (Figure 3B and 3C). These results suggested that there might be a fibroblast "quiescence program," a set of gene-expression changes central to any long-term quiescent state and independent of the specific arrest signal (Figure 4).

Definition of a Quiescence Program, and Its Expression during Prolonged Arrest

There were many gene-expression changes that were shared by cells reaching a longer transcriptional distance from growing cells—those arrested by an individual signal for 4 d and those grown to confluence and maintained for 4 d or 20 d (see below). We defined a "quiescence program" (see Materials and Methods) by identifying genes consistently regulated in these deeper arrests. There were 116 upregulated and 33 downregulated genes that met these criteria, while the expectation due to random chance was approximately 1 (Table 2). The quiescence program included not only genes involved in regulating cell growth and division, but also genes that suppressed apoptosis, suppressed differentiation, and were involved in intercellular communication; all of these are likely to be essential to maintaining cells and tissues in a reversible, viable arrested state (see Discussion).

Previous studies had shown that cells deprived of serum for weeks rather than days took longer to reenter the cell cycle [10,11]. At the molecular level this might be manifest as an even greater change in the expression of those genes that had been regulated during shorter arrests. We allowed the cells to grow to confluence and then maintained them in an arrested state with regular medium changes for approximately 20 d. We compared them with cells arrested by the same protocol for only 4 d. Prolonged quiescence resulted in the longest transcriptional distance from growing cells of any arrested state (Figure 5A). There was a 50% increase in the transcriptional distance to growing cells in cells arrested by growth to confluence for 20 d versus 4 d. Notably, 82% (27/33) of the downregulated genes and 77% (89/116) of the upregulated genes in the quiescence program were repressed or induced more strongly at 20 d of quiescence than at 4 d (Figure 5B–5D). Thus, arrest for longer periods of time, which results in a "deepening" of the quiescent state, is associated with an exaggeration of the quiescence program (Figure 4).

Prolonged arrest by growth to confluence also resulted in a

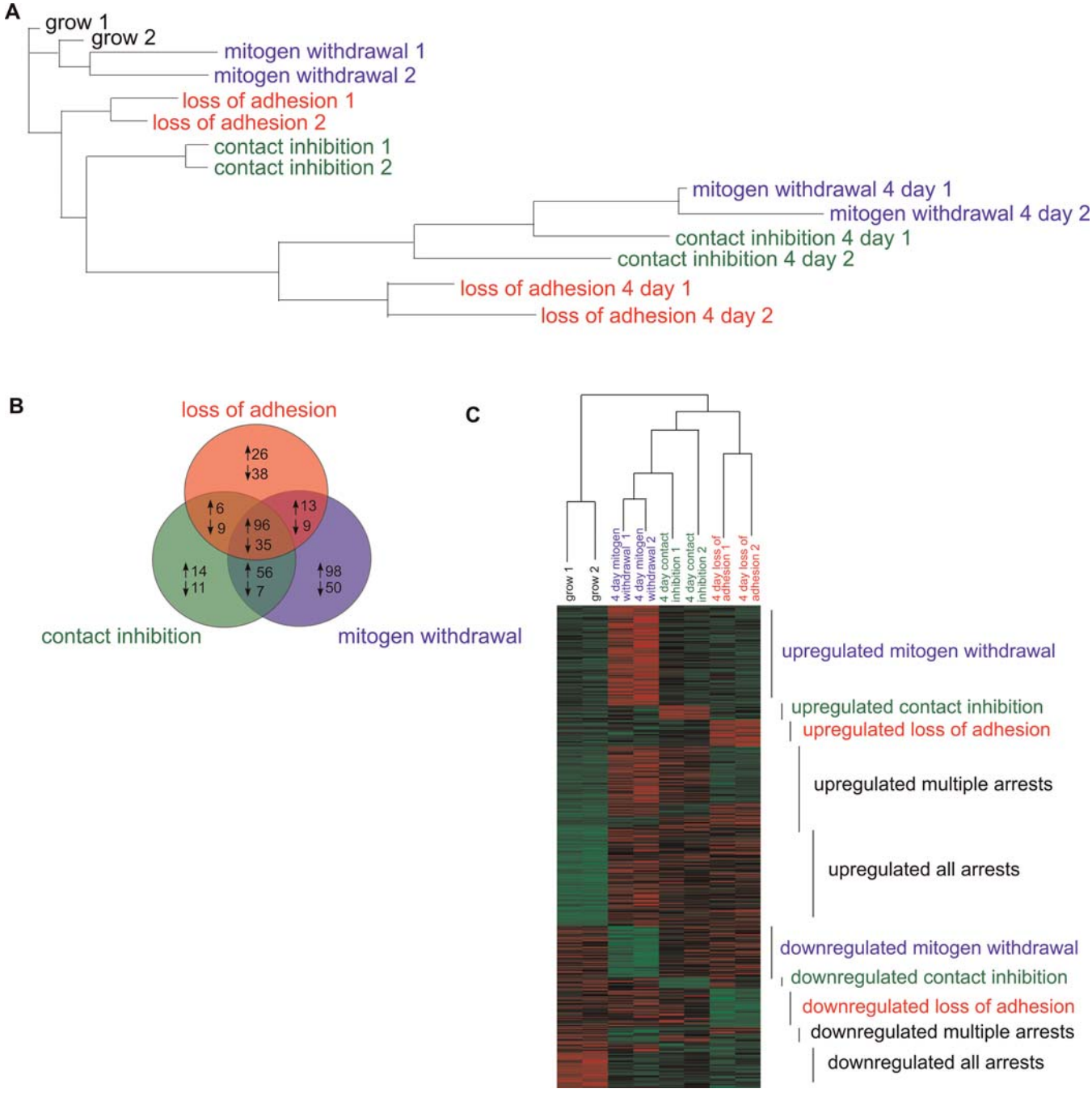

Figure 3. Quiescence Maintenance

(A) Neighbor-joining tree of quiescence initiation and maintenance. Neighbor-joining tree of the transcriptional distance between samples after overnight and 4 d of quiescence generated as described in Figure 2A.

(B) Venn diagrams depicting the number of genes regulated by one, two, or three arrest signals during quiescence maintenance. Methods are as described in (Figure 2B), except that cells were arrested for 4 d. The number of genes downregulated specifically by mitogen withdrawal or by loss of adhesion was significantly greater than the expectation (7.8 genes) at $p < 0.001$. The number of genes downregulated by contact inhibition was not statistically significant. The expected number of genes upregulated by a single signal was 5.1, and the signature for genes upregulated by each of the signals individually was statistically significant ($p < 0.001$). The expected number of genes downregulated by two signals was one, and the observed values for all three combinations were significant at $p < 0.001$. The expected number of genes downregulated by all three arrest signals was 0.85, and the observed value of 33 was highly significant ($p < 0.001$). All combinations of upregulation by two signals were statistically significantly elevated ($p < 0.001$) as compared with the expectation of 0.75 genes. The number of genes expected to be upregulated by all three signals was 1.2, and the observed value of 96 was significantly different ($p < 0.001$).

(C) ''Heat maps'' of genes regulated during quiescence maintenance. ''Heat map'' of the genes regulated after 4 d of quiescence and dendrogram are as described in Figure 2C.

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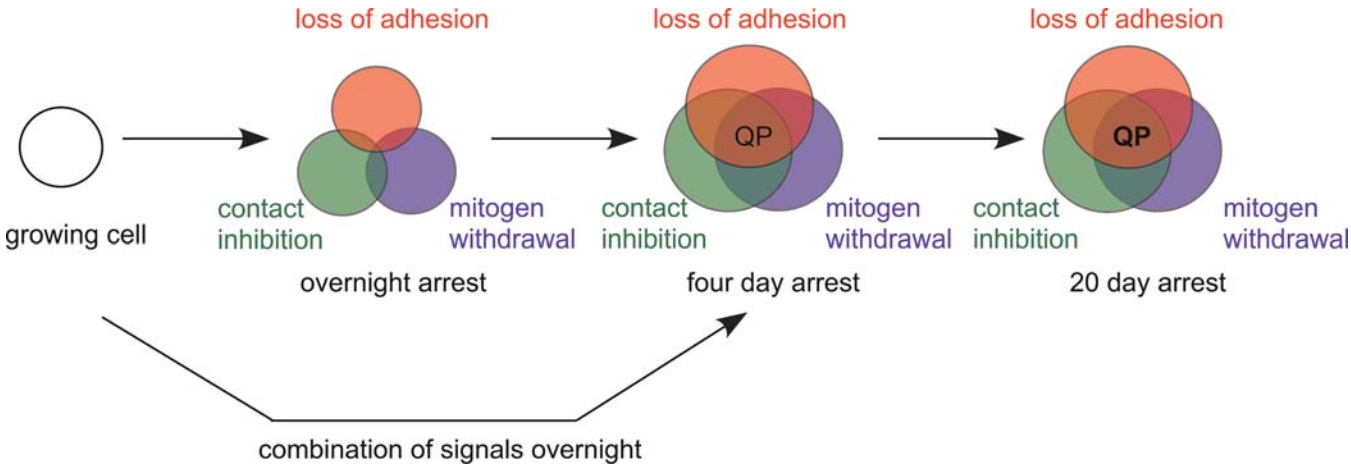

Figure 4. Summary of the Relationships among Quiescent States

In cells arrested overnight by a specific arrest signal, gene expression changes are largely signal-specific with a small amount of overlap. When cells have been arrested for 4 d, there continue to be signal-specific changes, but now there are a large number of commonly regulated genes that we refer to as a "quiescence program." In cells arrested for 20 d, the intensity of the changes in the quiescence program are magnified. The three circles are intended to reflect the enhancement of the quiescence program at 20 d. We have not analyzed all the three arrest conditions at this late time point. When cells are arrested by a combination of extracellular signals, quiescence program gene expression changes appear after only an overnight arrest.

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signal-specific gene expression profile in addition to an enhancement of the quiescence program. We identified 26 upregulated and 87 downregulated genes that were consistently regulated in cells grown to confluence for 20 d but not by mitogen withdrawal, contact inhibition, or loss of adhesion for 4 d (Figure 5E and Table 3).

Combination of Arrest Signals; Rapid Onset of Quiescence

Fibroblasts are most effectively made quiescent by exposing them to combinations of arrest signals. We hypothesized that a molecular explanation might involve activation of the quiescence program. To test this, we arrested cells for 14 h by withdrawing mitogens and simultaneously either plating them at high density or arresting them by loss of adhesion. Manipulating two pathways together had a synergistic effect; cells arrested by either combination were even farther from growing cells than the sum of the distances to the individual arrest states (Figure 6A and 6B).

The fraction of upregulated quiescence program genes that were also 2-fold upregulated in cells arrested overnight by a single signal was small—5% for mitogen withdrawal, 9% for contact inhibition, and 3% for loss of adhesion. In contrast, a much higher fraction of the upregulated quiescence program genes were regulated in cells arrested overnight by a combination of signals—32% for mitogen withdrawal plus contact inhibition, and 43% for mitogen withdrawal plus loss of adhesion (Figure 6C). Therefore, combining arrest signals shortcircuited the pathway to cellular quiescence, resulting in a more rapid expression of the quiescence program (Figure 4).

Cell-Cycle Arrest by Overexpression of Cyclin-Dependent Kinase Inhibitors

The observed induction of quiescence program genes might have been a consequence of cell-cycle arrest. For instance, different arrest signals might all directly downregulate cell-cycle proteins, and expression of quiescence program genes would then be a downstream, secondary effect. Alternatively, expression of the quiescence program could be the primary event that is induced in common by each of the extracellular anti-proliferative signals. Cell-cycle arrest would be one downstream effect of the quiescence program, being induced by a subset of the quiescence program genes.

To distinguish between these models, we bypassed extracellular signal transduction pathways, and directly arrested the cell cycle by inhibition of cyclin-dependent kinases (CDKs). The first model predicts that cell-cycle arrest should be sufficient to induce the quiescence program, while the second predicts that it would not. We used retroviral-mediated gene transduction to ectopically overexpress the CDK inhibitors (CKI) p21 or p27. This efficiently blocked cell proliferation, and did not induce cellular senescence as monitored by expression of senescence-associated β-galactosidase (Figure 7).

Cells arrested by CKI overexpression formed a clade that was distinct from the clade comprised of cells arrested by extracellular signals (Figure 8A). The transcriptional pattern induced by CKI overexpression did not closely resemble the quiescent state induced by any particular extracellular signal. Only a small proportion of all of the genes regulated by all signals or an individual signal were also regulated by CKIs, with the greatest overlap among genes downregulated by all signals (Figure 8B–8E). Indeed, although downregulated quiescence program genes were likely also to be downregulated by CDK inhibition, many upregulated genes in the quiescence program were not induced by CDK inhibition. More precisely, genes regulated by four different anti-proliferative signals were extremely likely (78%) to be regulated in a fifth condition. Among genes that were consistently downregulated 2-fold in all of the quiescent states, a significant fraction (54%) was also downregulated in cells arrested by p21 and p27 (Table 3). These commonly regulated genes clustered into the functional category of cell-cycle regulators. Thus, there was a self-reinforcing component to cell-cycle arrest, in the sense that CDK inhibition caused downregulation of additional genes that also directly affected the cell cycle.

Table 2. Genes in the Quiescence Program

Upregulated or Downregulated	Gene Name	GO Term
Upregulated	***PEPD:* peptidase D**	Amino acid metabolism
	DOC1: downregulated in ovarian cancer 1	Biological process unknown
	***HYAL2:* hyaluronoglucosaminidase 2**	Carbohydrate metabolism
	***GLB1:* galactosidase, beta 1**	Carbohydrate metabolism
	***NAGLU:* N-acetylglucosaminidase**	Carbohydrate metabolism
	***TIMP3:* tissue inhibitor of metalloproteinase 3**	Cell communication
	***CDH11:* cadherin 11, type 2**	Cell communication, cell adhesion
	***VCAM1:* vascular cell adhesion molecule 1**	Cell communication, cell adhesion
	***LAMC1:* laminin, gamma 1 (formerly LAMB2)**	Cell communication, cell adhesion
	***CTNNA1:* catenin (cadherin-associated protein), alpha 1, 102kDa**	Cell communication, cell adhesion
	***TNC:* tenascin C (hexabrachion)**	Cell communication, cell adhesion
	***FAT:* FAT tumor suppressor homolog 1**	Cell communication, cell adhesion
	***PLEKHC1:* pleckstrin homology domain containing, family C**	Cell communication, cell adhesion, cytoskeleton organization, and biogenesis
	***FGD1:* Faciogenital dysplasia**	Cell communication, cytoskeleton organization, and biogenesis
	***BRE:* brain and reproductive organ-expressed (TNFRSF1A modulator)**	Cell communication, signal transduction
	TNFRSF1A: tumor necrosis factor receptor superfamily, member 1A	Cell communication, signal transduction
	***ROR1:* receptor tyrosine kinase-like orphan receptor 1**	Cell communication, signal transduction
	***PRDX4:* peroxiredoxin 4**	Cell communication, signal transduction
	***PTPRG:* protein tyrosine phosphatase, receptor type, G**	Cell communication, signal transduction
	***GPR37:* G protein-coupled receptor 37**[a]	Cell communication, signal transduction
	TIP-1: Tax interaction protein 1	Cell communication, signal transduction
	CHN1: Chimerin (chimaerin) 1	Cell communication, signal transduction
	MAPK7: mitogen-activated protein kinase 7	Cell communication, signal transduction
	AGT: angiotensinogen (serine (or cysteine) proteinase inhibitor)	Cell communication, signal transduction
	FGFR1: fibroblast growth factor receptor 1	Cell communication, signal transduction
	***APP:* amyloid beta (A4) precursor protein**	Cell communication, signal transduction, cell adhesion
	***PTK7:* PTK7 protein tyrosine kinase 7**	Cell communication, signal transduction, cell adhesion
	***LIMK1:* LIM domain kinase 1**	Cell communication, signal transduction, cytoskeleton organization and biogenesis
	***IL1R1:* interleukin 1 receptor, type I**	Cell communication, signal transduction, immune response
	***IL6:* interleukin 6**	Cell communication, signal transduction, immune response
	BDKRB2: bradykinin receptor B2[a]	Cell communication, signal transduction, immune response
	***AP3S1:* adaptor-related protein complex 3, sigma 1 subunit**	Cell communication, signal transduction, intracellular transport
	***ITPR1:* inositol 1,4,5-triphosphate receptor, type 1**	Cell communication, signal transduction, metal ion transport
	***CPE:* carboxypeptidase E**	Cell communication, signal transduction, protein catabalism
	***MADH1:* MAD, mothers against decapentaplegic homolog 1**	Cell communication, signal transduction, regulation of transcription
	***SEPT6:* septin 6**[a]	Cell cycle, cell division
	***MYH10:* myosin, heavy polypeptide 10, non-muscle**	Cell division
	SERPINF1: serine proteinase inhibitor pigment epithelium derived factor	Cell proliferation
	***ERBB2:* v-erb-b2 erythroblastic leukemia viral oncogene homolog 2**	Cell proliferation, cell communication, signal transduction
	***LRP1:* low density lipoprotein-related protein 1**	Cell proliferation, lipid metabolism
	***A2M:* alpha-2-macroglobulin**	Cellular metabolism, establishment of localization, intracellular transport
	***PTGDS:* prostaglandin D2 synthase 21kDa**	Cellular metabolism, lipid metabolism
	TFRC: transferrin receptor (p90, CD71)	Cellular metabolism, metal ion transport
	CTSO: cathepsin O	Cellular metabolism, protein catabalism
	***PPP2CB:* protein phosphatase 2 (formerly 2A)**	Cellular protein metabolism
	***PTPRK:* Protein tyrosine phosphatase, receptor type, K**	Cellular protein metabolism
	***NMT1:* N-myristoyltransferase 1**	Cellular protein metabolism
	CDR2: cerebellar degeneration-related protein 2	Cellular protein metabolism
	HERC1: Hect (homologous to E6-AP and RCC1-like domain)	Cellular protein metabolism
	UROS: uroporphyrinogen III synthase	Cofactor metabolism
	***TPM1:* tropomyosin 1 (alpha)**	Development
	***COL3A1:* collagen, type III, alpha 1**	Development, establishment of localization, transport
	ACTA2: actin, alpha 2, smooth muscle, aorta	Development, morphogenesis
	***MGP:* matrix Gla protein**[a]	Development, morphogenesis, response to external stimulus
	***TNFAIP1:* tumor necrosis factor, alpha-induced protein 1**[a]	Establishment of localization, transport
	***COL4A5:* collagen, type IV, alpha 5**	Establishment of localization, transport
	***LUM:* lumican**	Extracellular matrix organization and biogenesis
	***PROS1:* protein S (alpha)**	Homeostasis, response to external stimulus
	***HLA-E:* major histocompatibility complex, class I, E**[a]	Immune response
	***PCCA:* propionyl Coenzyme A carboxylase, alpha polypeptide**	Lipid metabolism
	***ECH1:* Enoyl Coenzyme A hydratase 1, peroxisomal**	Lipid metabolism
	***GM2A:* GM2 ganglioside activator protein**	Lipid metabolism
	***AFAP:* actin filament associated protein**	No swiss prot identifiers

Table 2. Continued

Upregulated or Downregulated	Gene Name	GO Term
	***RBMS1:* RNA binding motif, single stranded interacting protein 1**	No swiss prot identifiers
	***NR2F1:* nuclear receptor subfamily 2, group F, member 1**	No swiss prot identifiers
	***HIC1:* hypermethylated in cancer 1**	No swiss prot identifiers
	***PL1:* endogenous retrovirus envelope region mRNA (PL1)**	No swiss prot identifiers
	***CEBPD:* CCAAT/enhancer binding protein (C/EBP), delta**	No swiss prot identifiers
	***LAMP2:* lysosomal-associated membrane protein 2**	No swiss prot identifiers
	***NNMT:* Nicotinamide N-methyltransferase**	No swiss prot identifiers
	***SAT:* Spermidine/spermine N1-acetyltransferase**	No swiss prot identifiers
	***RRAGA:* Ras-related GTP binding A**	No swiss prot identifiers
	***SPHAR:* S-phase response (cyclin-related)**	No swiss prot identifiers
	***OBRGRP:* leptin receptor gene-related protein**[a]	No swiss prot identifiers
	***PIK3C3:* phosphoinositide-3-kinase, class 3**	No swiss prot identifiers
	ITGA1: integrin, alpha 1	No swiss prot identifiers
	STOM: stomatin	No swiss prot identifiers
	***MUC1:* mucin 1, transmembrane**	Nothing listed
	TGFBR2: transforming growth factor, beta receptor II[a]	Nothing listed
	GARP: glycoprotein A repetitions predominant	Nothing listed
	AMPD3: adenosine monophosphate deaminase (isoform E)	Nucleotide metabolism
	***ABCD3:* ATP-binding cassette, sub-family D (ALD)**	Organelle organization and biogenesis
	***PEX19:* peroxisomal biogenesis factor 19**	Organelle organization and biogenesis
	***SOD3:* superoxide dismutase 3, extracellular**	Oxygen and reactive oxygen species metabolism
	***BMP1:* bone morphogenetic protein 1, (pro collagen C proteinase)**	Protein catabolism
	***SPG7:* spastic paraplegia 7, paraplegin**	Protein catabolism
	PLAT: plasminogen activator, tissue	Protein catabolism, response to external stimulus
	***PAM:* peptidylglycine alpha-amidating monooxygenase**	Protein metabolism
	TRB2: tribbles homolog 2	Protein metabolism
	MYLK: myosin, light polypeptide kinase	Protein metabolism
	***IFITM1:* Interferon induced transmembrane protein 1 (9–27)**	Regulation of cell cycle, cell communication
	***STAT1:* signal transducer and activator of transcription 1, 91kDa**	Regulation of cell cycle, cell communication, regulation of transcription
	***TCF7L2:* transcription factor 7-like 2 (TCF-4)** [a]	Regulation of cell cycle, cell communication, regulation of transcription
	***CCND2:* cyclin D2**	Regulation of cell cycle, cell division
	***TP53:* tumor protein p53 (Li-Fraumeni syndrome)**	Regulation of cell cycle, regulation of transcription
	GPNMB: glycoprotein (transmembrane) nmb	Regulation of cell proliferation
	***BCL6:* B-cell CLL/lymphoma 6 (zinc finger protein 51)**	Regulation of cell proliferation, regulation of transcription
	***IGBP1:* immunoglobulin (CD79A) binding protein 1**	Regulation of signal transduction
	***MXI1:* MAX interacting protein 1**[a]	Regulation of transcription
	***ATBF1:* AT-binding transcription factor 1**	Regulation of transcription
	***ZNF143:* zinc finger protein 143**	Regulation of transcription
	***TRIM22:* tripartite motif-containing 22**	Regulation of transcription
	PIPPIN: RNA-binding protein pippin	Regulation of transcription, RNA metabolism
	***SERPING1:* serine (or cysteine) proteinase inhibitor**	Response to external stimulus, immune response
	***F3:* coagulation factor III (thromboplastin, tissue factor)**[a]	Response to external stimulus, immune response
	***MX2:* myxovirus (influenza virus) resistance 2**[a]	Response to external stimulus, immune response
	***CFHL1:* complement factor H-related 1**	Response to external stimulus, immune response
	***FCGRT:* Fc fragment of IgG, receptor, transporter, alpha**	Response to external stimulus, immune response
	C1R: complement component 1, r subcomponent	Response to external stimulus, immune response
	C1S: complement component 1, s subcomponent	Response to external stimulus, immune response
	ADAR: adenosine deaminase, RNA-specific	RNA metabolism
	***STK25:* serine/threonine kinase 25 (STE20 homolog, yeast)**	Signal transduction
	***NFKB2:* nuclear factor of kappa light polypeptide gene enhancer**	Signal transduction, regulation of transcription
	CRBP: cellular retinol binding protein	Vitamin metabolism
	RBP1: retinol binding protein 1, cellular	Vitamin metabolism, establishment of localization
	EPHX1: epoxide hydrolase 1, microsomal (xenobiotic)	Xenobiotic metabolism, response to external stimulus
Downregulated	***GGH:* gamma-glutamyl hydrolase**[a]	Amino acid metabolism
	DLG7: discs, large homolog 7 (*Drosophila*)[a]	Cell communication
	HIST1H4C: histone 1, H4c	Cell communication, chromosome organization, and biogenesis
	***NPTX1:* neuronal pentraxin I**	Cell communication, establishment of localization
	GNAT1: guanine nucleotide binding protein (G protein)	Cell communication, signal transduction
	***OPRK1:* opioid receptor, kappa 1**	Cell communication, signal transduction
	***STMN1:* stathmin 1/oncoprotein 18**	Cell communication, signal transduction
	***PLCB4:* phospholipase C, beta 4**	Cell communication, signal transduction, lipid metabolism
	CDKN3: cyclin-dependent kinase inhibitor 3[a]	Cell cycle arrest, cell cycle, regulation of cell proliferation
	***CUL1:* cullin 1**	Cell cycle arrest, negative regulation of cell proliferation, cell cycle

Table 2. Continued

Upregulated or Downregulated	Gene Name	GO Term
	***RCC1:* chromosome condensation 1**	Cell cycle, cell division
	***CKS2:* CDC28 protein kinase regulatory subunit 2**[a]	Cell cycle, cell proliferation, signal transduction, cell communication
	***CNAP1:* chromosome condensation-related SMC-associated protein 1**[a]	Cell division, cell cycle
	UBE2S: ubiquitin-conjugating enzyme E2S[a]	Cellular protein metabolism
	***CENPA:* centromere protein A, 17kDa**[a]	Chromosome organization and biogenesis
	***TK1:* thymidine kinase 1, soluble**[a]	DNA replication, nucleobase, nucleoside, nucleotide, and nucleic acid metabolism
	***TYMS:* thymidylate synthetase**[a]	DNA replication, nucleobase, nucleoside, nucleotide, and nucleic acid metabolism
	***DBI:* diazepam binding inhibitor (acyl-Coenzyme A binding protein)**[a]	Establishment of localization, transport
	***NAPG:* N-ethylmaleimide–sensitive factor attachment protein**	Establishment of protein localization, transport
	***KIAA0101:* KIAA0101 gene product**[a]	No swiss prot identifiers
	***LMNB1:* lamin B1**[a]	No swiss prot identifiers
	TMPO: thymopoietin[a]	No swiss prot identifiers
	***HAS2:* hyaluronan synthase 2**	No swiss prot identifiers
	***HNRPM:* heterogeneous nuclear ribonucleoprotein M**	No swiss prot identifiers
	***KIAA0114:* KIAA0114 gene product**	No swiss prot identifiers
	***PSME4:* proteasome (prosome, macropain) activator subunit 4**	No swiss prot identifiers
	***S100A4:* S100 calcium binding protein A4**	No swiss prot identifiers
	***PPN:* porcupine**[a]	Nothing listed
	***USP14:* ubiquitin specific protease 14**[a]	Protein catabolism
	***MRPL23:* mitochondrial ribosomal protein L23**	Protein metabolism, protein biosynthesis
	***CDC20:* CDC20 cell division cycle 20 homolog**[a]	Regulation of cell cycle, cell division
	***CCNB1:* cyclin B1**[a]	Regulation of cell cycle, cell division, mitosis
	***FOXM1:* forkhead box M1**[a]	Regulation of transcription

Genes consistently regulated by multiple arrest signals were defined as described in the text. Functional category information was assigned as described in Table 1.
Genes indicated in boldface are regulated even further in cells arrested by growth to confluence for 20 d as compared with 4 d.
[a]Genes also regulated in models of T cell activation.
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In contrast, only 12% of the quiescence program genes that were upregulated 2-fold in all arrest conditions were also upregulated by CKI overexpression, and these genes did not fall into any particular functional class (Table 2). Therefore, a single molecule that causes cell-cycle arrest could not recapitulate the complete, functionally diverse quiescence program.

Quiescent Cells, but Not Cell Cycle Arrested Cells, Are Resistant to Differentiation

Reversibility is the defining characteristic that distinguishes quiescence from other nondividing cell states. Based on our gene expression results, we hypothesized that quiescent cells remain in this reversible state by activating pathways that prevent the inappropriate adoption of nondividing states associated with irreversible cell-cycle arrest, such as senescence and terminal differentiation. To test this hypothesis, we asked whether quiescent fibroblasts were resistant to terminal differentiation. We transduced human dermal fibroblasts (strain 91-SF5) with a retroviral vector expressing a MyoD-estrogen receptor fusion protein [12] and subsequently treated the cells with β-estradiol (10^{-7} M) to activate MyoD and induce the program of gene expression that results in myogenic differentiation [13], (see Materials and Methods for details). Muscle differentiation was monitored by real-time RT-PCR for expression of the myogenesis markers myogenin and myosin heavy chain. As can be seen in Figure 9, activation of MyoD in proliferating cells dramatically induced myogenin (Figure 9A) and myosin heavy-chain expression (Figure 9D), whereas fibroblasts that were made quiescent for 4 d by either serum withdrawal (Figure 9A and 9D) or contact inhibition (Figure 9B and 9E) were resistant to differentiation, as evidenced by an approximately 10-fold decrease in both myogenin and myosin heavy-chain expression levels. Quiescence did not affect MyoD-ER expression (unpublished data).

Our results had indicated that direct inhibition of CDKs arrested the cell cycle, but did not induce the quiescence program of gene expression. We therefore hypothesized that CKI-arrested cells would not share the same functional properties as naturally quiescent cells. Specifically, CDK inhibition did not induce the expression of genes that were associated with inhibition of differentiation (see Discussion), and we therefore tested their ability to undergo MyoD-induced myogenic differentiation. Fibroblasts expressing the MyoD-ER fusion were transduced with a retroviral vector expressing the CKI $p21^{Cip1}$, or with an empty vector as a control. The p21 expressing cells stopped proliferating, whereas the control cells did not (unpublished data). After 3 d, cells were induced to differentiate by exposure to β-estradiol. As shown in Figure 9C and 9F, nonproliferating cells transduced with the CKI p21 and control cells expressed similar amounts of myogenin and myosin heavy chain. Thus, the cell-cycle arrest induced by p21 was compatible with myogenesis, which was in contrast to the differentiation resistance exhibited by fibroblasts that were quiescent due to contact inhibition or growth-factor deprivation. This sup-

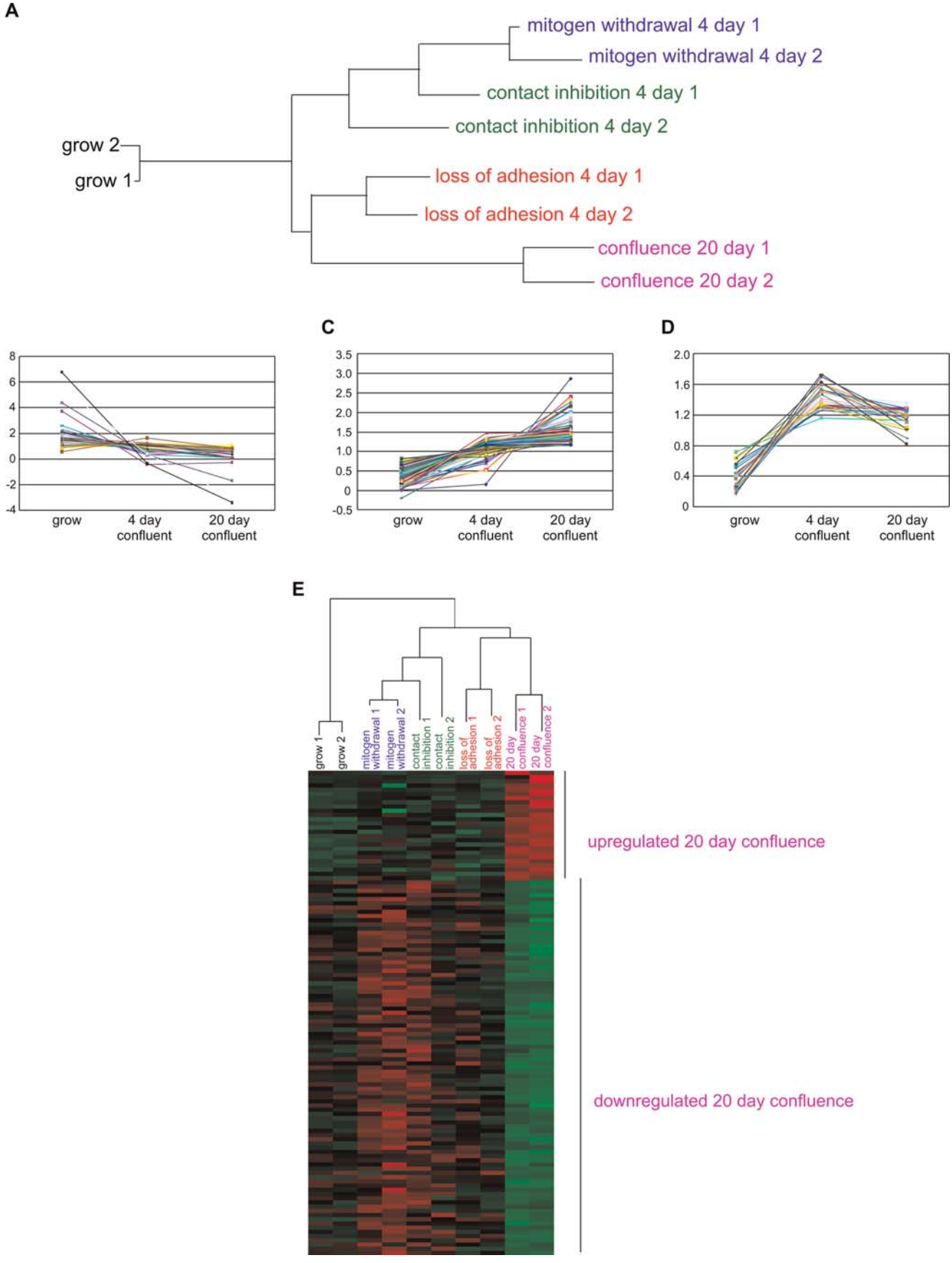
A
mitogen withdrawal 4 day 1
mitogen withdrawal 4 day 2
contact inhibition 4 day 1
contact inhibition 4 day 2
loss of adhesion 4 day 1
loss of adhesion 4 day 2
confluence 20 day 1
confluence 20 day 2
grow 2
grow 1
B
C
D
grow
4 day confluent
20 day confluent
E
grow 1
grow 2
mitogen withdrawal 1
mitogen withdrawal 2
contact inhibition 1
contact inhibition 2
loss of adhesion 1
loss of adhesion 2
20 day confluence 1
20 day confluence 2
upregulated 20 day confluence
downregulated 20 day confluence

Figure 5. Change in Expression with Longer Arrest Time

(A) Neighbor-joining tree defining the relationship between growing cells, cells arrested by mitogen withdrawal, contact inhibition, or loss of adhesion for 4 d, and by growth to confluence for 20 d. Transcriptional distances (Genechip suite 4.0) were determined and used to construct the distance matrix and neighbor-joining tree as described in Figure 2A.

(B–D) Plot of expression levels in quiescence program genes in growing, 4-d confluent and 20-d confluent cells. Mean expression values in growing cells, cells arrested by growth to confluence for 4 d and cells arrested by growth to confluence for 20 d were normalized by dividing by the average value. The data are plotted for (B), all downregulated quiescence program genes; (C), the 89 upregulated quiescence program genes that were more strongly upregulated at 20 d than 4 d; and (D), the 26 upregulated quiescence program genes that were more strongly upregulated at 4 d than 20 d.

(E) Heat maps of gene expression changes characteristic of longer arrest. Gene expression patterns for genes downregulated or upregulated in 20-d confluent cells but not cells arrested by mitogen withdrawal, contact inhibition, or loss of adhesion for 4 d are shown. Heat maps were generated as described for Figure 2C. The expected number of genes, 1.3 upregulated genes and 0.5 downregulated genes, were determined by permutation testing. The observed values of 26 and 87, respectively, are highly significant ($p \ll 0.001$).

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ports our conclusion that the quiescence program of gene expression, which is induced in cells that become quiescent in response to external signals but not by direct CDK inhibition, ensures the reversibility of the quiescent state by inhibiting terminal differentiation.

Discussion

The conceptually important points that emerge from our work are: 1) that quiescence is not a unique state but rather a collection of states that are determined by the initiating signal; 2) that it is possible to identify genes that are universally characteristic of quiescence (i.e., signal independent) implying the existence of a genetic program of quiescence; and 3) that quiescence is functionally different from cell-cycle arrest.

Quiescence Is Not a Unique State

Our first major observation was that different quiescence signals arrested growth and division by different mechanisms, and on this basis we concluded that cells enter different signal-dependent quiescent "states." The unique features of these different quiescent states were evident both in the pathways by which cells exited the cell cycle in response to antimitogenic signals, as well as in the pathways by which growth and proliferation were suppressed after the cells had entered the nondividing states. More specifically, we observed that different antiproliferative signals initiated cell-cycle exit by affecting the expression of different growth regulatory genes (Table 1). For example, contact inhibition resulted in downregulation of *cul-2, PCNA, lamin B* and its receptor, *CENPA,* biosynthetic enzymes in nucleotide metabolism, splicing factors, and different members of the inositol triphosphate signaling pathway. Loss of adhesion caused downregulation of *cyclin E1, Cul-1,* a protein kinase C isoform, another MAP kinase, and *FGF4*. Mitogen withdrawal caused the downregulation of G-protein–signaling molecules, and kinases from the MAP kinase and inositol-triphosphate–signaling pathways, as well as upregulation of *IGFBP3*. The importance of all of these proteins for controlling cell proliferation has been established, but their unique relationships to specific quiescence signals were not known. We do not mean to imply that the altered expression of any one gene alone would be sufficient to explain the mechanism of cell cycle exit in response to any particular anti-mitogenic signal. We suggest that it is the combinatorial impact of these multiple gene expression changes that defines the unique features of each quiescent state.

Interestingly, many of the genes that initially responded in a signal-specific manner during the initiation of quiescence became more uniformly regulated, in a signal-independent manner, after cells had been quiescent for 4 d. Many of these genes ultimately made up part of the quiescence program and are discussed below. Nevertheless, even at this late time point each anti-proliferative signal continued to maintain cells in unique quiescent states as evidenced by the regulation of new signal-specific genes that suppressed growth and division in different ways (Table 1). Thus genes essential for DNA synthesis *(POLD2)*, RNA synthesis *(POLR2I)*, and protein synthesis *(EIF3S9, HARS)* were uniquely downregulated by mitogen withdrawal after 4 d of quiescence. Many genes essential for various aspects of intermediary metabolism were uniquely downregulated after 4 d of loss of adhesion, including genes crucial for lipid, carbohydrate, and nucleotide metabolism (Table 1). Moreover, anti-proliferative pathways uniquely upregulated in mitogen-starved quiescent cells included those governed by *TGF-beta 2* and *-3*. In contrast, loss of adhesion induced the phosphatases *Dusp4* and *-5* (Map-kinase phosphatases), which are inhibitors of the MAP-kinase signaling pathway, whereas contact inhibition induced the quiescent cell-specific transcription inhibitor *E2F4*.

One implication of our work is that it will be important to consider the specific quiescent state from which cells are emerging when analyzing the effects of particular proteins on cell proliferation. It will be of interest to determine which particular quiescent states are found in vivo, as this should provide new insights into the mechanisms that control cell dynamics and tissue homeostasis during embryonic development and in mature animals. We speculate that the tissue-specific effects of particular oncogenes and tumor suppressors might be understood in terms of their functional relationship to different quiescent states.

Quiescence Program

Superimposed on this diversity of states is a set of transcriptional responses that is shared by all quiescent fibroblasts. While on average only 9% of genes are regulated in any given arrest state, 78% of those regulated in four transcriptionally distant arrest states were also regulated in the fifth. Since these changes in gene expression are characteristic of quiescence per se, rather than of a particular mechanism of cell cycle arrest, we suggest that they may represent a fibroblast "quiescence program." We further define below the characteristics of quiescent cells, in addition to cell cycle arrest, that emerge from these commonly regulated genes.

Many of the quiescence program genes have already been shown to play a role in controlling cell proliferation. The upregulated protein *MXI1* has been shown to control cell proliferation in mouse models [14]. The downregulated

Table 3. Genes Regulated by Quiescence for Longer Periods of Time

Upregulated or Downregulated	Gene Name	GO Term
Upregulated in 20-d confluent cells	*ACO1:* aconitase 1, soluble	Carbohydrate metabolism
	B4GALT1: UDP-Gal:betaGlcNAc b-1,4- galactosyltransferase, polypeptide 1	Carbohydrate metabolism
	ME1: malic enzyme 1, NADP(+)-dependent, cytosolic	Carbohydrate metabolism
	IL32: interleukin 32	cell communication, cell adhesion, immune response
	PPP2R5A: protein phosphatase 2, regulatory subunit B (B56), alpha isoform	Cell communication, signal transduction
	RAB32: RAB32, member RAS oncogene family	Cell communication, signal transduction
	BAG1: BCL2-associated athanogene	Cell communication, signal transduction
	RRAS: related RAS viral (r-ras) oncogene homolog	Cell communication, signal transduction, intracellular transport
	CLOCK: clock homolog	Cell communication, signal transduction, regulation of transcription
	ELN: elastin	Cell proliferation, development, morphogenesis
	DYRK4: dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 4	Cellular protein metabolism
	MLF1: myeloid leukemia factor 1	Development
	KCNA4: potassium voltage-gated channel, member 4	Establishment of localization, ion transport
	AP3M2: adaptor-related protein complex 3, mu 2 subunit	Intracellular transport
	WARS: tryptophanyl-tRNA synthetase	Negative regulation of cell proliferation
	Hel-N1: ELAV-like neuronal protein 1	Nothing listed
	TWIST1: twist homolog 1	Nothing listed
	KIAA0276	Nothing listed
	FAS: Fas (TNF receptor superfamily, member 6)	Nothing listed
	G0S2: G0/G1switch 2	Regulation of cell cycle,
	E2F5: E2F transcription factor 5, p130-binding	Regulation of cell cycle, regulation of transcription
	TSG101: tumor susceptibility gene 101	Regulation of cell growth
	HHEX: hematopoietically expressed homeobox	Regulation of transcription, development
	PTX3: pentraxin-related gene	Response to external stimulus, immune response
	HNRPA1: heterogeneous nuclear ribonucleoprotein A1	RNA metabolism
	CYP3A7: cytochrome P450, family 3, subfamily A, polypeptide 7	Xenobiotic metabolism, response to external stimulus
Downregulated in 20-d confluent cells		
	IGHM: immunoglobulin heavy constant mu	Biological process unknown
	ESD: esterase D	Biological process unknown
	ATXN10: ataxin 10	Biological process unknown
	LPP: LIM domain containing preferred translocation partner in lipoma	Biological process unknown
	SMAP: small acidic protein	Biological process unknown
	COL6A2: collagen, type VI, alpha 2	Cell communication, cell adhesion
	ITGA9: integrin, alpha 9	Cell communication, cell adhesion, signal transduction
	LIFR: leukemia inhibitory factor receptor	Cell communication, signal transduction
	CSNK1A1: casein kinase 1, alpha 1	Cell communication, signal transduction
	ANK2: ankyrin 2, neuronal	Cell communication, signal transduction
	RALGDS: ral guanine nucleotide dissociation stimulator	Cell communication, signal transduction
	GNB1: guanine nucleotide binding protein	Cell communication, signal transduction
	BMPR1A: bone morphogenetic protein receptor	Cell communication, signal transduction
	RAB11B: RAB11B, member RAS oncogene family	Cell communication, signal transduction
	GNAQ: guanine nucleotide binding protein (G protein), q polypeptide	Cell communication, signal transduction
	TRIO: triple functional domain (PTPRF interacting)	Cell communication, signal transduction
	CDC2L2: cell division cycle 2-like 2 (PITSLRE proteins)	Cell communication, signal transduction
	VIPR2: vasoactive intestinal peptide receptor 2	Cell communication, signal transduction
	IQGAP1: IQ motif containing GTPase activating protein 1	Cell communication, signal transduction
	ALCAM: activated leukocyte cell adhesion molecule	Cell communication, signal transduction, cell adhesion, immune response
	NCK1: NCK adaptor protein 1	Cell communication, signal transduction, cytoskeleton organization, and biogenesis
	IL13: interleukin 13	Cell communication, signal transduction, immune response
	PLAU: plasminogen activator, urokinase	Cell communication, signal transduction, protein catabolism
	ELK3: ELK3, ETS-domain protein	Cell communication, signal transduction, regulation of transcription
	CRK: v-crk sarcoma virus CT10 oncogene homolog	Cell communication, signal transduction, regulation of transcription
	PRKAR1A: protein kinase, cAMP-dependent, regulatory, type I, alpha	Cell communication, signal transduction, regulation of transcription
	STAT6: signal transducer and activator of transcription 6	Cell communication, signal transduction, regulation of transcription

Table 3. Continued

Upregulated or Downregulated	Gene Name	GO Term
	PPP6C: protein phosphatase 6, catalytic subunit	Cell cycle
	CUL4A: cullin 4A	Cell cycle arrest, negative regulation of cell cycle
	CSPG4: chondroitin sulfate proteoglycan 4	Cell motility
	UROD: uroporphyrinogen decarboxylase	Cofactor metabolism
	CALD1: caldesmon 1	Development
	CALD1: caldesmon 1	Development, morphogenesis
	RPA4: replication protein A4, 34kDa	DNA replication
	MTMR3: myotubularin related protein 3	Lipid metabolism
	C8A: complement component 8, alpha polypeptide	Immune response
	GDF1: growth differentiation factor 1	Lipid metabolism
	CHKA: choline kinase alpha	Lipid metabolism
	VAMP3: vesicle-associated membrane protein 3	Localization, transport
	COPS8: COP9 constitutive photomorphogenic homolog subunit 8	Metabolism
	GSTA2: glutathione S-transferase A2	Metabolism
	LAMP1: lysosomal-associated membrane protein 1	Nothing listed
	Basic Transcription Factor 2, 34 Kda Subunit	Nothing listed
	PGRMC1: progesterone receptor membrane component 1	Nothing listed
	BCR: breakpoint cluster region	Nothing listed
	TAL2: T-cell acute lymphocytic leukemia 2	Nothing listed
	Ptg-12	Nothing listed
	TSNAX: translin-associated factor X	Nothing listed
	Nadh-Ubiquinone Oxidoreductase,51 Kda Subunit	Nothing listed
	MATR3: matrin 3	Nothing listed
	CTSE: cathepsin E	Nothing listed
	PSMD5: proteasome (prosome, macropain) 26S subunit, non-ATPase, 5	Nothing listed
	Ras Inhibitor Inf	Nothing listed
	MYO1C: myosin IC	Nothing listed
	SLC5A3: solute carrier family 5 (inositol transporters), member 3	Nothing listed
	Zinc Finger Protein, Kruppel-Like	Nothing listed
	ADSS: adenylosuccinate synthase	Nucleotide metabolism
	ATP6V1A: ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A	Nucleotide metabolism, ion transport
	PEX1: peroxisome biogenesis factor 1	Organelle organization and biogenesis
	MMP14: matrix metallopeptidase 14	Protein catabolism
	FBXO21: F-box protein 21	Protein catabolism
	RBP3: retinol binding protein 3, interstitial	Protein catabolism, lipid metabolism
	ELA3A: elastase 3A, pancreatic	Protein catabolism, lipid metabolism
	SUMO1: SMT3 suppressor of mif two 3 homolog 1	Protein metabolism
	PPP2R3A: protein phosphatase 2, regulatory subunit B, alpha	Protein metabolism
	PPIF: peptidylprolyl isomerase F	Protein metabolism
	SUMO3: SMT3 suppressor of mif two 3 homolog 3	Protein metabolism
	SUMO2: SMT3 suppressor of mif two 3 homolog 2	Protein metabolism
	PIGR: polymeric immunoglobulin receptor	Protein secretion
	BCL2L7P1: BCL2-like 7 pseudogene 1	Regulation of apoptosis
	CCNE1: cyclin E1	Regulation of cell cycle
	HTATIP: HIV-1 Tat interacting protein, 60kDa	Regulation of cell growth, regulation of transcription
	TCF12: transcription factor 12	Regulation of transcription
	CITED2: Cbp/p300-interacting transactivator	Regulation of transcription
	MAZ: MYC-associated zinc finger protein	Regulation of transcription
	ATRX: alpha thalassemia/mental retardation syndrome X-linked	Regulation of transcription
	CDK8: cyclin-dependent kinase 8	Regulation of transcription
	PRKCBP1: protein kinase C binding protein 1	Regulation of transcription
	ZNF266: zinc finger protein 266	Regulation of transcription
	ATF2: activating transcription factor 2	Regulation of transcription
	RNF2: ring finger protein 2	Regulation of transcription
	ZNF9: zinc finger protein 9	Regulation of transcription
	GATAD1: GATA zinc finger domain containing 1	Regulation of transcription
	ZNF161: zinc finger protein 161	Regulation of transcription
	CDX2: caudal type homeo box transcription factor 2	Regulation of transcription, development
	MSX2: msh homeo box homolog 2	Regulation of transcription, development, morphogenesis
	HBA2: hemoglobin, alpha 2	Transport, establishment of localization
	ASNA1: arsA arsenite transporter, ATP-binding, homolog 1	Transport, response to external stimulus

Genes consistently regulated at 20 d of quiescence by growth to confluence but not by mitogen withdrawal, loss of adhesion, or contact inhibition for 4 d are listed along with relevant GO classifications assigned as described in Table 1.
DOI: 10.1371/journal.pbio.0040083.t003

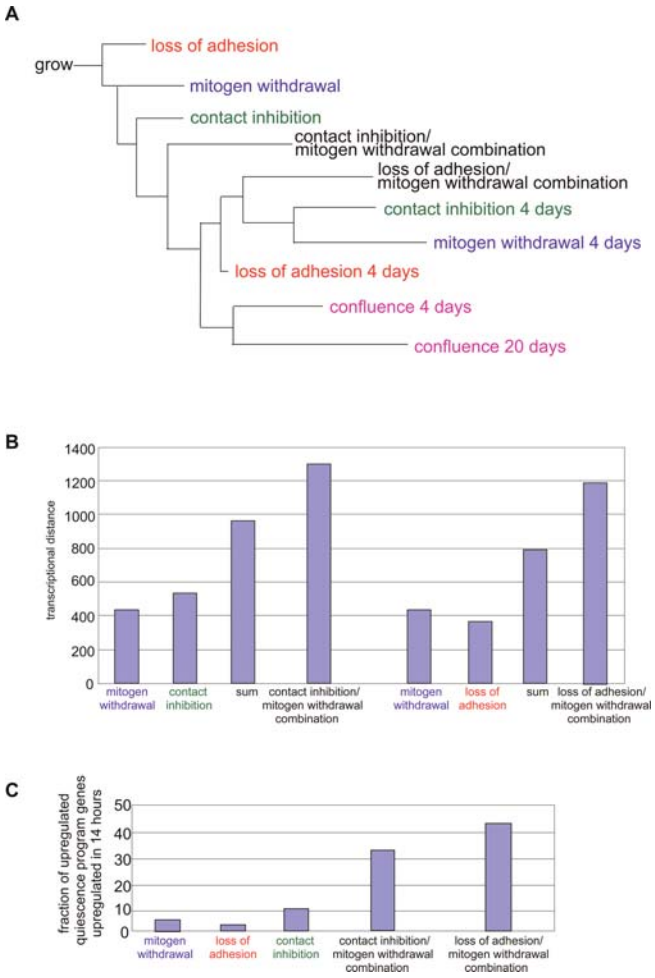

Figure 6. Combination of Arrest Signals Results in Synergistic Effects

(A) Neighbor-joining tree defining the relationship among growing cells, cells arrested by a single signal overnight, by a combination of signals, by single signals for 4 d and by longer arrest. Transcriptional distances (Genechip suite 4.0) were determined and the mean transcriptional distances for the two examples of each arrest condition were used to construct the distance matrix and neighbor-joining tree.

(B) Mean transcriptional distance from growing cells to cells arrested by individual signals overnight, and the sum of the distances, is plotted, and compared with the transcriptional distance from growing cells to cells arrested overnight by a combination of signals.

(C) The fraction of upregulated quiescence program genes that were upregulated by each individual signal and by combinations of signals after an overnight arrest.

DOI: 10.1371/journal.pbio.0040083.g006

proteins include *FOXM1,* which has also been shown to be rate-limiting for cell proliferation in animal models [15]. Also downregulated was *CKS2* [16] and other proteins known to be associated with cell proliferation, including *cyclin B1, CENPA, thymidine kinase*, and *thymidylate synthase*. Thus, the functional relevance of the quiescence-program gene-expression changes with regard to cell-cycle arrest is clear.

Among the downregulated quiescence program genes were both *Cdc20* and *Cul-1*, indicating that quiescence involves decreased activity of two major cell-cycle, proteolytic pathways, those governed by APC *Cdc20* and the SCF. Note that a different form of the APC, APC-*Cdh1,* is activated in quiescent cells [17], suggesting that quiescence involves a switch in the regulation and function of the APC.

Many of the genes upregulated in the quiescence program

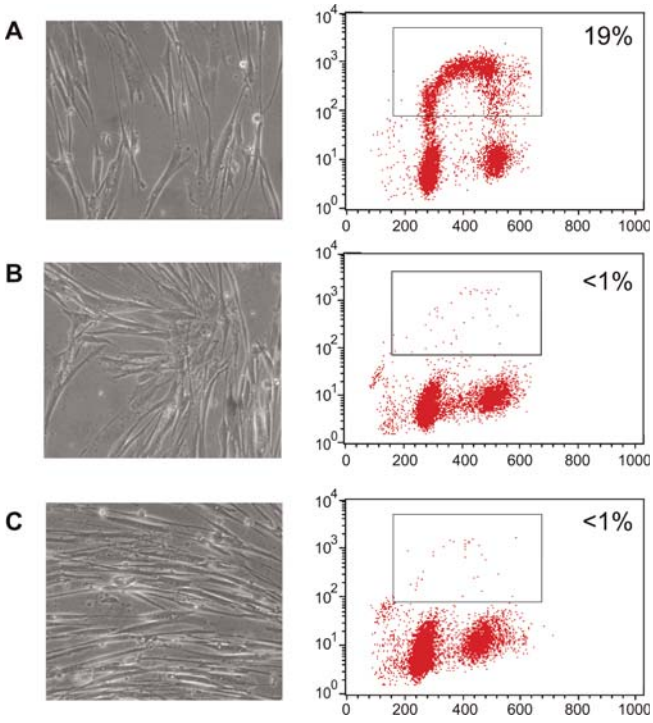

Figure 7. Cells Arrested by CKI Overexpression

Photographs of cells transduced with an empty vector (A), or a vector encoding p21 (B), or p27 (C). Methods and axes are as in Figure 1. Tetraploid cells were gated out. In cells transduced with an empty vector, 19% of the nuclei incorporated BrdU, while less than 1% of cells expressing p21 or p27 incorporated BrdU during the same time period.

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are also involved in negatively controlling cell division. Indeed, some of the upregulated genes that may be involved in actively maintaining cell cycle arrest encode established and putative tumor-suppressor proteins including *MXI1, TP53, FAT*, transmembrane receptor *PTK7*, downregulated in ovarian cancer 1 *(DOC1)*, tyrosine phosphatase receptor *(PTPRK)*, N-myristoyltransferase 1 *(NMT1)*, hypermethylated in cancer (*HIC-1*), tissue inhibitor of metalloproteinases-3 *(TIMP3)*, and cadherin 11 *(CDH11)*. This underscores the idea that tumorigenesis, in part, involves escape from an actively maintained nondividing state.

The genes and pathways in the fibroblast quiescence program are also important for quiescence in other cell types. Twenty-four of the genes we identified as being downregulated in the fibroblast quiescence program were also studied in T lymphocytes, and 17 of those were downregulated in quiescent versus proliferating T cells ($p \ll .001$ by chi-squared analysis) [18] (Table 2). Moreover, 81 genes we identified as being upregulated in the fibroblast quiescence program were also studied in T lymphocytes, and 12 were expressed at higher levels in quiescent lymphocytes ($p < .01$ by chi-squared analysis). It is not unexpected that there would be less overlap among upregulated quiescence genes, because many of them participate in cell-type specific pathways such as intercellular communication and suppression of differentiation. We also highlight specific pathways that are shared between quiescent fibroblasts and T lymphocytes. One is downregulation of myc, which in fibroblasts is achieved by upregulation of the myc antagonist *MXI1*, and in

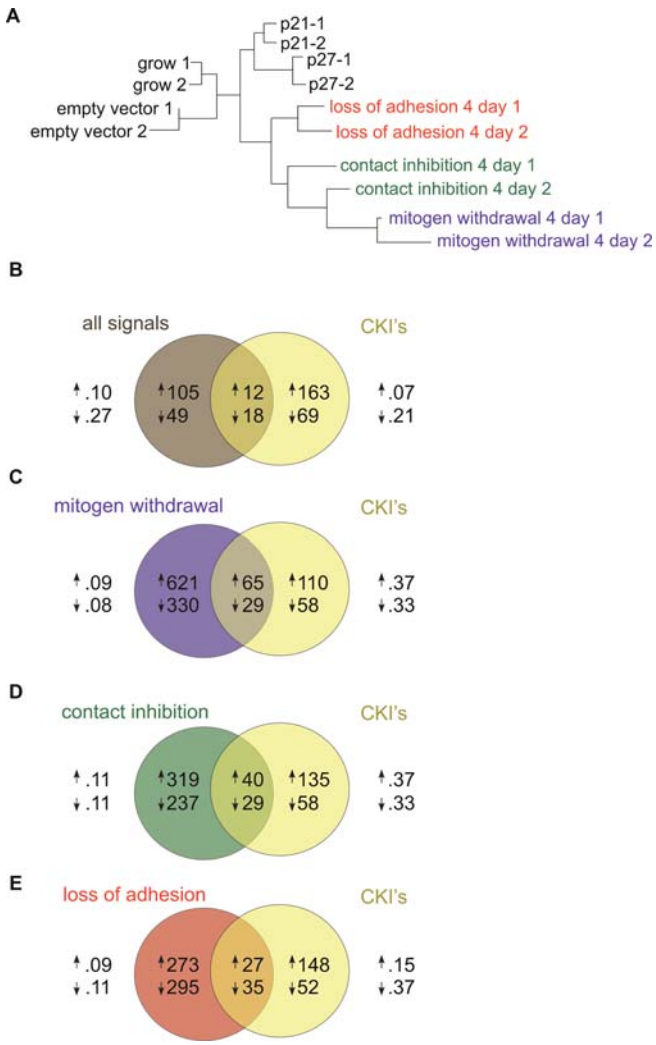

Figure 8. CKI Overexpression Does Not Closely Resemble the Quiescent State Induced by Any Extracellular Signal

(A) Neighbor-joining tree of growing cells, cells arrested by extracellular signals for 4 d, cells transduced with an empty vector, and cells transduced with a CKI. Transcriptional distances were determined for all pairwise comparisons (Genechip suite 5.0), and neighbor-joining trees were drawn as described in Materials and Methods.

(B–E) The overlap between genes regulated by CKI overexpression and genes consistently regulated by all signals (B), mitogen withdrawal (C), contact inhibition (D), or loss of adhesion (E) was determined. The number of regulated genes is shown within the circle. The fraction of regulated genes within the overlap area is provided outside the circles. Genes consistently downregulated by all three arrest signals were most likely to be regulated by CKI overexpression.

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T cells by transcriptional repression of the *myc* gene itself [19]. Another is modulation of the TGF-β pathway. An important component of T cell quiescence is the TGF-β agonist *TOB1* [20], and the related proteins *TOB2* and *BTG-2* are upregulated in quiescent fibroblasts.

Quiescence Is Not Only Cell-Cycle Arrest

The genes universally expressed in quiescent fibroblasts can be broadly divided into two groups. One group includes the genes that are regulated as a downstream consequence of cell-cycle exit, for example by inhibition of CDKs. These genes are mostly repressed during cell-cycle arrest and many of them encode proteins that directly participate in cell growth and division. This suggests that feedback regulation of cell-cycle genes is an important component of exit from the cell cycle. In contrast, cell-cycle arrest was not sufficient to recapitulate the regulation of the second group of quiescence genes, which included most of the upregulated quiescence program genes. Induction of these genes was more likely to reflect the engagement of a signaling pathway shared by these extracellular arrest signals, that culminates not only in the inhibition of proliferation, but also in other gene expression changes that are required to actively maintain cells in a viable and reversible state of arrest.

Reversible Arrest

The cardinal feature of quiescence that distinguishes it from other nondividing cell states, such as senescence, apoptosis, and terminal differentiation, is that quiescence is reversible. Some of the genes regulated in quiescent cells may, therefore, play roles in suppressing entry into these irreversible "out-of-cycle" states. In particular, we showed that both contact inhibition and serum starvation inhibited MyoD-induced myogenesis, while cells arrested by p21 overexpression entered a myogenesis pathway with an efficiency similar to controls. These results are consistent with the classical literature on myogenesis, in which it was demonstrated that cultured myoblasts can only initiate terminal myogenic differentiation when they are proliferating, whereas quiescent myoblasts are resistant to myogenic signals [21–23]. In the C2C12 myoblast cell line, MyoD-dependent differentiation involves induction of myogenin while the cells are still proliferating [24] followed by p21-dependent exit from the cell cycle [25–27]. Moreover, in vivo, quiescent myogenic stem cells (satellite cells) first proliferate in response to muscle injury before initiating myogenesis and differentiating to form new myotubes [28]. Our results extend these findings to fibroblasts and show that the block to myogenesis imposed by quiescence is at the level of responsiveness to MyoD. The p21-induced nonproliferating state remains permissive for myogenesis, which may therefore explain why the p21 mode of cell-cycle exit is utilized as part of the myogenic program.

Some of the genes regulated in quiescent cells but not cells arrested by CDK inhibition may mediate the resistance to differentiation through the Notch, bone morphogenetic protein (BMP), or Wnt pathways. The upregulated gene *HES1* (hairy and enhancer of split homolog-1), a basic helix-loop-helix (bHLH) transcriptional repressor, is a key target in Notch signaling. It blocks nerve growth factor-induced differentiation [29] and the myogenic differentiation properties of MyoD [30,31]. *SMAD1*, a signaling platform for the BMP pathway, was also consistently upregulated in quiescent cells. It can facilitate Notch signaling and thereby inhibit differentiation [32]. A number of genes in the Wnt pathway, which can suppress differentiation in stem cells [33], were upregulated in quiescent cells including *WNT2,* the frizzled receptor *FZD2, TCF7L2,* and p300/CBP-associated factor *(PCAF).* Other gene-expression changes that may play a role in suppressing differentiation include upregulation of *BCL6* [34], upregulation of matrix *Gla* protein, which inhibits chondrocyte differentiation [35], and downregulation of acyl coenzyme A binding protein *(DBI),* which promotes adipocyte differentiation [36,37]. The latter two may be particularly important in quiescent fibroblasts, which can differentiate

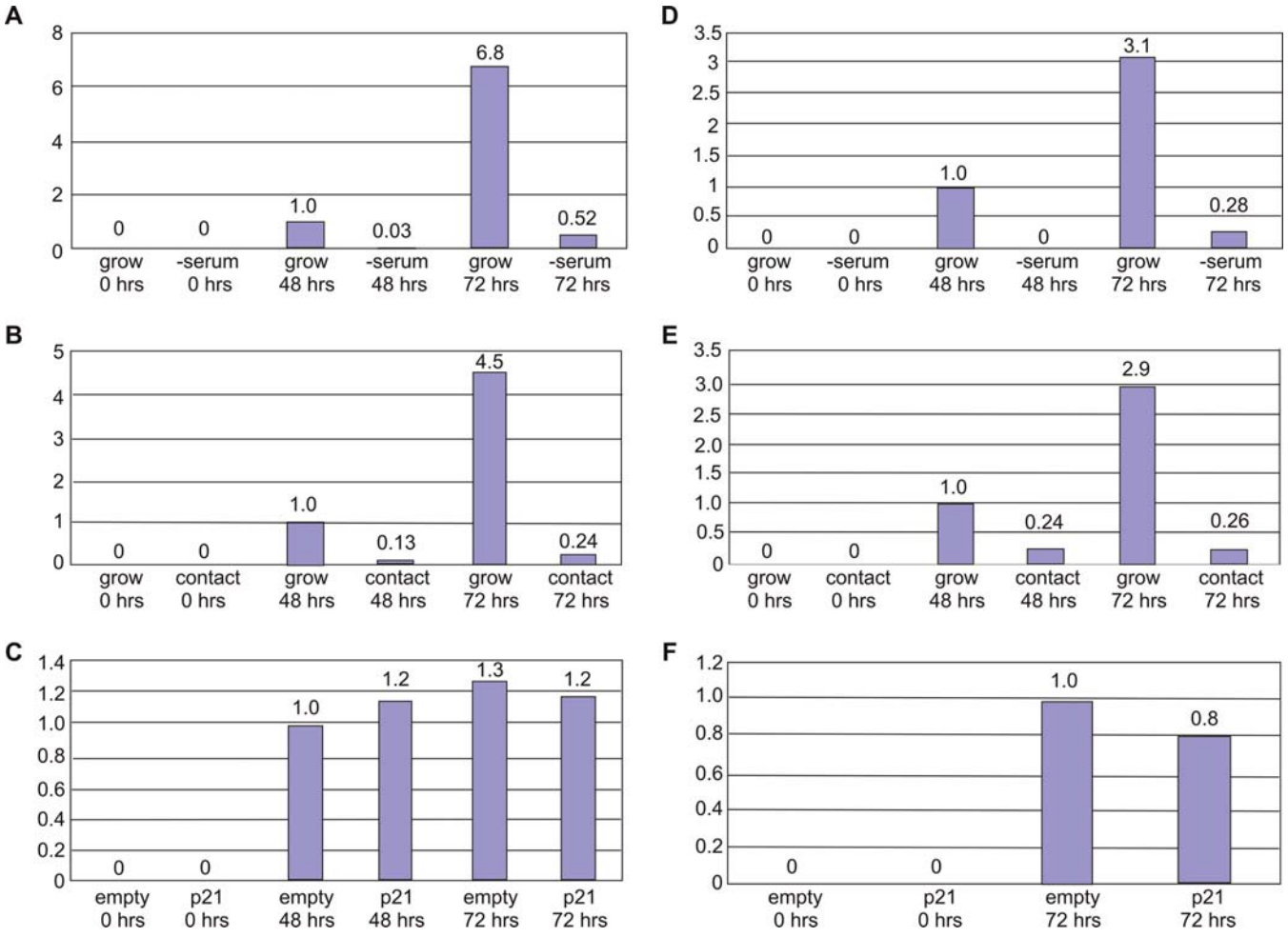

Figure 9. Quiescence, but Not Simply Cell-Cycle Arrest, Protects against Differentiation

Human dermal fibroblasts 91-SF5 were transduced with a MyoD-estrogen receptor fusion, selected for stably transduced cells, and differentiated. At the indicated time points after differentiation, myogenin (A–C) and myosin heavy chain (D–F) expression levels were determined with real time RT-PCR using GAPDH as an internal standard and normalized values were plotted. Myogenesis marker expression induced by differentiation was inhibited in cells that were made quiescent for 4 d by serum withdrawal (A and D) or contact inhibition (B and E) as compared with growing cells. In contrast, in cells arrested by overexpression of the CKI p21, there was no reduction in myogenesis marker expression as compared with control cells transduced with an empty vector (C and F).

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along the chondrocyte and adipocyte lineages, whereas other types of quiescent cells may express different lineage-specific inhibitors of differentiation.

Other regulated genes may inhibit apoptosis. Upregulation of NF-kappa B family member p49/p100 *(NFKB2),* which is an inhibitor of apoptosis, was observed in cells arrested for 4 d by all signals. Cells arrested by mitogen withdrawal upregulated *MET,* which inhibits apoptosis [38], while downregulating *Bak*, an apoptosis inducer [39,40]. Cells arrested overnight by loss of adhesion upregulated *BRAG-1*, a *Bcl-2* homologue [41]. Cells arrested by contact inhibition upregulated *FYN,* which may play a role in IGF-1–mediated survival [42].

Other upregulated genes that may function to preserve the reversibility of quiescence are those that protect the cells from accumulating damage. These include superoxide dismutase-3 *(SOD3),* which scavenges free radicals [43], peroxiredoxin 4 *(PRDX4),* which protects against hydroperoxides [44], and epoxide hydrolase *(EPHX1),* which detoxifies environmental chemicals.

Surprisingly, some of the genes upregulated in the quiescence program have been previously characterized as oncogenes, including *NFKB2* [45], *BCL6,* and *cyclin D2* [47–50]. This paradox can be resolved by appreciating that tumorigenic cells share with quiescent cells the ability to suppress entry into a terminally arrested state, and therefore that oncogenic transformation should not simply be viewed as a negation of quiescence.

Intercellular Signaling

A large number of quiescence program genes were linked to intercellular signaling (Table S1), including cell surface receptors that affect how quiescent fibroblasts respond to their environment, proteins involved in remodeling of the extracellular microenvironment, secreted proteins that could affect the proliferation of surrounding cells, and extracellular inhibitors of angiogenesis. These gene-expression changes suggest that quiescent fibroblasts may create a growth-suppressing microenvironment that affects not only other fibroblasts but also other neighboring cell types. Thus, quiescence might be an organized property of the collection

of cells that comprise a tissue or organ, rather than a strictly cell-autonomous process. Fibroblasts in tumors, unlike fibroblasts in normal tissues, frequently express genes characteristic of proliferating as opposed to serum-starved, quiescent cells [51]. Tumor progression, then, could be viewed as a disruption in the quiescent state maintained in the entire tissue, and reflect changes not only in the tumor cells themselves, but also in the surrounding stroma and the vasculature.

Materials and Methods

Cells. Fetal human lung diploid fibroblasts from American Type Culture Collection (CCL153) were grown in FBM medium (Biowhittaker, Walkersville, Maryland, United States) supplemented with 2% fetal calf serum, 5 μg/ml insulin, and 1 ng/ml fibroblast growth factor (FGM medium). Cells were grown at 37 °C in 5% CO_2 and routinely split 1 to 3 every 3 d. For each condition, frozen aliquots of 5×10^6 cells were expanded for five passages, and approximately 4×10^7 cells were plated in the given condition.

Arrest conditions. For growing cultures, cells were plated at a density of 2×10^4 cells per cm^2 in FGM medium and allowed to attach to the tissue culture plate. Because we wanted to explore the effects of mitogen withdrawal on the cell cycle without the many other effects of changes in serum concentrations, cells were grown or arrested in low-serum conditions in the presence or absence of the mitogen platelet-derived growth factor (PDGF). The medium was changed to FBM with 0.1% fetal calf serum, 25 ng/ml IGF-1 (R&D Systems, Minneapolis, Minnesota, United States), and 30 ng/ml PDGF (R&D Systems). Cells arrested by mitogen withdrawal were plated at a concentration of 10^4 cells/cm^2 and allowed to attach to the tissue culture plate. The medium was changed to FBM, 0.1% serum, and IGF-1. To arrest cells by loss of adhesion, tissue culture plates were coated with FBM medium containing 1% agarose that was allowed to solidify to prevent cells from attaching to the plate. Cells were placed in a suspension of FBM, 0.1% serum, IGF-1 and PDGF supplemented with 0.4% methylcellulose (Fisher Scientific, Fair Lawn, New Jersey, United States). For cells arrested by contact inhibition, the cells were plated at 2×10^5 cells/cm^2 and allowed to attach before the medium was changed to 0.1% serum, IGF-1 and PDGF. Cells were incubated either for 14 h or for 4 d with medium changes every 2 d. For arrest by growth to confluence, cells were plated at a density of 2×10^4 cells per cm^2 in FGM and allowed to grow to confluence. Medium was changed every 2 to 3 d for 4 d or approximately 20 (17–22) d. The night before cells were collected for flow cytometry, the medium was changed to FBM, 0.1% serum, IGF-1 and PDGF. For each condition, two independent replicates were performed.

Arrest via CKI overexpression. To arrest CCL153 by CKI overexpression, Phoenix cells (American Type Culture Collection, Manassas, Virginia, United States) were transfected with QCXIP empty vector or QCXIP into which the coding sequence of p21 or p27 had been introduced with fugene (Roche, Indianapolis, Indiana, United States). Due to a change in the termination codon, the p21 vector expresses a protein that is 6-kDa larger than p21 at its C terminus. The viral supernatants from transduced Phoenix cells were collected 48 h later and used to transduce fibroblasts. Two days after transduction, the cells were selected with 5 μg/ml puromycin for 2 d. The night before cells were collected for flow cytometry, the medium was changed to FBM, 0.1% serum, IGF-1 and PDGF.

Bromodeoxyuridine labeling. Cells were labeled with 100-μM bromodeoxyuridine (BrdU) for 6 h. Cells were collected with trypsin. The trypsin was inactivated with serum, and cells were fixed in PBS with 67% cold ethanol. Cell membranes were lysed at 37 °C in 0.08% pepsin for 20 min, and nuclei were treated with 2M HCl for 20 min. Samples were neutralized with 0.1M sodium borate, then incubated in a buffer of 10 mM Hepes, (pH7.4), 150 mM NaCl, 4% fetal bovine serum, and 2 μg of anti-BrdU-FITC antibody (Pharmingen, San Diego, California, United States) on ice for 2 h. The antibody was removed and cells were incubated in the same buffer with 0.5% Tween-20, 250 μg/ml RNase A, 100 μg/ml propidium iodide, and analyzed on a Beckton Dickinson FACS Calibur (Becton Dickinson, Palo Alto, California, United States).

Flow cytometry. Cells were incubated with approximately 5 μg/ml Hoescht 33342 (Calbiochem-Novabiochem, San Diego, California, United States) for 45 min. Cells were treated with trypsin and collected into 1 mg/ml soybean trypsin inhibitor (Sigma, St. Louis, Missouri, United States), centrifuged and resuspended in Hanks balanced saline solution supplemented with BSA (100 mg/ml), gelatin (0.04%), and EGTA (5M). Propidium iodide (0.5 μg/ml) was added, and the cells were incubated on ice. For cells arrested by mitogen withdrawal for 4 d, Hoescht was not applied because it induced cell death. Samples were run on a Beckton Dickinson FACS Vantage SE.

Microarray analysis. RNA was converted into target and hybridized to HuGENE-FL microarrays as described previously [52]. Microarray results were analyzed with the GENECHIP software (Affymetrix suite 4.0 or 5.0, Affymetrix, Santa Clara, California, United States). Each chip was scaled such that the average intensity was 1,000 while masking outliers (suite 4.0) or 500 (suite 5.0). Because of differences in the overall intensity of the different chips used in these experiments, only genes with average difference values in the first growing sample between ten and 6,700 were considered for analysis. This resulted in the systematic exclusion of classes of proteins expressed at high levels, for instance, ribosomal proteins.

Neighbor-joining trees. The transcriptional distance between two samples was determined for each pair of samples as the sum of the log base two of the absolute values of the fold changes for each gene, or the sum of the signal log ratios. This value was assigned a zero for a particular gene if there was a "no change" call between the samples or if the gene was absent in both samples. The distance matrix was used to create neighbor-joining trees with the Phylip software suite version 3.5 (available at http://www.evolution.genetics.washington.edu/phylip/software.html).

Identifying regulated genes. In the first method, we required that all of the gene expression values were higher or lower in the regulated samples as compared with all samples in which the gene was not regulated. We then required that the change in gene expression met a 2-fold cutoff under regulated conditions but did not change 2-fold in unregulated conditions. 2-fold cutoffs were assessed by comparing arrested state replicate 1 to growing sample replicate 1 and arrested state replicate 2 to growing sample replicate 2. To avoid dividing by small or negative numbers, average difference values less than 20 were thresholded to a value of 20. The number of genes expected by chance was determined by performing the same analysis on the same data set with permuted column headings. For prolonged arrest, we only used the 2-fold cutoff approach.

In the second method, we used a template-matching approach described in more detail previously [52]. An idealized template vector consisting of zeroes and ones was compared with the vector of average difference values for all of the genes that passed the filter, and the Pearson correlation coefficient was computed [53]. Genes that matched the template better than was ever observed in 20 permutations of the entire data set (0.05 genes expected) were included in the list of consistently regulated genes.

The total number of false positives expected (the sum of the number expected from both methods) was compared with the observed number of regulated genes based on a Poisson distribution.

Defining a quiescence program: the expression values in all 4-d or longer samples were required to be higher or lower than the values in the growing samples or the growing samples with an empty vector. In permuted data, 1.4 upregulated and 3.4 downregulated genes met these criteria. In contrast, 205 genes were expressed at a higher level in all longer arrested states, and 59 genes were expressed at a lower level in all longer arrested states. We then required that the gene expression value in each of the longer arrested states was consistently 2-fold higher than in growing cells. There were 43 upregulated genes that met these criteria and 16 downregulated genes, whereas 0.1 upregulated and 0.1 downregulated genes were expected by chance.

To this list of consistently regulated genes, we added genes that met the template-matching criteria for eight different templates. The templates were defined by higher (or lower) expression in all arrests for 4 or 20 d, and all combinations of regulation in the same direction in subsets of overnight samples. For each template, genes with Pearson correlation coefficients closer to −1 or 1 than observed for any gene when the entire data set was permuted 20× were included. These eight different template-matching tests resulted in a total of 98 upregulated genes and 30 downregulated genes when 0.4 ($8 \times .05$) genes were expected by chance. The total number of quiescence program genes when the two lists were merged was 116 upregulated and 33 downregulated.

Myogenesis. For myogenesis experiments, Phoenix cells were transfected with pBabe-MyoD-ER, and the supernatant was used to transduce human dermal fibroblasts 91-SF5 (generous gift of T. Norwood, University of Washington, Seattle, Washington, United States), as described above. 91-SF5 cells were grown in phenol red-free DMEM, supplemented with 10% bovine growth serum (Hyclone, Logan, Utah, United States), and were selected with 2 μg/ml puromycin for 2 d. To induce differentiation, 91-SF5 cells transduced with pBabe-MyoD-ER were washed twice with PBS and the medium

was changed to phenol-red free DMEM with insulin (10 μg/ml, Sigma), transferrin (10 μg/ml, Sigma), horse serum (0.5%, or 0.2% for serum-starved samples, Gibco/Invitrogen, Carlsbad, California, United States), and β-estradiol (10^{-7} M; Sigma). For serum-starved samples, cells were incubated in phenol red-free DMEM with no serum for 4 d prior to differentiation. For contact-inhibited samples, cells were plated at high density (2×10^5 cells/cm^2) and incubated for 4 d prior to differentiation. For CDK inhibition, 91-SF5 cells were transduced with a pBabe-MyoD-ER vector containing a gene-encoding hygromycin resistance, and selected with hygromycin (250 μg/ml). Cells were then transduced with either the QCXIP empty vector or QCXIP containing the p21 coding sequence, and selected with puromycin (2 μg/ml). Cells were then induced to differentiate. Samples were collected into Trizol reagent at the indicated times for real-time RT-PCR.

For real-time RT-PCR, RNA was isolated as described above. cDNA was synthesized from 2 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) primed with poly-T oligonucleotides according to the manufacturer's instructions. One-tenth the final volume was used for real-time PCR with master-mix–containing primers and probes specific for myosin heavy chain (ID number Hs00428600__m1, Applied Biosystems, Foster City, California, United States). Real-time PCR was performed on an ABI Prism 7900 instrument. Samples were normalized for expression levels of human GAPDH (Applied Biosystems).

Supporting Information

Figure S1. Persistence of Gene Expression Changes from 14 h to 4 d

Using template-matching, the number of genes upregulated and downregulated by mitogen withdrawal (A), contact inhibition (B), and loss of adhesion (C) was determined based on a Pearson correlation coefficient threshold of two genes by chance. For each arrest signal, the number of genes regulated by only an overnight arrest, by both overnight and 4 d, and only 4 d was determined. A signature was considered present if more than seven genes were included. Data are normalized and depicted as heat maps generated with Java Treeview.

Found at DOI: 10.1371/journal.pbio.0040083.sg001 (2.2 MB PDF).

Figure S2. Genes Regulated in Opposite Directions by Mitogen Withdrawal and Loss of Adhesion

Genes upregulated by mitogen withdrawal and downregulated by loss of adhesion, and vice versa, were identified by template-matching using a threshold of two genes by chance. The signatures at quiescence initiation and quiescence maintenance are shown as heat maps.

Found at DOI: 10.1371/journal.pbio.0040083.sg002 (2.6 MB TIF).

Table S1. Quiescence Genes Involved in Intercellular Communication

Genes regulated by one, two, or three signals at 14 h, 4 d, or 20 d that are involved in intercellular communication are listed, along with their classification by functional category.

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References

1. Williams JG, Penman S (1975) The messenger RNA sequences in growing and resting mouse fibroblasts. Cell 6: 197–206.
2. Schneider C, King RM, Philipson L (1988) Genes specifically expressed at growth arrest of mammalian cells. Cell 54: 787–793.
3. Coppock DL, Kopman C, Scandalis S, Gilleran S (1993) Preferential gene expression in quiescent human lung fibroblasts. Cell Growth Differ 4: 483–493.
4. Coppock D, Kopman C, Gudas J, Cina-Poppe DA (2000) Regulation of the quiescence-induced genes: Quiescin Q6, decorin, and ribosomal protein S29. Biochem Biophys Res Commun 269: 604–610.
5. Iyer VR, Eisen MB, Ross DT, Schuler G, Moore T, et al. (1999) The transcriptional program in the response of human fibroblasts to serum. Science 283: 83–87.
6. Venezia T, Merchant A, Ramos C, Whitehouse N, Young A, et al. (2004) Molecular signatures of proliferation and quiescence in hematopoietic stem cells. PLoS Biol 2: e301. DOI: 10.1371/journal.pbio.0020301
7. Whitfield M, Sherlock G, Saldanha AJ, Murray JI, Ball CA, et al. (2002) Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol Biol Cell 13: 1977–2000.
8. Enard W, Khaitovich P, Klose J, Zollner S, Heissig F, et al. (2002) Intra- and interspecific variation in primate gene expression patterns. Science 296: 340–343.
9. Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 95: 14863–14868.
10. Augenlicht LH, Baserga R (1974) Changes in the GO state of WI-38 fibroblasts at different times after confluence. Exp Cell Res 89: 255–262.
11. Soprano KJ (1994) WI-38 cell long-term quiescence model system: A valuable tool to study molecular events that regulate growth. J Cell Biochem 54: 405–414.
12. Hollenberg SM, Cheng PF, Weintraub H (1993) Use of a conditional MyoD transcription factor in studies of MyoD trans-activation and muscle determination. Proc Natl Acad Sci U S A 90: 8028–8032.
13. Weintraub H, Tapscott SJ, Davis RL, Thayer MJ, Adam MA, et al. (1989) Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc Natl Acad Sci U S A 86: 5434–5438.
14. Schreiber-Agus N, Meng Y, Hoang T, Hou H Jr, Chen K, et al. (1998) Role of Mxi1 in ageing organ systems and the regulation of normal and neoplastic growth. Nature 393: 483–487.
15. Wang X, Hung NJ, Costa RH (2001) Earlier expression of the transcription factor HFH-11B diminishes induction of p21(CIP1/WAF1) levels and accelerates mouse hepatocyte entry into S-phase following carbon tetrachloride liver injury. Hepatology 33: 1404–1414.
16. Urbanowicz-Kachnowicz I, Baghdassarian N, Nakache C, Gracia D, Mekki Y, et al. (1999) ckshs expression is linked to cell proliferation in normal and malignant human lymphoid cells. Int J Cancer 82: 98–104.
17. Fang G, Yu H, Kirschner MW (1998) Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. Mol Cell 2: 163–171.
18. Diehn M, Alizadeh AA, Rando OJ, Liu CL, Stankunas K, et al. (2002) Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. Proc Natl Acad Sci U S A 99: 15245.
19. Buckley A, Kuo CT, Leiden JM (2001) Transcription factor LKLF is sufficient to program T cell quiescence via a c-Myc-dependent pathway. Nat Immunol 2: 698–704.
20. Tzachanis D, Freeman GJ, Hirano N, van Puijenbroek AA, Delfs MW, et al. (2001) Tob is a negative regulator of activation that is expressed in anergic and quiescent T cells. Nat Immunol 2: 1174–1182.
21. Nameroff M, Holtzer H (1969) Contact-mediated reversible suppression of myogenesis. Dev Biol 19: 380–396.
22. Dienstman SR, Holtzer H (1977) Skeletal myogenesis. Control of proliferation in a normal cell lineage. Exp Cell Res 107: 355–364.
23. Yeoh GC, Holtzer H (1977) The effect of cell density, conditioned medium and cytosine arabinoside on myogenesis in primary and secondary cultures. Exp Cell Res 104: 63–78.
24. Andres V, Walsh K (1996) Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. J Cell Biol 132: 657–666.
25. Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, et al. (1995) Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science 267: 1018–1021.
26. Parker SB, Eichele G, Zhang P, Rawls A, Sands AT, et al. (1995) p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells. Science 267: 1024–1027.
27. Skapek SX, Rhee J, Spicer DB, Lassar AB (1995) Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. Science 267: 1022–1024.

28. Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, et al. (2005) Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122: 289–301.
29. Castella P, Wagner JA, Caudy M (1999) Regulation of hippocampal neuronal differentiation by the basic helix-loop-helix transcription factors HES-1 and MASH-1. J Neurosci Res 56: 229–240.
30. Fre S, Huyghe M, Mourikis P, Robine S, Louvard D, et al. (2005) Notch signals control the fate of immature progenitor cells in the intestine. Nature 435: 964–968.
31. Kageyama R, Ohtsuka T, Hatakeyama J, Ohsawa R (2005) Roles of bHLH genes in neural stem cell differentiation. Exp Cell Res 306: 343–348.
32. Dahlqvist C, Blokzijl A, Chapman G, Falk A, Dannaeus K, et al. (2003) Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. Development 130: 6089–6099.
33. Reya T, Clevers H (2005) Wnt signalling in stem cells and cancer. Nature 434: 843–850.
34. Albagli-Curiel O (2003) Ambivalent role of BCL6 in cell survival and transformation. Oncogene 22: 507–516.
35. El-Maadawy S, Kaartinen MT, Schinke T, Murshed M, Karsenty G, et al. (2003) Cartilage formation and calcification in arteries of mice lacking matrix Gla protein. Connect Tissue Res 44: 272–278.
36. Hansen H, Andreasen PH, Mandrup S, Kristiansen K, Knudsen J. (1991) Induction of acyl-CoA-binding protein and its mRNA in 3T3-L1 cells by insulin during preadipocyte-to-adipocyte differentiation. Biochem J 277: 341–344.
37. Mandrup S, Sorensen RV, Helledie T, Nohr J, Baldursson T, et al. (1998) Inhibition of 3T3-L1 adipocyte differentiation by expression of acyl-CoA-binding protein antisense RNA. J Biol Chem 273: 23897–23903.
38. Huh C, Factor VM, Sanchez A, Uchida K, Conner EA, et al. (2004) Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. Proc Natl Acad Sci U S A 101: 4477–4482.
39. Farrow S, White JH, Martinou I, Raven T, Pun KT, et al. (1995) Cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K. Nature 374: 731–733.
40. Chittenden T, Harrington EA, O'Connor R, Flemington C, Lutz,RJ, et al. (1995) Induction of apoptosis by the Bcl-2 homologue Bak. Nature 374: 733–736.
41. Das R, Reddy EP, Chatterjee D, Andrews DW (1996) Identification of a novel Bcl-2 related gene, BRAG-1, in human glioma. Oncogene 12: 947–951.
42. Cui QL, Zheng WH, Quirion R, Almazan G (2005) Inhibition of Src-like kinases reveals Akt-dependent and -independent pathways in insulin-like growth factor I-mediated oligodendrocyte progenitor survival. J Biol Chem 280: 8918–8928.
43. Serra V, von Zglinicki T, Lorenz M, Saretzki G (2003) Extracellular superoxide dismutase is a major antioxidant in human fibroblasts and slows telomere shortening. J Biol Chem 278: 6824–6830.
44. Shen C, Nathan C (2002) Nonredundant antioxidant defense by multiple two-cysteine peroxiredoxins in human prostate cancer cells. Mol Med 8: 95–102.
45. Lin A, Karin M (2003) NF-kappaB in cancer: A marked target. Semin Cancer Biol 13: 107–114.
46. Ohno H (2004) Pathogenetic role of BCL6 translocation in B-cell non-Hodgkin's lymphoma. Histol Histopathol 19: 637–650.
47. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, et al. (2004) Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 103: 275–282.
48. Schmidt BA, Rose A, Steinhoff C, Strohmeyer T, Hartmann M, et al. (2001) Up-regulation of cyclin-dependent kinase 4/cyclin D2 expression but down-regulation of cyclin-dependent kinase 2/cyclin E in testicular germ cell tumors. Cancer Res 61: 4214–4221.
49. Faussillon M, Monnier L, Junien C, Jeanpierre C (2005) Frequent overexpression of cyclin D2/cyclin-dependent kinase 4 in Wilms' tumor. Cancer Lett 221: 67–75.
50. Zhang J, Hu S, Schofield DE, Sorensen PH, Triche TJ (2004) Selective usage of D-Type cyclins by Ewing's tumors and rhabdomyosarcomas. Cancer Res 64: 6026–6034.
51. Chang HY, Sneddon JB, Alizadeh AA, Sood R, West RB, et al. (2004) Gene expression signature of fibroblast serum response predicts human cancer progression: Similarities between tumors and wounds. PLoS Biol 2: e7. DOI: 10.1371/journal.pbio.0020007
52. Smith L, Coller HA, Roberts JM (2003) Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. Nat Cell Biol 5: 474–479.
53. Pavlidis P, Noble WS (2001) Analysis of strain and regional variation in gene expression in mouse brain. Genome Biol 2: RESEARCH0042.
54. Saldanha AJ (2004) Java Treeview—Extensible visualization of microarray data. Bioinformatics 20: 3246–3248.
55. Boyle EI, Weng S, Gollub J, Jin H, Botstein D, et al. (2004) GO:TermFinder—Open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. Bioinformatics 20: 3710–3715.
56. Camon E, Magrane M, Barrell D, Lee V, Dimmer E, et al. (2004) The Gene Ontology Annotation (GOA) Database: Sharing knowledge in Uniprot with Gene Ontology. Nucleic Acids Res 32: D262–D266.