Identification of potential anticancer drug targets through the selection of growth-inhibitory genetic suppressor elements

Thomas Primiano,[1] Mirza Baig,[1,3] Anil Maliyekkel,[1] Bey-Dih Chang,[1,3] Stacey Fellars,[1] Justin Sadhu,[1] Sergey A. Axenovich,[2] Tatyana A. Holzmayer,[2,‡] and Igor B. Roninson[1,3,*]

[1]Department of Molecular Genetics, University of Illinois at Chicago, Chicago, Illinois 60607
[2]PPD Discovery Inc., Menlo Park, California 94025
[3]Present address (after August 1, 2003): Cancer Center, Ordway Research Institute, Inc., 150 New Scotland Avenue, Albany, New York 12208
*Correspondence: roninson@ordwayresearch.org
‡Dr. Tatyana Holzmayer, who played a key role in developing the GSE methodology, tragically died on February 27, 2002. This article is dedicated to her memory.

Summary

To identify human genes required for tumor cell growth, transcriptome-scale selection was used to isolate genetic suppressor elements (GSEs) inhibiting breast carcinoma cell growth. Growth-inhibitory GSEs (cDNA fragments that counteract their cognate gene) were selected from 57 genes, including known positive regulators of cell growth or carcinogenesis as well as genes that have not been previously implicated in cell proliferation. Many GSE-cognate genes encode transcription factors (such as STAT and AP-1) and signal transduction proteins. Monoclonal antibodies against a cell surface protein identified by GSE selection, neural cell adhesion molecule L1CAM, strongly inhibited the growth of several tumor cell lines but not of untransformed cells. Hence, selection for growth-inhibitory GSEs allows one to find potential targets for new anticancer drugs.

Introduction

The completion of the draft sequence of the human genome has provided us with a partial list of known and putative human genes, the total number of which is estimated between 30,000 and 45,000 (Lander et al., 2001; Venter et al., 2001). These genes provide many potential targets for drugs, some of which may be useful in stopping the growth of cancers. The development of new anticancer drugs could be greatly facilitated by the ability to narrow down the list of human genes to those that are necessary for the growth of tumor cells. To find such genes, we have used a general strategy, which is based on the principle that genes required for cell proliferation should give rise to genetic suppressor elements (GSEs) that inhibit cell growth.

GSEs are short biologically active cDNA fragments that interfere with the function of their cognate gene (Gudkov et al., 1999; Roninson et al., 1995). GSEs may encode antisense RNA molecules that inhibit gene expression or peptides corresponding to protein domains that act as dominant negative inhibitors. The general strategy for the isolation of biologically active GSEs involves the preparation of an expression library containing randomly fragmented DNA of the target gene or genes. This library is then introduced into recipient cells, followed by selection for the desired phenotype and the recovery of biologically active GSEs from the selected cells. By using a single cDNA as the starting material for GSE selection, one can generate specific inhibitors of the target gene and map functional domains in the target protein. By using a mixture of multiple genes or the entire transcriptome as the starting material, GSE selection allows one to identify genes responsible for a specific cellular function, since such genes will give rise to GSEs inhibiting this function. For example, GSE selection has been used to identify viral genes that are essential for the infectious cycle of bacteriophage lambda (Holzmayer et al., 1992) or human immunodeficiency virus (Dunn et al., 1999), and to clone tumor suppressor genes (Garkavtsev et al., 1996), genes that mediate cellular sensitivity to anticancer drugs (Gudkov et al., 1994; Levenson et al., 1999), and regulators of transcription (Novoa et al., 2001).

GSEs derived from genes involved in cell proliferation are expected to inhibit cell growth. To select for growth-inhibitory GSEs, we have used bromodeoxyuridine (BrdU) suicide as the selection strategy. This strategy has been previously used to isolate three growth-inhibitory GSEs from a mixture of 19 cDNAs associated with the G_0/G_1 transition (Pestov and Lau, 1994),

SIGNIFICANCE

The development of new anticancer drugs can be greatly facilitated by determining which of the human genes are required for tumor cell growth. The present study shows that function-based selection of GSEs inhibiting the growth of tumor cells allows one to generate an extensive list of potential targets for cancer treatment. 57 target genes, many of which were previously unknown to play a role in cell proliferation, have been identified through this selection. Antibodies against one of the identified gene products, L1CAM, show a growth-inhibitory activity specific for tumor cells, which demonstrates the power of our strategy for elucidating promising new targets for cancer treatment.

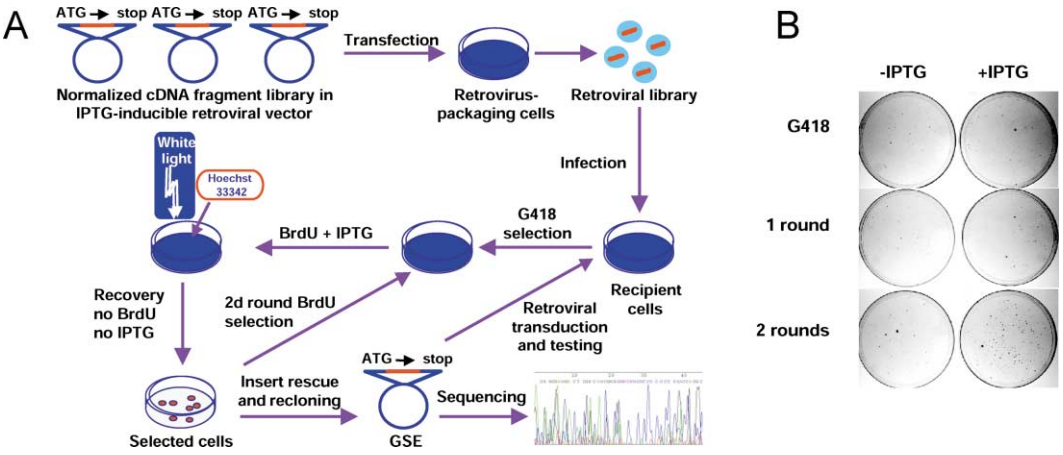

Figure 1. Selection of growth-inhibitory GSEs from a normalized cDNA fragment library

A: Scheme of selection.

B: Survival of BrdU suicide selection by library-transduced MDA-MB231 cells, tested immediately after G418 selection, after one round and after two rounds of BrdU selection in the presence of IPTG. For testing, cells were plated at 200,000 per P100 and subjected to one round of BrdU selection in the absence or in the presence of 50 μM IPTG, and allowed to form colonies for 12–14 days in the absence of IPTG.

which were fragmented and cloned in a regulated plasmid expression vector. To carry out such selection from a library that would represent the entire transcriptome of breast carcinoma cells, we have now generated a large library of normalized (reduced redundancy) fragments of total cellular cDNA in a retroviral vector that allows for high-efficiency transduction and isopropyl-β-thio-galactoside (IPTG)-regulated gene expression in mammalian cells (Chang and Roninson, 1996). With this library, we have succeeded in identifying a large set of genes that give rise to GSEs inhibiting breast carcinoma cell growth. Monoclonal antibodies against a cell surface protein L1CAM, encoded by one of these genes, inhibit the growth of several transformed cell lines but not of untransformed cells, demonstrating that GSE-cognate genes can be regarded as potential new targets for cancer treatment.

Results and Discussion

Library construction and selection of growth-inhibitory GSEs

The selection of growth-inhibitory GSEs is schematized in Figure 1A. The normalized cDNA fragment library was generated from MCF-7 breast carcinoma cell line (estrogen receptor-positive, wild-type for p53). The cDNA fragments in this library (50–400 bp size) were tagged by different adaptors at the ends corresponding to the 5′ and 3′ direction of the original mRNA. The 5′ adaptor contains translation initiation codons in three open reading frames, and the 3′ adaptor contains stop codons in all the frames. The cDNA fragment mixture was subjected to normalization based on $C_o t$ fractionation, using a modified procedure of Patanjali et al. (1991) (Gudkov and Roninson, 1997). The normalized cDNA preparation was cloned into IPTG-inducible retroviral vector LNXCO3 in both sense and antisense orientations, producing a library of approximately 50 million clones, 87% of which were recombinant. This library was transduced into breast carcinoma cell line MDA-MB231, which represents a more malignant class of breast cancers; it is estrogen receptor-negative and p53-deficient. The choice of different cell lines as the source of RNA and as the recipient was aimed at identifying target genes that are more likely to be essential in different types of breast cancer. Prior to transduction, the recipient cell line was modified by the introduction of murine ecotropic receptor, which renders cells highly infectable with ecotropic retroviruses, and of modified bacterial LacI repressor that allows for IPTG-regulated gene expression from promoters containing Lac operator.

Eighty million library-transduced MDA-MB231 cells were used for two consecutive rounds of BrdU suicide selection. This selection (Stetten et al., 1977) is based on the destruction of cells that replicate their DNA in the presence of BrdU, a photoactive nucleotide that incorporates into DNA and causes lethal DNA crosslinking upon white light illumination. Prior to BrdU addition, cells are treated with IPTG to induce transcription of library-derived cDNA fragments. Cells containing IPTG-inducible growth inhibitory GSEs do not replicate their DNA and do not incorporate BrdU in the presence of IPTG. Such cells therefore survive the selection and will proliferate upon the removal of IPTG. The advantages of this method include its sensitivity for weak growth-inhibitory GSEs; i.e., even if only a small fraction of GSE-containing cells fail to replicate in the presence of IPTG, such cells will survive BrdU suicide and will give rise to a recovering clone. This technique also allows one to identify and exclude spontaneously arising BrdU-resistant mutants, since their survival will not depend on the presence of IPTG. As shown in Figure 1B, two consecutive rounds of selection increased the number of colonies that survive BrdU suicide in the presence of IPTG relative to the absence of IPTG, which is consistent with successful selection of IPTG-inducible growth-inhibitory GSEs.

Identification of growth-inhibitory GSEs

Genomic DNA was isolated after BrdU suicide selection and used to recover the inserts from integrated proviruses by PCR, using vector-derived sequences flanking the inserts as primers (Gudkov and Roninson, 1997). The PCR-amplified mixture was

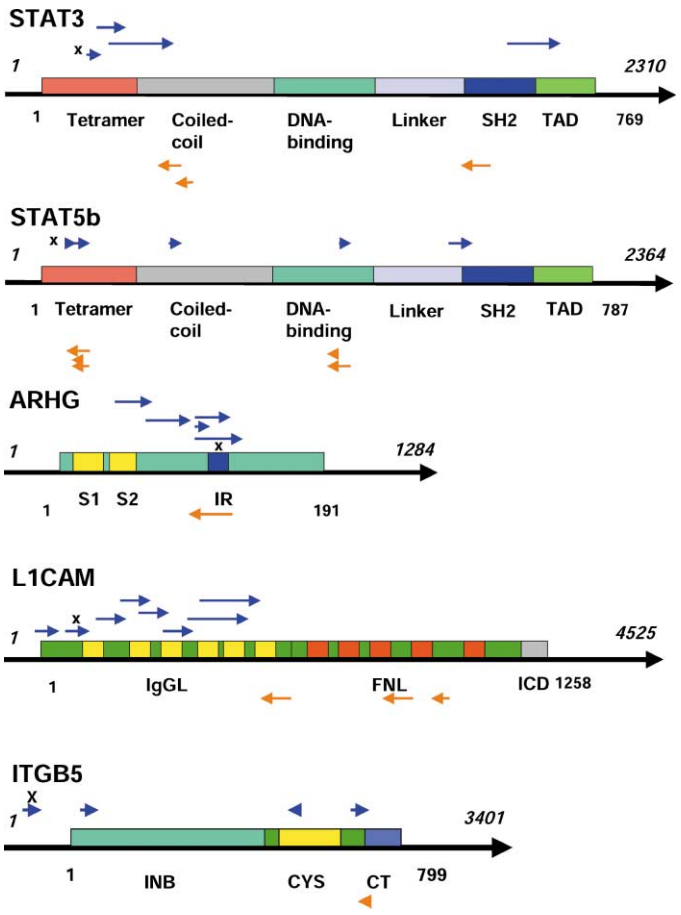

Figure 2. Distribution of selected fragments in individual cDNAs

Positions of the selected sense-oriented (top arrows) and antisense-oriented (bottom arrows) fragments relative to the cDNA sequence and protein domains of STAT3, STAT5b, ARHG (RhoG), L1CAM, and ITGB5. GSEs verified by functional assays and used to generate cell lines are identified by an X. Domain abbreviations: TAD, transcriptional activation domain; S1, site 1; S2, site 2; IR, insert region; IgGL, immunoglobulin-like; FNL, fibronectin-like; ICD, intracellular domain; INB, integrin-β; CYS, cystein-rich; CT, C-terminal.

directionally cloned into LNXCO3 to yield a GSE-enriched library that was then analyzed by high-throughput sequencing. cDNA sequences were obtained for 1482 inserts. To determine the identity of the corresponding genes, these sequences were analyzed using the BLASTMask program that we wrote for sequential BLAST homology searches in NCBI sequence databases. Through this analysis, 64 known and unknown genes were found to give rise to three or more identical clones or to two or more different cDNA sequences in the sequenced set. Of these, 21 genes were represented by a single cDNA sequence, and 43 genes were represented by 2 to 11 different cDNA sequences.

As in earlier studies (where multiple GSEs were selected from a single gene or a viral genome), both sense and antisense-oriented fragments of the same gene tend to form clusters. Such clusters indicate selectivity for specific protein domains or for specific RNA regions susceptible to antisense inhibition. In some cases, sense-oriented fragments were localized to protein domains involved in known functional interactions, suggesting that peptides encoded by these fragments disrupt the corresponding protein interactions. Figure 2 shows the position of

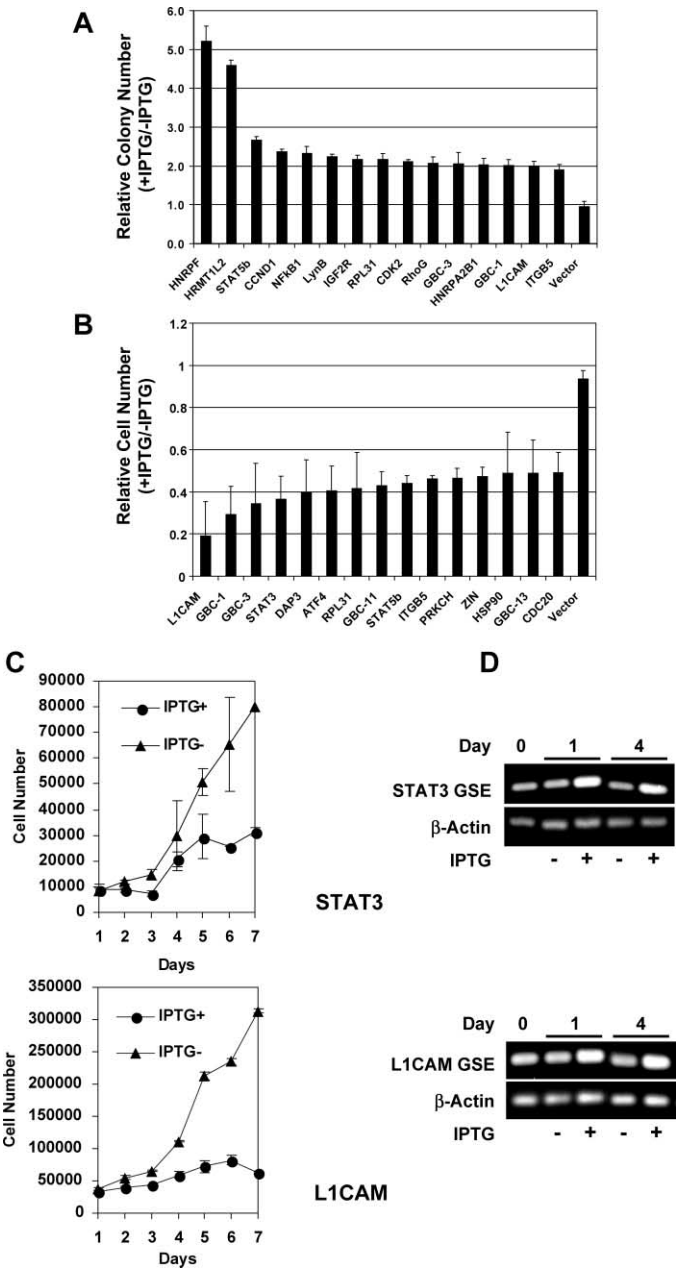

Figure 3. Functional assays for growth-inhibitory activity of representative GSEs

Results are shown as mean ± standard error for two separate experiments, each performed in triplicate.

A: Clonogenic recovery from BrdU suicide selection in cell populations transduced with the indicated GSEs in the presence relative to the absence of IPTG.

B: Relative cell number of clonal cell lines transduced with the indicated GSEs after 7-day growth in the presence relative to the absence of IPTG.

C: Growth curves of clonal cell lines transduced with the GSEs from STAT3 and L1CAM, in the presence or in the absence of IPTG.

D: Reverse transcription-PCR analysis of RNA expression of STAT3 and L1CAM GSEs in the cell lines of **C**, on the indicated days in the presence or in the absence of IPTG. β-actin was used as a normalization standard.

Table 1. Genes giving rise to growth-inhibitory GSEs

Gene	Accession Number	# Sequences (s/as)	# clones	Gene function	Association with cancer	Functional assays*
Transcription factors						
STAT5b	NM_012448	4(s), 4(as)	152	Transcription factor (involved in hematopoietic cell growth)	Implicated in lymphomagenesis	A, B
STAT3	NM_003150	4(s), 3(as)	25	Transcription factor (proliferation)	Upregulated in breast cancer	A, B
C-FOS	NM_005252	3(s), 3(as)	17	AP-1 component	Protooncogene	A
ATF4	NM_001675	5(as)	369	Activating transcription factor (involved in hematopoietic cell growth)	Induced in breast cancer by heregulin	A
FOSL2	NM_005253	1(s), 1(as)	2	AP-1 component	FOS-related	A
MBD1	NM_015847	1(s), 1(as)	2	Methylated DNA binding protein, transcription inhibitor		B
HES6	XM_043579	1(s)	6	Transcription cofactor, differentiation inducer		B
NFκB-1	NM_003998	1(as)	5	Transcription factor (stress, apoptosis, proliferation)	Upregulated in several types of cancer	A, B
NR3C1	NM_000176	1(s)	5	Glucocorticoid receptor		A
TAF7	XM_049114	1(s)	5	Transcription initiation factor		A
JunB	NM_002229	1(s)	4	AP-1 component	Protooncogene	B
Signal transduction						
ARHG	NM_001665	5(s), 1(as)	43	RhoG, small GTPase, cytoskeletal reorganization	Ras family, interacts with ect2 oncogene, activates Rac1	A
PRKCH	NM_006255	3(s), 2(as)	5	Serine/threonine protein kinase	Stimulated by tumor promoters	A, B
PRKCZ	NM_002744	2(s), 1(as)	6	Serine/threonine protein kinase	Stimulated by tumor promoters	B
PRKCD	NM_006254	2(s), 1(as)	5	Serine/threonine protein kinase	Stimulated by tumor promoters	C
AP1B1/BAM22	NM_001127	2(s)	5	Clathrin-associated adaptor protein		A
PPP2R1B	NM_002716	2(as)	3	Protein phosphatase 2 regulatory subunit Aβ	Putative tumor suppressor	B
Rab5B	NM_002868	2(as), 1(s), 1(as)	3	Small GTPase, involved in vesicle transport	Ras family	C
MAP2K2	NM_030662	2(as)	2	MAP kinase kinase	Implicated in medulloblastoma metastasis	A
ZIN	NM_013403	1(as)	6	Calmodulin-binding WD repeat protein		A, B
14-3-3ζ	NM_145690	1(s)	2	14-3-3 protein, phospholipase A2	Regulates Raf	B
Cell cycle progression genes						
MCM-3	NM_002388	3(s), 4(as)	38	DNA replication		C
FEN1	NM_004111	2(s), 2(as)	12	DNA replication and repair		A
CCN D1	NM_001758	2(s), 2(as)	9	Cyclin, G1/S transition	Amplified in cancers	A
CDK2	NM_001798	2(s)	3	Cyclin-dependent kinase, S phase	Amplified in cancers	A, B
CDK10	NM_003674	2(s)	2	Cell cycle, G2/M		B
CDC20	NM_001255	1(as)	9	CDC2-related kinase, mitosis		B
RPA3	NM_002947	1(s)	6	DNA replication, excision repair		A
PCNA	NM_002592	1(s)	3	DNA replication	Upregulated in cancers	C
Growth factors/growth factor receptors						
EFN A1	NM_004428	1(s), 3(as)	9	Receptor tyrosine kinase ligand	RAS pathway regulator	A
FGFR1	NM_000604	2(s), 1(as)	6	Fibroblast growth factor receptor, tyrosine kinase	Amplified in breast cancers	A
LynB	NM_002350	2(as)	8	Tyrosine kinase	YES protooncogene	A
IGF2R	NM_000876	2(s)	3	Insulin-like growth factor 2 receptor	Mutated in breast cancers	A, B
Cell adhesion						
L1CAM	NM_000425	8(s), 3(as)	20	Cell adhesion, neural		A, B
ITGβ5	NM_002213	4(s), 1(as)	30	Cell adhesion, vitronectin receptor		A, B
ICAM2	NM_000873	2(s), 1(as)	8	Cell adhesion, intercellular		A
ITG α4	NM_000885	1(s), 1(as)	4	Cell adhesion	Involved in Src pathway	B
Protein synthesis						
EIF3S10	NM_003750	3(s)	3	Translation initiation factor 3, subunit 10		A
RPL31	NM_000993	2(s)	9	Ribosomal protein L31	Overexpressed in colorectal tumors	A, B
RPL35	NM_007209	2(as)	4	Ribosomal protein L35		C
KIAA1270	XM_044835	1(as)	9	Alanyl-tRNA synthetase homolog		A
Protein processing						
Hsp90α	NM_005348	2(s)	27	Chaperone, protein folding	Overexpressed in breast cancer, activates oncogenic tyrosine kinases and Raf	B

(*continued*)

Table 1. Continued

Gene	Accession Number	# Sequences (s/as)	# clones	Gene function	Association with cancer	Functional assays*
ADPRT	NM_001618	1(s), 1(as)	10	Poly (ADP ribosyl) transferase		C
GRP58	NM_005313	1(s), 1(as)	4	Chaperone, interacts with STAT3		B
NIN283	NM_032268	1(s)	11	Lysosomal, putative role in protein ubiquitination		A
RNA processing and transport						
HNRPF	NM_004966	1(s)	5	Heterogeneous nuclear ribonucleoprotein F		A, B
HRPMT1L2	NM_001536	1(s)	5	Hnrp arginine methyltransferase-like 2		A, B
HNRPA2B1	NM_002137	1(s)	4	Heterogeneous nuclear ribonucleoprotein A2/B1		A
Others						
VWF	NM_000552	6(s), 5(as)	39	Blood clotting		B
DAP-3	NM_004632	2(as)	3	Positive/negative apoptosis regulator	Overexpressed in gliomas	A, B
MYL6	NM_021019	2(s)	2	Contractility		A
IF1	NM_016311	1(s)	4	Inhibitor of Fo/F1 mitochondrial ATPase		A
Unknown function						
GBC-1	NM_031221	2 (s)	70	Contains helical repeat peptide		A, B
GBC-3	AA443027	1(s)	12	HC 3q29		A, B
GBC-11		1(s)	4	HC 14		A, B
GBC-12		1(s)	3	HC 1		A, B
GBC-13		1(s)	3	HC 16		B

*A, confirmed by BrdU suicide assay; B, gave rise to cell line inhibited by IPTG; C, low survival of infected cells.

selected cDNA fragments relative to the cDNA sequence and to the corresponding protein domains for some of the enriched genes. As shown in this figure, two related transcription factors STAT3 and STAT5b gave rise to clusters of fragments from the tetramerization domain (involved in dimer-dimer interactions), and to a fragment of the SH2 domain that interacts with phosphotyrosine residues. In another example, all eight sense-oriented GSEs of L1 cell adhesion molecule (L1CAM) mapped to its immunoglobulin-like domains (IgGL), which are involved in multiple *cis* and *trans* interactions.

Selected cDNA fragments representing the 64 enriched genes were individually tested for biological activity in MDA-MB231 cells, using two assays. In the first assay, LNXCO3 retroviruses carrying the tested sequences were transduced into MDA-MB231-3′SS31 cells, and the transduced populations were tested for IPTG-dependent resistance to BrdU suicide. IPTG had no effect on the survival of control cells transduced with insert-free LNXCO3 vector, but cDNA fragments derived from 38 genes have so far shown statistically significant and reproducible increases in BrdU survival in the presence of IPTG relative to its absence. Results of representative assays with a subset of these GSEs are shown in Figure 3A.

In theory, IPTG-dependent survival of BrdU suicide selection could result not only from growth inhibition by IPTG-regulated GSEs but also from the ability of such GSEs to confer resistance to the selective procedure through some other mechanism. Therefore, in the second assay, we determined whether GSE induction by IPTG directly inhibits cell growth, a much more stringent criterion than an increase in IPTG-dependent survival of BrdU suicide. In the initial experiments, IPTG produced no significant growth inhibition in mass populations of GSE-transduced cells, suggesting that GSE activity in most of the infected cells was insufficient for this effect. The same lack of effect in a mass population was previously observed with another BrdU-selected growth inhibitor, ubiquitin-conjugating enzyme UbcM2 (Pestov et al., 1998). To obviate this problem, we picked and expanded 12 cell colonies from each GSE- or vector-transduced cell population after BrdU selection. These clonal cell lines were then individually tested for growth inhibition by IPTG. IPTG treatment neither affected the growth of untransduced cells in more than 40 assays nor had any effect on the 12 cell lines transduced with an insert-free vector. IPTG, however, inhibited the growth of one or more cell lines carrying individual GSEs from 30 of the tested genes, with 17 genes giving rise to two or more IPTG-inhibited lines. The results for some of these GSEs are shown in Figure 3B. Figure 3C shows the time course of IPTG growth inhibition in two of the selected cell lines; this growth inhibition was associated with the induction of GSE expression at the RNA level in IPTG-treated cells (Figure 3D). Altogether, GSEs from 51 genes have been confirmed so far by either one or both of the two assays. In addition, GSEs from 6 other genes consistently yielded very few stably transduced cells, suggesting that even "leaky" basal-level expression of these GSEs is growth-inhibitory.

Nature of the selected genes

Table 1 describes 57 genes that gave rise to growth-inhibitory GSEs, including 52 genes with known or tentative functions and 5 novel genes; the latter were designated GBC (for growth of breast cancer). Strikingly, close to 60% of these genes (33/57) are already known to act as positive regulators of cell growth or neoplastic transformation, and some of the genes have been implicated specifically in breast cancer (see Table 1), providing biological validation of our experimental strategy. These include bona fide protooncogenes, such as C-FOS, JunB, and LynB (c-YES), as well as other growth-promoting genes that are ampli-

fied in cancers, such as CCND1, CDK2, and FGFR1. Eight genes in Table 1 are directly involved in cell cycle transitions or DNA replication, and four other genes encode growth factors or growth factor receptors.

The largest functional category of the selected genes includes 11 transcription regulatory proteins, some of which are known to be involved in cell proliferation, including AP-1 components C-FOS, FOSL2, and JUNB, and proliferation-promoting factors STAT5b and STAT3, the latter of which is upregulated in breast cancers (Garcia et al., 1997). Another highly enriched transcription factor, ATF4, was recently shown to be a positive regulator of hematopoietic cell proliferation (Masuoka and Townes, 2002). ATF4 expression and function are augmented by heregulin, a factor that stimulates the growth of breast cancers (Talukder et al., 2000). Notably, ATF4 has been shown to dimerize with Jun (Hai et al., 1999), and STAT3 and Jun were shown to interact in regulating Fas expression (Ivanov et al., 2002). In addition, concerted activation of STAT, AP-1, and NFκB (another transcription factor that gave rise to growth-inhibitory GSEs) was found to be a frequent and early event in human hepatocellular carcinomas (Liu et al., 2002).

Table 1 also includes ten regulators of signal transduction, many of which are involved in cell growth, including three isoforms of protein kinase C that mediates the tumor-promoting effect of phorbol esters, small GTPase ARHG (RhoG), and the regulatory subunit of protein phosphatase 2 (PPP2R1B). Although the latter gene is considered a putative tumor suppressor, protein phosphatase 2 inhibitors were reported to induce differentiation in breast carcinoma cell lines, including MDA-MB231 (Kiguchi et al., 1992). Four other genes in Table 1 encode cell adhesion molecules and integrins, which mediate intracellular signaling, such as integrin α4 (ITGA4) that plays a role in the Src pathway. Remarkably, many of the identified genes interact with each other in signal transduction pathways that regulate the activities of transcription factors STAT, AP-1, and ATF4. Such interactions are illustrated in Figure 4 for 17 proteins and protein families identified in the present study.

About 40% of the GSE-cognate genes have not been previously implicated in cell growth, including five novel genes identified in the present study. Some genes in this category were among those that were most highly enriched by GSE selection (Table 1), including von Willebrand factor (VWF) involved in blood clotting, L1CAM, a cell adhesion molecule of immunoglobulin superfamily, integrin β5 (ITGB5), and novel gene GBC-1 that contains a GSE-encoded helical repeat peptide and GBC-3, a gene from chromosome 3q29 with no detected homologies. How these novel genes regulate cell proliferation remains a subject for future studies.

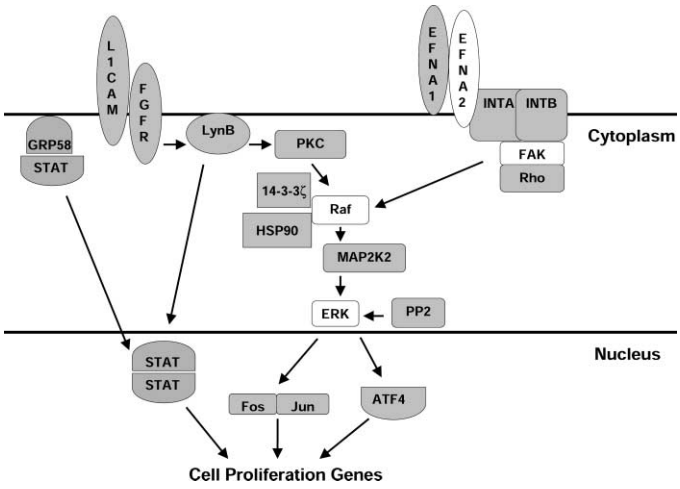

Figure 4. Interactions of some of the gene products selected in the present study in proliferation-associated signal transduction pathways

Several proteins that are not represented by the selected GSEs are shown as unshaded symbols. The relations between different proteins in Figure 4 are based on the following references: FGFR1-STAT (Hart et al., 2000; Smedley et al., 1999), GRP58-STAT (Sehgal et al., 2002), LynB-PKC (Dibirdik et al., 1998; Joseloff et al., 2002), HSP90-Raf (Schulte et al., 1995), 14-3-3ζ-Raf (Light et al., 2002), ERK-Fos (Wagstaff et al., 2000), Fos-Jun (Johnson et al., 2000), ERK-ATF4 (Hayes and McMahon, 2001), Mek-ERK (Seddighzadeh et al., 1999), INT-Rho (Jo et al., 2002), FAK-Rho (Hildebrand et al., 1996), EFNA-INT (Huai and Drescher, 2001), PP2-ERK (Kiguchi et al., 1992), LynB-STAT (Tilbrook et al., 2001), L1CAM-FGFR (Williams et al., 1994).

Cellular effects of L1CAM inhibition

One of the most highly enriched genes that has not been previously implicated in cell proliferation encodes neural cell adhesion molecule L1CAM involved in signal transduction (Figure 4). L1CAM is expressed primarily in the brain, but its expression has also been seen in some other normal tissues and in several types of cancer, including breast cancer (Perou et al., 2000). L1CAM expression in malignant melanoma was found to correlate with the metastatic phenotype (Thies et al., 2002), but to the best of our knowledge there is as yet no evidence that L1CAM plays any role in cell proliferation. Germline mutations in human L1CAM have been associated with neural system abnormalities, and similar neurological disorders have been reproduced in L1CAM null mice, which show apparently normal development in other respects (Kamiguchi et al., 1998). This limited biological function of L1CAM, combined with its frequent expression in cancers and easy accessibility on the cell surface, make this molecule an interesting potential target for cancer treatment. We have therefore investigated the effects of L1CAM inhibition in more detail.

We have used a cell line that carries an IPTG-inducible sense-oriented GSE from L1CAM (Figure 2) and undergoes growth inhibition upon GSE induction (Figures 3B and 3C) to determine if the GSE has an effect on L1CAM expression on the cell surface. Concurrently, we have asked the same question for a cell line carrying a GSE from another cell-surface protein, integrin β5 (ITGB5). FACS analysis using L1CAM- and ITGB5-specific antibodies (Figure 5A) demonstrated that induction of both GSEs by IPTG decreased the amounts of their cognate proteins on the cell surface but had no effect on the expression of the other protein. Interestingly, the GSE that inhibits ITGB5 expression is a sense-oriented fragment derived from the 5′ untranslated region of the corresponding cDNA (Figure 2); the mechanism of action of this novel type of GSE remains to be determined.

L1CAM GSE also induced pronounced changes in cell morphology, leading to the formation of filopodia and apparent focal adhesion plaques (Figure 5B). Another reproducible morphological effect of L1CAM GSE induction was the appearance of cells with fragmented or lobulated nuclei (Figure 5C). The fraction of such micronucleated (MN) cells reached 8.3% after 7 days of IPTG treatment, as compared to 3.3% observed in this cell line cultured for the same period of time in the absence of IPTG, or

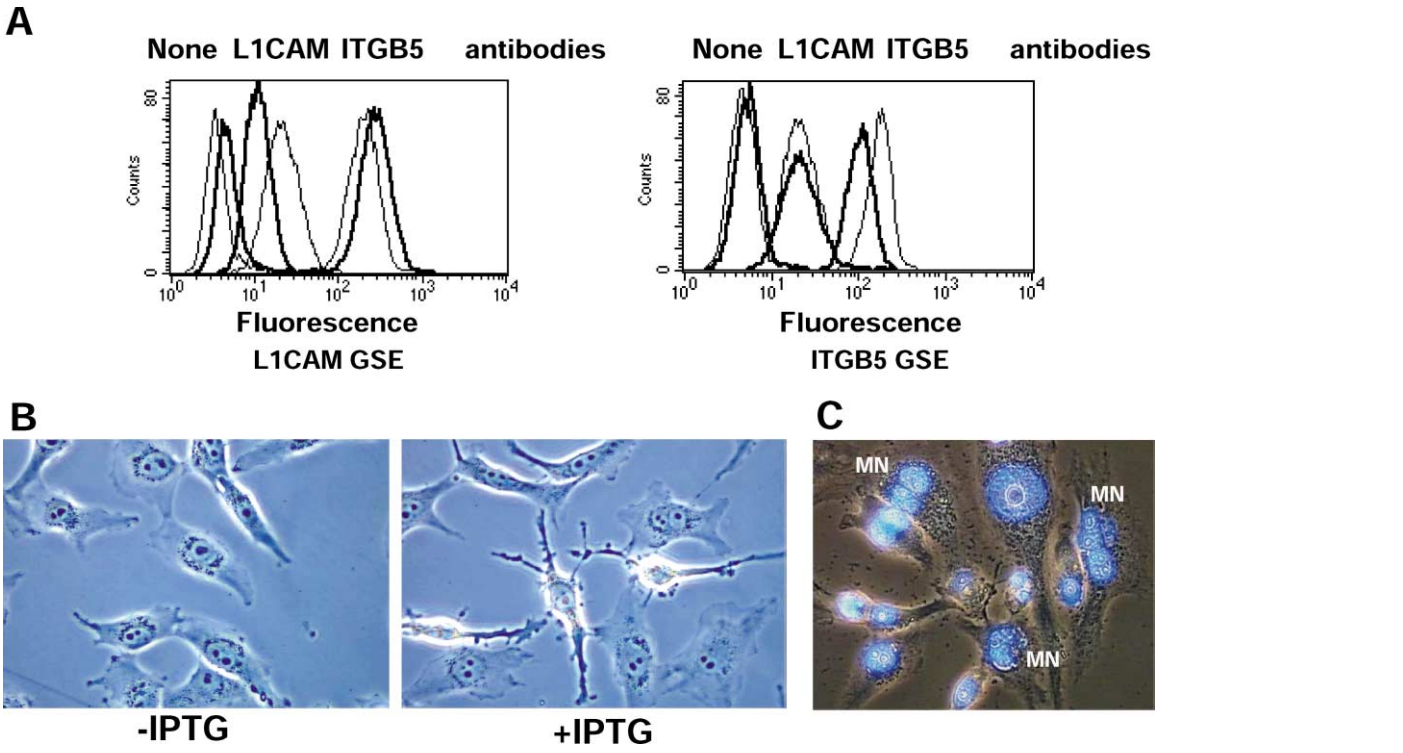

Figure 5. Cellular effects of a GSE from L1CAM

A: FACS analysis of the binding of L1CAM or ITGB5-specific antibodies to the surface of cells expressing the L1CAM GSE (left) or ITGB5 GSE (right) indicated in Figure 2, in the presence (thick lines) or absence (thin lines) of ITPG.

B: Morphological changes of cells expressing the L1CAM GSE in the absence (left) or presence (right) of ITPG, photographed using phase-contrast microscopy at 100× magnification.

C: Micronuclei (MN) in cells expressing L1CAM GSE, in the presence of IPTG, photographed after DAPI staining at 400× magnification.

1.8% in vector-transduced cells (in the presence or absence of IPTG). These changes in nuclear morphology are characteristic of mitotic catastrophe, a major form of tumor cell death, which is potentiated by various checkpoint deficiencies characteristic of tumor cells (Roninson et al., 2001). This finding suggested that L1CAM inhibition has not only cytostatic but also cytotoxic effect on breast carcinoma cells.

L1CAM-specific antibodies inhibit the growth of different tumor cell lines, but not normal cells

We have investigated the effects of two different monoclonal antibodies against the extracellular domain of L1CAM, UJ127, and 5G3 on the growth of MDA-MB231 and MCF-7 breast carcinoma cell lines, HeLa cervical carcinoma and HCT116 colon carcinoma lines, telomerase-immortalized hTERT-BJ1 normal human fibroblasts, and three cultures of normal human mammary epithelial cells (HMEC), 48RS, 184, and 161. All the tumor cell lines as well as the normal hTERT-BJ1, 48RS, and 184 cells expressed L1CAM on their surface as determined by FACS analysis, but 161 HMEC cells showed no detectable L1CAM (Figure 6A). In the experiment shown in Figure 6B, we have investigated the effects of UJ127 and 5G3 antibodies, native or heat-denatured, as well as their corresponding isotype controls, on the growth of all the cell lines for a period of four days, which corresponds to 1.9–2.5 population doublings (the doubling times for each cell line are indicated in the legend to Figure 6B). The addition of either UJ127 or 5G3 antibody to the culture media at 20 nM resulted in 3- to 6-fold decrease in the cell number of all four tumor cell lines relative to their corresponding isotype controls, but the monoclonal antibodies produced little or no growth inhibition in any of the four normal cell cultures. The growth-inhibitory activity of both monoclonal antibodies was abolished by heat denaturation (Figure 6B). Microscopic examination of tumor cells remaining on the plate after 4 days of incubation with anti-L1CAM monoclonal antibodies showed the appearance of micronucleated or apoptotic cells, indicative of the induction of cell death (Figure 7). These findings suggest that anti-L1CAM monoclonal antibodies or their derivatives may potentially be used as anticancer agents.

GSE-cognate genes as potential drug targets

The example of L1CAM illustrates the utility of GSE-cognate genes as potential targets for future chemotherapeutic drugs. In fact, some of these genes are already targeted by new drugs that are entering the clinical armamentarium. These include CCND1 and CDK2 targeted by small-molecule CDK inhibitors, and HSP90α, a member of HSP90 family of chaperone proteins (Csermely et al., 1998). While the chaperone function by itself does not imply a role in cell growth, HSP90 was found to stabilize several proteins involved in oncogenic pathways and to serve as the target of an antitumor antibiotic geldanamycin. Furthermore, HSP90α is expressed in several cancers (including breast) at higher levels than in nonmalignant tissues (Csermely et al., 1998). HSP90-inhibiting geldanamycin analog 17-AAG has been

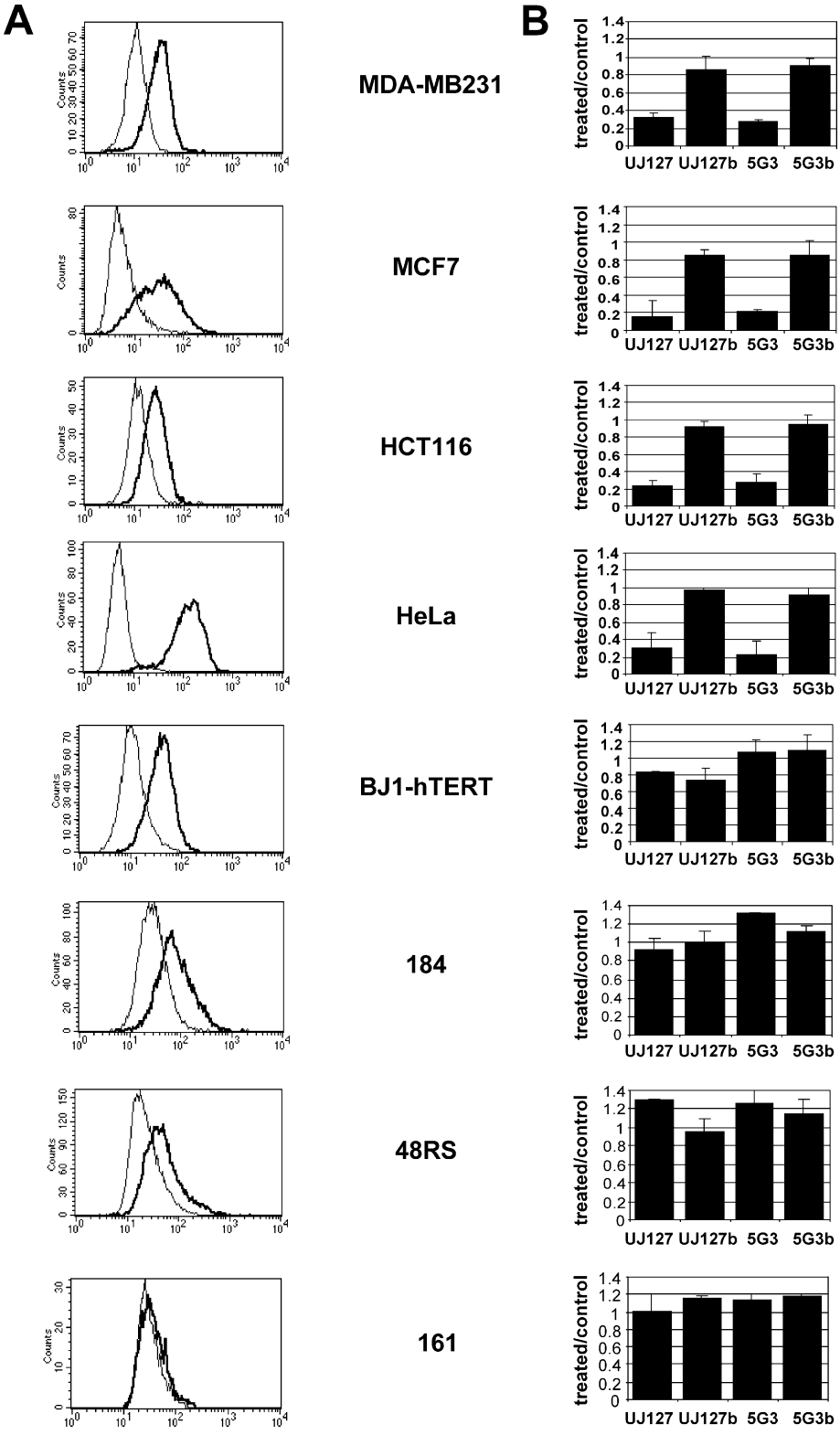

Figure 6. Effects of monoclonal antibodies against L1CAM on different types of tumor and normal cells

A: FACS analysis of the binding of L1CAM-specific UJ127 antibody to the surface of the indicated cell lines.

B: Effects of L1CAM-specific UJ127 (IgG1) and 5G3 (IgG2a) antibodies on the growth of MDA-MB231 and MCF7 breast carcinomas, HCT116 colon carcinoma, HeLa cervical carcinoma, hTERT-BJ1 normal immortalized fibroblasts, and 184, 161, and 48RS normal mammary epithelial cell cultures. Cells were grown in the presence of 20 nM of the antibodies or the corresponding isotype controls, native or boiled (b), and counted after 4 days (in triplicates). Each bar represents the mean and standard deviation for the number of cells in the presence of the indicated antibodies relative to the isotype controls. The doubling times for each cell line were as follows: MDA-MB231, 42 hrs; MCF7, 41 hrs; HCT116, 38 hrs; HeLa, 39 hrs; BJ1-hTERT, 50 hrs; 184, 41 hrs; 161, 42 hrs; 48RS, 51 hrs.

shown to arrest the growth of breast carcinoma cell lines including MDA-MB231 (Munster et al., 2001), and it is currently in clinical trial for breast cancer.

Many of the genes implicated in tumor cell growth are also likely to be essential for normal cells. The most promising potential targets for anticancer drugs would be those genes that are required for the growth of tumor but not normal cells. The apparent tumor specificity of growth inhibition by L1CAM antibodies suggests that L1CAM belongs in this category of targets. Indirect evidence from mouse knockout studies indicates that

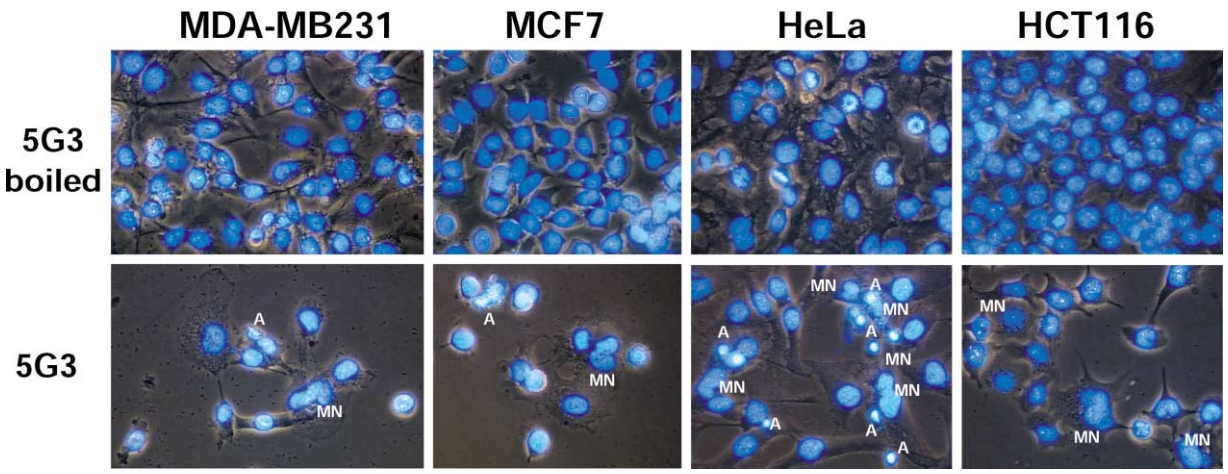

Figure 7. Induction of cell death by an antibody against L1CAM

Changes in nuclear morphology of tumor cells exposed for 4 days to 5G3 antibody, heat-inactivated (top) or native (bottom), photographed after DAPI staining at 400× magnification. MN: micronucleated cells, A: apoptotic cells.

many other genes identified in the present study may also be inessential for normal cells. The phenotypes of homozygous knockout mice have been described in the literature for 20 of the GSE-cognate genes (Table 2). Although GSE-mediated inhibition of these genes suppresses the growth of breast carcinoma cells, the knockout of only six genes (STAT3, FGFR1, IGF2R, ITGA4, JUNB, NR3C1) results in embryonic lethal phenotype, indicating that these genes are required for the growth or development of normal tissues. Mice null for L1CAM and 13 other genes (CCND1, C-FOS, LYN, PRKCD, PRKCZ, PRKCH, STAT5b, NFκB-1, ATF4, ITGB5, ICAM2, VWF, and ADPRT) are viable, with variable degrees of limited developmental abnormalities. Although the effects of germline knockout are not equivalent to those of protein inhibition in mature somatic cells, these results suggest nevertheless that inhibitors of the latter genes may not be growth-inhibitory for most of normal tissues. L1CAM and some of the other proteins with potentially tumor-specific functions are found on the cell surface (ICAM2, ITGB5) or secreted (VWF), making the development of drugs targeting these proteins relatively straightforward.

The nature of GSE selection used in the present study required that the GSEs induce reversible growth arrest rather than cell death. This does not mean, however, that more potent drugs targeting the GSE-cognate genes should have only cytostatic

Table 2. Phenotypes of Mice Null for GSE-Cognate Genes

Gene	Accession #	Mouse phenotype	References
ATF4	NM_001675.1	Viable, severe microthalmia	(Tanaka et al., 1998)
STAT5b	NM_012448.1	Viable, loss of dimorphic GH signaling and cytokine responses	(Udy et al., 1997)
VWF	NM_000552.2	Viable, circulatory and bleeding disorders	(Denis et al., 1998)
ITGB5	NM_002213.1	Viable, fertile, impaired cell adhesion	(Huang et al., 2000)
STAT3	NM_003150.1	Lethal during early embryogenesis	(Takeda et al., 1997)
L1CAM	NM_000425.2	Shorter lifespan than WT, malformed hindbrain	(Dahme et al., 1997); (Cohen et al., 1998)
C-FOS	NM_005252.2	Viable, fertile, lack osteoclasts, lymphopenia	(Johnson et al., 1992)
ADPRT	NM_001618	Viable, increased sensitivity to radiation	(Agarwal et al., 1997)
CCN D1	NM_001758.1	Viable, abnormal breast development	(Sicinski et al., 1995)
ICAM2	NM_000873	Viable, lung inflammation	(Sultan et al., 1997)
LYN	NM_002350	Viable, splenomegaly (myeloid cells)	(Chan et al., 1997)
FGFR1	NM_000604.2	Embryonic lethal, failure of mesoderm and endoderm formation	(Ciruna et al., 1997)
PKCζ	NM_002744	Viable, fertile, malformed lymph glands	(Leitges et al., 2001b)
NFκB-1	NM_003998.1	Viable, fertile, decreased response to ionizing radiation	(Sha et al., 1995)
NR3C1	NM_000176	Lethal perinatally	(Cole et al., 1995)
PKCΔ	NM_006254.1	Viable, fertile, failure of apoptosis in muscle cells, increased arteriosclerosis	(Leitges et al., 2001a)
ITGA4	NM_000885	Embryonic lethal	(Arroyo et al., 1996)
JunB	NM_002229.1	Embryonic lethal (E8.5–10)	(Schorpp-Kistner et al., 1999)
PKCη	NM_006255.1	Viable, fertile, attenuated immune responses	(Castrillo et al., 2001)
IGF2R	NM_000876	Lethal perinatally	(Ludwig et al., 1996)

rather than cytotoxic activity. In fact, most of the cancer chemotherapeutic drugs produce cytostatic inhibition at low doses and cell death at higher doses. Since most GSEs are relatively weak inhibitors, one can expect them to mimic the cytostatic effects of low drug doses. We have previously investigated the mechanism of the cytostatic/cytotoxic transition in the case of a human fibrosarcoma cell line with inducible expression of a cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1/Sdi1}$ (Chang et al., 2000). In that study, we observed that many cells that reenter cell cycle after p21-induced cytostatic arrest die as a result of mitotic catastrophe. p21-induced abnormal mitosis was associated with asynchronous resynthesis of different mitotic proteins, leading to a failure of mitotic spindle checkpoint control, which is deficient in many tumor cell lines (Chang et al., 2000). The finding that some of the GSEs isolated in the present study, including L1CAM, induce not only cytostatic inhibition but also mitotic catastrophe indicates the cytotoxic potential of drugs that will target the GSE-cognate genes.

In summary, we have identified numerous genes that give rise to GSEs that inhibit the growth of human breast carcinoma cells and verified the growth-inhibitory activity of GSEs from 57 of these genes by functional testing. The example of L1CAM demonstrates that the inhibition of some GSE-cognate genes produces not only cytostatic but also cytotoxic effect, and that non-GSE agents targeting such genes may have a tumor-specific growth inhibitory activity. Most of the identified genes were represented by several different GSE sequences; this redundancy suggests that we have selected GSEs from the majority of genes that are amenable to BrdU suicide selection in MDA-MB231 cells. The approach used in the present study is readily applicable to other types of cancer and can also be used with normal cell cultures (such as telomerase-immortalized cell lines). Extension of this analysis should allow us to generate an extensive database of genes that regulate the growth of different types of normal and tumor cells and to select potential new targets for the treatment of cancer.

Experimental procedures

Preparation of the normalized cDNA fragment library

PolyA+ RNA was fragmented by heating at 100°C for 9 min. Double-stranded cDNA was generated from this heat-fragmented RNA using the Gibco Superscript kit with the reverse-transcription primer 5′-GTGGATCCTCACTCACTC ANNNNNNNN-3′. This primer contains a random octamer sequence at its 3′ end for random priming, and it carries a unique sequence (termed "stop adaptor"), which provides TGA stop codons in all three open reading frames at the 3′ end of cDNA fragments, as well as BamHI restriction site. The resulting double-stranded cDNA fragments were ligated to the following adaptor:

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5′-GTACCTGAGTTATAGGATCCCTGCCATGCCATGCCATG-3′
                3′-CCTAGGGACGGTACGGTACGGTAC-5′
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This "start adaptor" contains ATG translation initiation codons in all three frames, together with a BamHI site. The adaptor-tagged, double-stranded cDNA was amplified by PCR with primers that anneal specifically to the start and stop adaptors. Normalization of the PCR-amplified double-stranded cDNA fragments was achieved by denaturing the cDNA fragments at 100°C for 10 min and reannealing for 96 hr at 68°C. Single-stranded cDNA fragments were isolated after reannealing by hydroxyapatite chromatography, converted to double-stranded fragments by PCR, and cloned into the Bgl II site of the LNXCO3 vector (Chang and Roninson, 1996). Normalization was established by Southern hybridization of PCR-amplified fragments and by colony hybridization of plasmid library, using probes corresponding to actin, c-*myc*, c-*fos*, and *mdr*1 mRNA species.

GSE selection and testing

MDA-MB231 cells were first modified by transduction with the murine ecotropic receptor (Albritton et al., 1989) in retroviral vector LXIHis (Levenson et al., 1998). At the next stage, cells were transfected with the 3′SS plasmid (Stratagene) that carries the LacI repressor and the hygromycin resistance marker and subcloned. 33 clonal cell lines were isolated and tested for IPTG-regulated expression of a LacI-inhibited promoter. One of the tested cell lines, called MDA-MB231-3′SS31, showed about 80% infectability with ecotropic retroviruses and 10-fold inducibility by IPTG. This line was chosen as the recipient for GSE selection.

The normalized cDNA fragment library was converted into a mixture of recombinant ecotropic retroviruses by transfection of BOSC-23 packaging cells, used to infect 2×10^8 MDA-MB231-3′SS31 as described (Gudkov and Roninson, 1997), and the infectants were selected with G418. For BrdU selection, 8×10^7 infected cells were plated at 10^6 per P150 and treated with 50 μM IPTG for 36 hr, then with 50 μM IPTG and 50 mM BrdU for 48 hr. Cells were then incubated with 10 μM Hoechst 33342 for 3 hr and illuminated with fluorescent white light for 15 min. Cells were washed twice with phosphate-buffered saline and allowed to recover in G418-containing medium without IPTG or BrdU, for 7–10 days. The surviving cells were pooled, replated, and subjected to a second step of identical BrdU selection.

The inserts of integrated proviruses were recovered by PCR from the genomic DNA of the selected cells and recloned into the pLNXCO3 vector essentially as described in Gudkov and Roninson (1997); the specific primers and procedures will be provided upon request. Sequence analysis of the recovered inserts was carried out using an Applied Biosystems 3700 DNA Analyzer (Foster City, CA). The analysis and attribution of the deduced sequences were carried out using BLAST alignment tool and BLASTMask program (which will be made available at our website at http://www2.uic.edu/~amaliy1/blastmask). Subsequent alignment and exclusion (masking) was carried out for *Escherichia coli* genome sequences, the NCBI UniVec vector segment database, the human Alu sequence database, and a set of rRNA and miscellaneous non-mRNA sequences. The partially masked dataset was then BLASTed against the RefSeq mammalian mRNA sequences. Sequences that contained unidentified stretches of at least 50 bp were then selected by BLASTMask and sequentially BLASTed against NCBI's NR, EST, and HTGS databases. The BLAST hit data from databases containing human and mammalian sequences were combined and used by BLASTMask to group related GSE sequences with their gene attributions.

For testing the GSE function by BrdU suicide survival assays, GSE-transduced cell populations (200,000 per P100, in triplicate) were treated with 50 μM IPTG for 72 hr, then with 50 μM IPTG and 50 μM BrdU for 48 hr. A parallel set of cells was treated in the same way but without IPTG. Cells were then illuminated with white light in the presence of Hoechst 33342 and allowed to recover in the absence of BrdU and IPTG for 7–10 days, prior to scoring the surviving colonies. For testing the GSE function in clonal lines of GSE-transduced cells by IPTG growth inhibition assay, such lines were plated at 50,000 cells per P100 (in triplicates) and allowed to grow for 7 days in the presence or absence of 500 μM IPTG. Cell numbers were determined using a Coulter counter. To monitor the induction of GSE RNA following addition of IPTG, RNA was extracted and analyzed by semiquantitative reverse transcription-PCR (Noonan et al., 1990), using primers corresponding to vector sequences flanking the GSE insert; β-actin was used as a normalization control.

To determine the morphological effects of a GSE, IPTG-treated and untreated cells were fixed in P100 plates with methanol/acetic acid (3:1), stained with DAPI (5 mg/ml), and examined for blue fluorescence and under phase contrast, using a Leica inverted fluorescence microscope. The fraction of micronucleated cells was determined under phase contrast, by counting 500–1000 cells per sample.

Antibody assays

MDA-MB-231, MCF-7, and HeLa cells were purchased from the ATCC. HTERT-BJ1 cell line was obtained from Clontech (Palo Alto, CA). Wild-type HCT116 cell line was a gift from Bert Vogelstein (Johns Hopkins Medical Institutions, Baltimore, MD). Normal human mammary epithelial cell (HMEC) cultures 184, 161 (both passage 7), and 48RS (passage 9), isolated from reduction mammoplasty, were provided by Dr. Martha Stampfer (Lawrence Berkeley National Labs, Berkeley, CA). HMECs were grown in MEGM, a serum-free medium containing human epidermal growth factor (10 ng/ml),

insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), and bovine pituitary extract (Cambrex BioScience, Walkersville, MD). The other cell lines were grown in DMEM with 10% fetal bovine serum. Monoclonal antibody to L1CAM from hybridoma 5G3 (IgG2a) was purchased from BD PharMingen (San Diego), and monoclonal antibody to L1CAM from hybridoma UJ127 (IgG1), as well as nonimmune IgG1 and IgG2a, were purchased from NeoMarkers (Fremont, CA). Rabbit polyclonal antibody against ITGB5 was from Chemicon (Temecula, CA). The corresponding secondary antibodies were labeled with fluorescein isothiocyanate (Santa Cruz, CA). FACS analysis was carried out using Becton Dickinson FACSort.

For cell culture assays, azide-free monoclonal antibodies or isotype controls were added to the media at the final concentration of 20 nM and sterilized with 0.22 micron polysulfone filters. As an additional control, monoclonal antibodies were denatured by heating at 95°C for 10 min in 0.1 ml PBS. To determine the effect of anti-L1CAM monoclonal antibodies on cell growth and morphology, 10,000–20,000 cells were plated into each well of 24-well plates, and incubated at 37°C for 4 days in the presence or absence of the corresponding antibody, with change of media after 2 days. Cell growth was measured by counting the cell number (in triplicate) using a Coulter counter. For nuclear morphology analysis, cells were fixed with methanol/acetic acid (5:1), stained with DAPI (5 μg/ml in PBS), and examined for blue fluorescence and under phase contrast, using a Leica inverted fluorescence microscope.

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References

Agarwal, M.L., Agarwal, A., Taylor, W.R., Wang, Z.Q., Wagner, E.F., and Stark, G.R. (1997). Defective induction but normal activation and function of p53 in mouse cells lacking poly-ADP-ribose polymerase. Oncogene *15*, 1035–1041.

Albritton, L.M., Tseng, L., Scadden, D., and Cunningham, J.M. (1989). A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell *57*, 659–666.

Arroyo, A.G., Yang, J.T., Rayburn, H., and Hynes, R.O. (1996). Differential requirements for α4 integrins during fetal and adult hematopoiesis. Cell *85*, 997–1008.

Castrillo, A., Pennington, D.J., Otto, F., Parker, P.J., Owen, M.J., and Bosca, L. (2001). Protein kinase C epsilon is required for macrophage activation and defense against bacterial infection. J. Exp. Med. *194*, 1231–1242.

Chan, V.W., Meng, F., Soriano, P., DeFranco, A.L., and Lowell, C.A. (1997). Characterization of the B lymphocyte populations in Lyn-deficient mice and the role of Lyn in signal initiation and down-regulation. Immunity *7*, 69–81.

Chang, B.D., and Roninson, I.B. (1996). Inducible retroviral vectors regulated by lac repressor in mammalian cells. Gene *183*, 137–142.

Chang, B.D., Broude, E.V., Fang, J., Kalinichenko, T.V., Abdryashitov, R., Poole, J.C., and Roninson, I.B. (2000). $p21^{Waf1/Cip1/Sdi1}$-induced growth arrest is associated with depletion of mitosis-control proteins and leads to abnormal mitosis and endoreduplication in recovering cells. Oncogene *19*, 2165–2170.

Ciruna, B.G., Schwartz, L., Harpal, K., Yamaguchi, T.P., and Rossant, J. (1997). Chimeric analysis of fibroblast growth factor receptor-1 (Fgfr1) function: a role for FGFR1 in morphogenetic movement through the primitive streak. Development *124*, 2829–2841.

Cohen, N.R., Taylor, J.S., Scott, L.B., Guillery, R.W., Soriano, P., and Furley, A.J. (1998). Errors in corticospinal axon guidance in mice lacking the neural cell adhesion molecule L1. Curr. Biol. *8*, 26–33.

Cole, T.J., Blendy, J.A., Monaghan, A.P., Krieglstein, K., Schmid, W., Aguzzi, A., Fantuzzi, G., Hummler, E., Unsicker, K., and Schutz, G. (1995). Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev. *9*, 1608–1621.

Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z., and Nardai, G. (1998). The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacol. Ther. *79*, 129–168.

Dahme, M., Bartsch, U., Martini, R., Anliker, B., Schachner, M., and Mantei, N. (1997). Disruption of the mouse L1 gene leads to malformations of the nervous system. Nat. Genet. *17*, 346–349.

Denis, C., Methia, N., Frenette, P.S., Rayburn, H., Ullman-Cullere, M., Hynes, R.O., and Wagner, D.D. (1998). A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc. Natl. Acad. Sci. USA *95*, 9524–9529.

Dibirdik, I., Kristupaitis, D., Kurosaki, T., Tuel-Ahlgren, L., Chu, A., Pond, D., Tuong, D., Luben, R., and Uckun, F.M. (1998). Stimulation of Src family protein-tyrosine kinases as a proximal and mandatory step for SYK kinase-dependent phospholipase Cγ2 activation in lymphoma B cells exposed to low energy electromagnetic fields. J. Biol. Chem. *273*, 4035–4039.

Dunn, S.J., Park, S.W., Sharma, V., Raghu, G., Simone, J.M., Tavassoli, R., Young, L.M., Ortega, M.A., Pan, C.H., Alegre, G.J., et al. (1999). Isolation of efficient antivirals: genetic suppressor elements against HIV-1. Gene Ther. *6*, 130–137.

Garcia, R., Yu, C.L., Hudnall, A., Catlett, R., Nelson, K.L., Smithgall, T., Fujita, D.J., Ethier, S.P., and Jove, R. (1997). Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. Cell Growth Differ. *8*, 1267–1276.

Garkavtsev, I., Kazarov, A., Gudkov, A., and Riabowol, K. (1996). Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. Nat. Genet. *14*, 415–420.

Gudkov, A., and Roninson, I.B. (1997). Isolation of genetic suppressor elements (GSEs) from random fragment cDNA libraries in retroviral vectors. In Methods in Molecular Biology: cDNA library protocols, I.G. Cowell and C.A. Austin, eds. (Totowa, N.J.: Humana Press), pp. 221–240.

Gudkov, A.V., Kazarov, A.R., Thimmapaya, R., Axenovich, S.A., Mazo, I.A., and Roninson, I.B. (1994). Cloning mammalian genes by expression selection of genetic suppressor elements: association of kinesin with drug resistance and cell immortalization. Proc. Natl. Acad. Sci. USA *91*, 3744–3748.

Gudkov, A., Roninson, I.B., and Brown, R. (1999). Functional approaches to gene isolation in mammalian cells. Science *285*, 1733–1737.

Hai, T., Wolfgang, C.D., Marsee, D.K., Allen, A.E., and Sivaprasad, U. (1999). ATF3 and stress responses. Gene Expr. *7*, 321–335.

Hart, K.C., Robertson, S.C., Kanemitsu, M.Y., Meyer, A.N., Tynan, J.A., and Donoghue, D.J. (2000). Transformation and Stat activation by derivatives of FGFR1, FGFR3, and FGFR4. Oncogene *19*, 3309–3320.

Hayes, J.D., and McMahon, M. (2001). Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention. Cancer Lett. *174*, 103–113.

Hildebrand, J.D., Taylor, J.M., and Parsons, J.T. (1996). An SH3 domain-containing GTPase-activating protein for Rho and Cdc42 associates with focal adhesion kinase. Mol. Cell. Biol. *16*, 3169–3178.

Holzmayer, T.A., Pestov, D.G., and Roninson, I.B. (1992). Isolation of dominant negative mutants and inhibitory antisense RNA sequences by expression selection of random DNA fragments. Nucleic Acids Res. *20*, 711–717.

Huai, J., and Drescher, U. (2001). An ephrin-A-dependent signaling pathway controls integrin function and is linked to the tyrosine phosphorylation of a 120-kDa protein. J. Biol. Chem. *276*, 6689–6694.

Huang, X., Griffiths, M., Wu, J., Farese, R.V., Jr., and Sheppard, D. (2000). Normal development, wound healing, and adenovirus susceptibility in β5-deficient mice. Mol. Cell. Biol. *20*, 755–759.

Ivanov, V.N., Krasilnikov, M., and Ronai, Z. (2002). Regulation of Fas expression by STAT3 and c-Jun is mediated by phosphatidylinositol 3-kinase-AKT signaling. J. Biol. Chem. *277*, 4932–4944.

Jo, M., Thomas, K.S., Somlyo, A.V., Somlyo, A.P., and Gonias, S.L. (2002). Cooperativity between the Ras-ERK and Rho-Rho kinase pathways in urokinase-type plasminogen activator-stimulated cell migration. J. Biol. Chem. *277*, 12479–12485.

Johnson, R.S., Spiegelman, B.M., and Papaioannou, V. (1992). Pleiotropic effects of a null mutation in the c-fos proto-oncogene. Cell *71*, 577–586.

Johnson, A.C., Murphy, B.A., Matelis, C.M., Rubinstein, Y., Piebenga, E.C., Akers, L.M., Neta, G., Vinson, C., and Birrer, M. (2000). Activator protein-1 mediates induced but not basal epidermal growth factor receptor gene expression. Mol. Med. *6*, 17–27.

Joseloff, E., Cataisson, C., Aamodt, H., Ocheni, H., Blumberg, P., Kraker, A.J., and Yuspa, S.H. (2002). Src family kinases phosphorylate protein kinase Cδ on tyrosine residues and modify the neoplastic phenotype of skin keratinocytes. J. Biol. Chem. *277*, 12318–12323.

Kamiguchi, H., Hlavin, M.L., and Lemmon, V. (1998). Role of L1 in neural development: what the knockouts tell us. Mol. Cell. Neurosci. *12*, 48–55.

Kiguchi, K., Giometti, C., Chubb, C.H., Fujiki, H., and Huberman, E. (1992). Differentiation induction in human breast tumor cells by okadaic acid and related inhibitors of protein phosphatases 1 and 2A. Biochem. Biophys. Res. Commun. *189*, 1261–1267.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., et al. (2001). Initial sequencing and analysis of the human genome. Nature *409*, 860–921.

Leitges, M., Mayr, M., Braun, U., Mayr, U., Li, C., Pfister, G., Ghaffari-Tabrizi, N., Baier, G., Hu, Y., and Xu, Q. (2001a). Exacerbated vein graft arteriosclerosis in protein kinase Cδ-null mice. J. Clin. Invest. *108*, 1505–1512.

Leitges, M., Sanz, L., Martin, P., Duran, A., Braun, U., Garcia, J.F., Camacho, F., Diaz-Meco, M.T., Rennert, P.D., and Moscat, J. (2001b). Targeted disruption of the ζPKC gene results in the impairment of the NF-κB pathway. Mol. Cell *8*, 771–780.

Levenson, V.V., Transue, E.D., and Roninson, I.B. (1998). Internal ribosomal entry site-containing retroviral vectors with green fluorescent protein and drug resistance markers. Hum. Gene Ther. *9*, 1233–1236.

Levenson, V.V., Lausch, E., Kirschling, D.J., Broude, E.V., Davidovich, I.A., Libants, S., Fedosova, V., and Roninson, I.B. (1999). A combination of genetic suppressor elements produces resistance to drugs inhibiting DNA replication. Somat. Cell Mol. Genet. *25*, 9–26.

Light, Y., Paterson, H., and Marais, R. (2002). 14–3-3 antagonizes Ras-mediated Raf-1 recruitment to the plasma membrane to maintain signaling fidelity. Mol. Cell. Biol. *22*, 4984–4996.

Liu, P., Kimmoun, E., Legrand, A., Sauvanet, A., Degott, C., Lardeux, B., and Bernuau, D. (2002). Activation of NF-κB, AP-1 and STAT transcription factors is a frequent and early event in human hepatocellular carcinomas. J. Hepatol. *37*, 63–71.

Ludwig, T., Eggenschwiler, J., Fisher, P., D'Ercole, A.J., Davenport, M.L., and Efstratiadis, A. (1996). Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. Dev. Biol. *177*, 517–535.

Masuoka, H.C., and Townes, T.M. (2002). Targeted disruption of the activating transcription factor 4 gene results in severe fetal anemia in mice. Blood *99*, 736–745.

Munster, P.N., Srethapakdi, M., Moasser, M.M., and Rosen, N. (2001). Inhibition of heat shock protein 90 function by ansamycins causes the morphological and functional differentiation of breast cancer cells. Cancer Res. *61*, 2945–2952.

Noonan, K.E., Beck, C., Holzmayer, T.A., Chin, J.E., Wunder, J.S., Andrulis, I.L., Gazdar, A.F., Willman, C.L., Griffith, B., Von Hoff, D.D., and Roninson, I.B. (1990). Quantitative analysis of *MDR*1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. Proc. Natl. Acad. Sci. USA *87*, 7160–7164.

Novoa, I., Zeng, H., Harding, H.P., and Ron, D. (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2α. J. Cell Biol. *153*, 1011–1022.

Patanjali, S.R., Parimoo, S., and Weissman, S.M. (1991). Construction of a uniform-abundance (normalized) cDNA library. Proc. Natl. Acad. Sci. USA *88*, 1943–1947.

Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A., et al. (2000). Molecular portraits of human breast tumours. Nature *406*, 747–752.

Pestov, D.G., and Lau, L.F. (1994). Genetic selection of growth-inhibitory sequences in mammalian cells. Proc. Natl. Acad. Sci. USA *91*, 12549–12553.

Pestov, D.G., Grzeszkiewicz, T.M., and Lau, L.F. (1998). Isolation of growth suppressors from a cDNA expression library. Oncogene *17*, 3187–3197.

Roninson, I.B., Broude, E.V., and Chang, B.D. (2001). If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. Drug Resistance Updates *4*, 303–313.

Roninson, I.B., Gudkov, A.V., Holzmayer, T.A., Kirschling, D.J., Kazarov, A.R., Zelnick, C.R., Mazo, I.A., Axenovich, S., and Thimmapaya, R. (1995). Genetic suppressor elements: new tools for molecular oncology–thirteenth Cornelius P. Rhoads Memorial Award Lecture. Cancer Res. *55*, 4023–4028.

Schorpp-Kistner, M., Wang, Z.Q., Angel, P., and Wagner, E.F. (1999). JunB is essential for mammalian placentation. EMBO J. *18*, 934–948.

Schulte, T.W., Blagosklonny, M.V., Ingui, C., and Neckers, L. (1995). Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. J. Biol. Chem. *270*, 24585–24588.

Seddighzadeh, M., Zhou, J.N., Kronenwett, U., Shoshan, M.C., Auer, G., Sten-Linder, M., Wiman, B., and Linder, S. (1999). ERK signalling in metastatic human MDA-MB-231 breast carcinoma cells is adapted to obtain high urokinase expression and rapid cell proliferation. Clin. Exp. Metastasis *17*, 649–654.

Sehgal, P.B., Guo, G.G., Shah, M., Kumar, V., and Patel, K. (2002). Cytokine signaling: STATS in plasma membrane rafts. J. Biol. Chem. *277*, 12067–12074.

Sha, W.C., Liou, H.C., Tuomanen, E.I., and Baltimore, D. (1995). Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell *80*, 321–330.

Sicinski, P., Donaher, J.L., Parker, S.B., Li, T., Fazeli, A., Gardner, H., Haslam, S.Z., Bronson, R.T., Elledge, S.J., and Weinberg, R.A. (1995). Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell *82*, 621–630.

Smedley, D., Demiroglu, A., Abdul-Rauf, M., Heath, C., Cooper, C., Shipley, J., and Cross, N.C. (1999). ANF198-FGFR1 transforms Ba/F3 cells to growth factor independence and results in high level tyrosine phosphorylation of STATS 1 and 5. Neoplasia *1*, 349–355.

Stetten, G., Davidson, R.L., and Latt, S.A. (1977). 33258 Hoechst enhances the selectivity of the bromodeoxyuridine–light method of isolating conditional lethal mutants. Exp. Cell Res. *108*, 447–452.

Sultan, A.A., Briones, M.R., Gerwin, N., Carroll, M.C., and Nussenzweig, V. (1997). Sporozoites of Plasmodium yoelii infect mice with targeted deletions in ICAM-1 and ICAM-2 or complement components C3 and C4. Mol. Biochem. Parasitol. *88*, 263–266.

Takeda, K., Noguchi, K., Shi, W., Tanaka, T., Matsumoto, M., Yoshida, N., Kishimoto, T., and Akira, S. (1997). Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. Proc. Natl. Acad. Sci. USA *94*, 3801–3804.

Talukder, A.H., Vadlamudi, R., Mandal, M., and Kumar, R. (2000). Heregulin induces expression, DNA binding activity, and transactivating functions of basic leucine zipper activating transcription factor 4. Cancer Res. *60*, 276–281.

Tanaka, T., Tsujimura, T., Takeda, K., Sugihara, A., Maekawa, A., Terada, N., Yoshida, N., and Akira, S. (1998). Targeted disruption of ATF4 discloses its essential role in the formation of eye lens fibres. Genes Cells *3*, 801–810.

Thies, A., Schachner, M., Moll, I., Berger, J., Schulze, H.J., Brunner, G., and Schumacher, U. (2002). Overexpression of the cell adhesion molecule L1 is associated with metastasis in cutaneous malignant melanoma. Eur. J. Cancer *38*, 1708–1716.

Tilbrook, P.A., Palmer, G.A., Bittorf, T., McCarthy, D.J., Wright, M.J., Sarna, M.K., Linnekin, D., Cull, V.S., Williams, J.H., Ingley, E., et al. (2001). Maturation of erythroid cells and erythroleukemia development are affected by the kinase activity of Lyn. Cancer Res. *61*, 2453–2458.

Udy, G.B., Towers, R.P., Snell, R.G., Wilkins, R.J., Park, S.H., Ram, P.A., Waxman, D.J., and Davey, H.W. (1997). Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc. Natl. Acad. Sci. USA *94*, 7239–7244.

Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandell, M., Evans, C.A., Holt, R.A., et al. (2001). The sequence of the human genome. Science *291*, 1304–1351.

Wagstaff, S.C., Bowler, W.B., Gallagher, J.A., and Hipskind, R.A. (2000). Extracellular ATP activates multiple signalling pathways and potentiates growth factor-induced c-fos gene expression in MCF-7 breast cancer cells. Carcinogenesis *21*, 2175–2181.

Williams, E.J., Furness, J., Walsh, F.S., and Doherty, P. (1994). Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. Neuron *13*, 583–594.