Evidence That Myc Isoforms Transcriptionally Repress Caveolin-1 Gene Expression via an INR-Dependent Mechanism†

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ABSTRACT: The c-Myc oncoprotein contributes to oncogenesis by activating and repressing a repertoire of genes involved in cellular proliferation, metabolism, and apoptosis. Increasing evidence suggests that the repressor function of c-Myc is critical for transformation. Therefore, identifying and characterizing Myc-repressed genes is imperative to understanding the mechanisms of Myc-induced tumorigenesis. Here, we employ NIH 3T3 cell lines harboring c-Myc-ER or N-Myc-ER to dissect the relationship between Myc activation and caveolin-1 expression. In this well-established inducible system, treatment with estrogen like molecules, such as tamoxifen, leads to activation of Myc, but in a tightly controlled fashion. Using this approach, we show that Myc activation induces the repression of caveolin-1 expression at the transcriptional level. We also provide two independent lines of evidence suggesting that caveolin-1 is a direct target of Myc: (i) the effect of Myc activation on caveolin-1 expression is independent of new protein synthesis, as revealed through the use of cycloheximide; and (ii) Myc-mediated repression of the caveolin-1 promoter is dependent on an intact INR sequence. Moreover, we show that expression of caveolin-1, via an adenoviral vector approach, can suppress cell transformation that is mediated by Myc activation. In support of these observations, treatment with an adenoviral vector harboring anti-sense caveolin-1 specifically potentiates transformation induced by Myc activation. Taken together, our results indicate that caveolin-1 is a direct target of Myc repression, and they also provide evidence for an additional mechanism by which Myc repression can elicit a malignant phenotype.

The Myc oncogene, first characterized in the avian myelocytomatosis virus, is overexpressed in a variety of human malignancies. This nuclear phosphoprotein plays an active role in directing cellular proliferation, differentiation, and apoptosis (*1*−*3*). Overexpression studies of c-Myc have demonstrated a shortening of the G1 phase of the cell cycle and an inhibition of differentiation leading to a transformed phenotype (*4*, *5*). It is believed that the ability of Myc to transactivate a repertoire of growth-stimulatory genes is the main mechanism for its ability to mediate cellular transformation. Classically, Myc heterodimerizes with Max and subsequently activates gene expression through E-box Myc-binding sites (EMS).

However, Myc also has trans-repressor capacity, and increasing evidence indicates that this function largely contributes to its role in oncogenesis. An expanding array of Myc-repressed genes that regulate the cell cycle, differentiation, and DNA damage is currently emerging. Although the mechanisms of Myc repression are not fully understood, Myc is known to bind and repress at initiator elements (INR),[1] i.e., pyrimidine-rich sequences (Y) with the loose consensus sequence, YYCAYYYYY (*6*). The INR is found in many TATA-less promoters and helps recruit factors for transcription initiation (*7*−*9*). Myc binding to the INR sites of specific genes depends on both the INR sequence and the myc heterodimerization partner (*10*−*12*).

Mutational analysis of the Myc protein has reinforced that the transrepression domain is critical for tumorigenesis. Myc mutants (i.e., Δ106−143) that cannot transrepress, but retain their ability to transactivate, are unable to elicit transformation (*9*). In addition, the Burkitt lymphoma-derived MycB2

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[1] Abbreviations: Myc-ER, Myc-estrogen receptor; INR, initiator element; OHT, 4-hydroxytamoxifen; TRD, transregulatory domain; HLH, helix−loop−helix.

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mutant, which retains normal transactivation function but increased repression capacity, has more potent transformation ability (*13*). Other mutants have further confirmed the direct correlation between the ability of Myc to repress and its transformation capacity.

Caveolin-1 (Cav-1), a principal component of cholesterol-rich caveolae membranes, has been proposed to function as a scaffolding protein to concentrate specific lipids as well as lipid-modified signaling molecules (Src-like kinases, H-Ras, eNOS, and G-proteins) (*14*−*18*). Caveolin-1 binding can functionally suppress the GTPase activity of heterotrimeric G-proteins and inhibit EGF-R, Neu, PKC, and Src-family kinases through the caveolin-scaffolding domain (CSD) (*19*−*22*). Caveolin-1 has been shown to be lost or reduced during cellular transformation by activated oncogenes (*23, 24*). We have previously demonstrated that down-regulation of caveolin-1 protein expression in NIH 3T3 cells using an anti-sense approach elicited anchorage-independent growth in soft agar and tumor formation in immunodeficient mice. Loss of caveolin-1 expression was also marked by hyperactivation of the p42/44 MAP kinase pathway (*25*). In addition, the human CAV-1 gene is localized to a suspected tumor suppressor locus (7q31.1) that is deleted in many forms of human cancer (*26, 27*).

We have previously reported that caveolin-1 expression is dramatically down-regulated in mammary tumors derived from MMTV-c-Myc transgenic mice (*22*). However, the mechanism by which c-Myc expression induces the down-regulation of caveolin-1 protein expression remains unknown. This initial finding prompted us to directly investigate the relationship between Myc and caveolin-1.

Here, we employ NIH 3T3 cell lines harboring c-Myc-ER or N-Myc-ER to dissect the relationship between Myc activation and caveolin-1 expression. In this well-established system, treatment with estrogen like molecules, such as tamoxifen, leads to activation of Myc, but in a tightly controlled fashion. Taken together, our results indicate that caveolin-1 is a direct target of Myc-repression, and they also provide evidence for an additional mechanism by which Myc repression can elicit a malignant phenotype.

MATERIALS AND METHODS

Materials. The caveolin-1 mouse mAb 2297 and caveolin-2 mouse mAb 65 [used for immunoblotting (*28, 29*)] were the gifts of Dr. Roberto Campos-Gonzalez, BD Transduction Laboratories, Inc. NIH 3T3 cell lines stably expressing c-Myc-ER, N-Myc-ER, and pBABE empty vector were as described previously (*30*). A variety of other reagents were purchased commercially as follows: cell culture reagents were from Life Technologies, Inc.; cycloheximide, puromycin, and 4-hydroxytamoxifen were purchased from Sigma.

Cell Culture. Myc-ER cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/mL penicillin, 100 mg/mL streptomycin (Life Technologies, Inc.), and 5 mg/mL puromycin at 37 °C and 5% CO_2. Prior to 4-hydroxytamoxifen (OHT; 250 nM) treatment, Myc-ER cell lines were incubated in phenol red free DMEM, 0.2% charcoal dextran-stripped FBS (PRF-DMEM/CD-FBS) for 24 h to prevent any basal activation of Myc-ER. NIH 3T3 cells were cultured similarly using medium containing 10% donor calf serum (JRH Biosciences).

Expression Vectors. The cDNAs encoding wild-type c-Myc, c-MycΔhelix−loop−helix (c-MycΔHLH), and c-MycΔtransregulatory domain (c-MycΔTRD) were generous gifts from Dr. Nissim Hay, University of Illinois at Chicago (*31*). The pSV-β-gal plasmid is an SV40-driven vector expressing β-galactosidase purchased from Promega.

Immunoblot Analysis. Cells were cultured in their respective media and allowed to reach ∼80−90% confluency. The cells were incubated in PRF DMEM/CD-FBS for 24 h and then treated with 4-hydroxytamoxifen (OHT) at 250 nM for varying amounts of time. Subsequently, the cells were washed with PBS and incubated with lysis buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1% Triton X-100, 60 mM octyl glucoside) containing protease inhibitors (Boehringer Mannheim). Protein concentrations were quantified using the BCA reagent (Pierce), and the volume required for 10 μg of protein was determined. Samples were separated by SDS−PAGE (12.5% acrylamide) and transferred to nitrocellulose. The nitrocellulose membranes were stained with Ponceau S (to visualize protein bands) followed by immunoblot analysis. All subsequent wash buffers contained 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20, which was supplemented with 1% bovine serum albumin (BSA) and 2% nonfat dry milk (Carnation) for the blocking solution and with 1% BSA for the antibody diluent. Primary antibodies were used at a 1:500 dilution. Horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution, Pierce) were used to visualize bound primary antibodies with the Supersignal chemiluminescence substrate (Pierce).

Northern Blot Analysis. Cells were cultured in 10 cm dishes and allowed to reach ∼80−90% confluency. The cells were then treated as described above for immunoblot analysis. Subsequently, the cells were washed in PBS, and total RNA was extracted using the QIA shredder and RNeasy Mini Kits (Qiagen). Twenty micrograms of total RNA for each sample was separated using a 1.2% agarose gel under RNase-free conditions and transferred to nylon membranes. The filters were hybridized using the ExpressHyb solution (Clontech). Blots were probed with the caveolin-1 cDNA.

Inhibition of Protein Synthesis with Cycloheximide. c-Myc-ER NIH 3T3 cells were cultured in DMEM, 10% FBS media and allowed to reach ∼80−90% confluency. The cells were incubated in PRF-DMEM/CD-FBS for 24 h. Prior to adding OHT (250 nM), the cells were treated with 10 μM cycloheximide for 30 min in order to completely inhibit protein synthesis. We have previously shown that this concentration of cycloheximide fully abrogates protein synthesis in these cell lines (*30*). After cycloheximide treatment, OHT was added at 0, 2, 4, and 8 h. Cells were treated with cycloheximide alone, OHT alone, or in combination. The cells in each time point were then lysed and subjected to Northern blot analysis.

In Vivo Reporter Assay for Caveolin-1 Gene Expression. A 13 kb DNA segment containing the caveolin-1 exons 1 and 2 was identified by screening a mouse genomic DNA library as previously described (*32*). A portion of caveolin-1 exon-1 in addition to intron-1 and the 3 kb upstream promoter was derived from this segment and subcloned into the vector pA3Luc, a promoter-less vector containing the luciferase cDNA as a reporter (*33, 34*). In this way, the effect of various signal transduction pathways on the regulation of caveolin-1 at the transcriptional level can be assessed. Transient

transfections were performed using the calcium phosphate precipitation method. Briefly, 150 000 NIH 3T3 were seeded in 6-well plates ~12−24 h prior to transfection. Each cell well was then transfected with 0.5 μg of either the c-Myc wild-type, c-MycΔbHLH/LZ, c-MycΔTRD, or empty vector, 1.0 μg of the luciferase reporter containing the caveolin-1 promoter, and 0.2 μg of pSV-β-gal (Promega). In the case of Myc-ER cells, only the reporter construct and the pSV-β-gal plasmid were transfected. The pSV-β-gal plasmid, an SV40-driven vector expressing β-galactosidase, was used as a control for transfection efficiency. Twelve hours post-transfection, the cells were rinsed once with PBS and incubated for an additional 12 h for wild-type NIH 3T3. In addition to the above, Myc-ER cells were treated with OHT at 250 nM for 36 h. The cells were lysed in 200 μL of extraction buffer, 100 μL of which was used to measure luciferase activity as described (*35*). Another 50 μL of the lysate was used to conduct a β-galactosidase assay, as previously described (*36*). Each experimental value has been normalized using its respective β-galactosidase activity and represents the average of at least three separate transfections performed in parallel; error bars represent the observed standard deviation. All experiments were performed at least 3 times independently and yielded virtually identical results.

Cav-1 Promoter Constructs. pA3Luc was the luciferase reporter plasmid into which the caveolin-1 promoters were cloned. For construction of Pr-3kb, a 2.2 kb *Kpn*I−*Hin*dIII fragment from pXP2 and a ~750 bp *Hin*dIII−*Hin*dIII fragment from pZero Blunt were combined in a three-part ligation into the *Kpn*I−*Hin*dIII site of pA3Luc. For construction of Pr-750bp, the 750 bp *Hin*dIII−*Hin*dIII fragment was subcloned into the *Hin*dIII site of pA3Luc. Based on TESS search analysis, serial deletions of the caveolin-1 promoter were made in the 5′ portion of the 3 kb caveolin-1 promoter using a PCR-based approach. The various segments were then subcloned into the pA3LUC vector using *Kpn*I−*Hin*dIII.

INR Mutational Analysis. To mutate the INR element in the caveolin-1 promoter, a 78 bp reverse primer containing the mutation (CCGGCCAAGCTTGCTGGCGCGTGGCTG-GCTGCAGGCTCTGAGGAGGTTTCCCTGGGCTGTG-ATTTAAGTATACTGAGG) was used as a primer for PCR to generate a 360 bp mutated fragment. The mutated caveolin-1 promoter PCR product was then subcloned into the pA3LUC plasmid to yield pCav-1 ΔINR-Luc.

Growth in Soft Agar Assay. Growth in soft agar was assayed as we previously described, with minor modifications (*37*). Conditions for adenoviral transduction of cells were optimized by immunofluorescence and immunoblot analysis, so that relatively high protein expression was achieved without toxicity to the cells (unpublished observations). Twenty-four hours prior to infection, ~100 000 c-Myc-ER cells were plated in 6-well plates. At the time of infection, cells were washed once with PBS and incubated for 1 h with serum-free media containing either Ad-cav-1 alone (500 pfu/cell), Ad-tTA alone (200 pfu/cell), Ad-cav-1 + Ad-tTA (500 + 200 pfu/cell, respectively), or Ad-GFP + Ad-tTA (500 + 200 pfu/cell, respectively). Cells were then washed with PBS and cultured for an 12 additional h in medium containing 10% FBS. Each cell population was then trypsinized and quantitated by counting twice using a hemocytometer. Approximately 1.5×10^4 cells were suspended in 3 mL of DMEM containing 10% FBS, 0.33% SeaPlaque low-melting temperature agarose (FMC Bioproducts), and 250 nM OHT. The suspension was plated onto a 60 mm dish containing a 2 mL layer of solidified DMEM, 10% FBS, and 0.5% SeaPlaque agarose. Three 60 mm dishes were used for each cell population. The cells were allowed to settle to the interface between these layers for 30 min at 37 °C, and plates were allowed to harden at room temperature for an additional 30 min before being returned to 37 °C. The cells were fed every 3−4 days by overlaying with 2 mL of medium containing 10% FBS, 0.33% agarose, and 250 nM OHT. After 2 weeks, the number of colonies was quantitated manually by counting foci at five predesignated locations on the dish using 10× magnification. Experimental values represent the average number of colonies in the three 60 mm plates for each condition; error bars represent the observed standard deviation between the three plates. Representative regions were also photographed under low magnification (4×).

Anti-sense experiments using Ad-Cav-1-AS were done as described above. The adenovirus harboring anti-sense caveolin-1 (Ad-Cav-1-AS) was constructed as follows. The cDNA encoding murine caveolin-1 was subcloned in the anti-sense orientation into the adenovirus type 5 shuttle vector, pΔE1-CMV-pA, under the control of a constitutive CMV-driven promoter. pΔE1-CMV-pA was constructed using the vectors pΔE1sp1A (Microbix Biosystems, Inc., Ontario, Canada) and pcDNA3 (Invitrogen, Inc.). Briefly, the *Cla*I/*Bgl*II fragment of pDE1sp1A was removed and replaced with the *Nru*I/*Pvu*II fragment of pcDNA3 containing the CMV promoter, the MCS, and the BGH pA signal.

RESULTS

Regulated Activation of c-Myc and N-Myc Down-Regulates Caveolin-1 mRNA and Protein Expression. The relationship between Myc activation and caveolin-1 expression was examined utilizing the Myc-estrogen receptor (Myc-ER) chimera system. Myc-ER, which was first characterized by Eilers and Bishop, has been used as a reliable system for elucidating new direct and indirect Myc target genes. Fusing Myc with the hormone-binding domain of the estrogen receptor allows for exquisite posttranslational control of Myc activity (*38*). In the presence of 4-hydroxytamoxifen (OHT), Myc is activated and targeted to the nucleus where it can turn on or off Myc-responsive genes. Utilizing this approach, approximately 50 Myc-regulated genes have now been identified.

NIH 3T3 cells stably transfected with c-Myc-ER, N-Myc-ER, or the pBABE empty vector control were grown to ~80% confluence, incubated in phenol red-free DMEM/charcoal dextran-stripped FBS (PRF-DMEM/CD-FBS), and treated with OHT (250 nM) to induce Myc activation. Figure 1A shows that caveolin-1 protein expression is dramatically down-regulated by c-Myc activation in a time-dependent fashion. At 12−24 h post-activation, caveolin-1 protein levels are decreased by ~50%, while near complete loss of caveolin-1 expression is achieved by 36 h post-activation. Virtually identical results were obtained with N-Myc activation as shown in Figure 1B, while no effect on caveolin-1 expression was observed in cells harboring empty vector alone (pBABE; Figure 1C). Immunoblotting with antibodies to β-tubulin was performed as a control for equal loading;

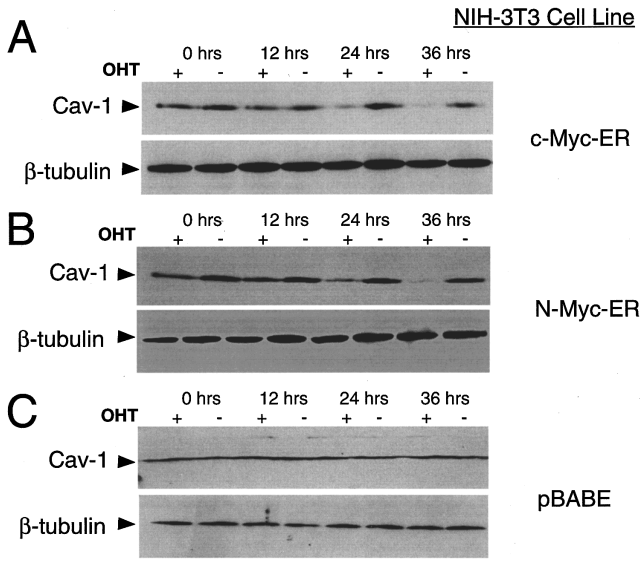

FIGURE 1: c-Myc and N-Myc activation down-regulates caveolin-1 protein expression. NIH 3T3 cell lines were grown to ~80% confluency in phenol red-free DMEM with 10% charcoal dextran-stripped fetal bovine serum (PRF-DMEM/CD-FBS) and treated with 4-hydroxytamoxifen (OHT; 250 nM) for 0, 12, 24, and 36 h. Cell lysates were prepared; the cellular proteins were then separated by SDS−PAGE and subjected to immunoblot analysis with anti-caveolin-1 IgG (mAb 2297) and anti-β-tubulin. (A) NIH 3T3 cells harboring c-Myc-ER; (B) NIH 3T3 cells harboring N-Myc-ER; and (C) NIH 3T3 cells harboring pBABE (empty vector).

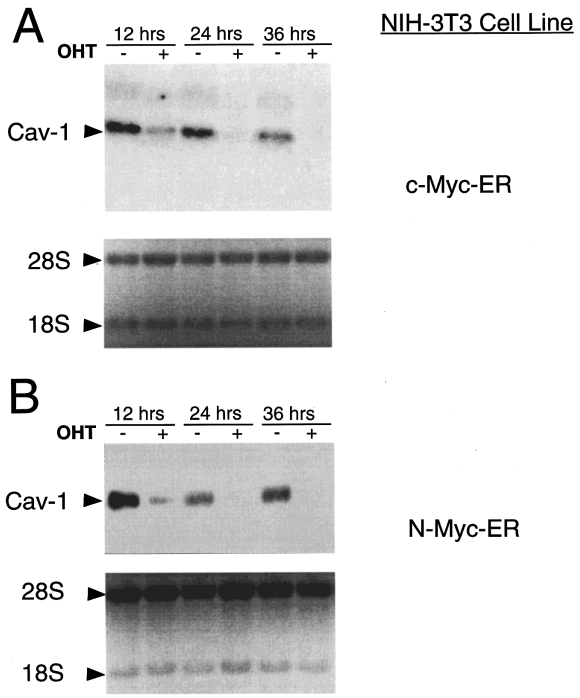

FIGURE 2: c-Myc and N-Myc activation down-regulates caveolin-1 mRNA levels. NIH 3T3 cell lines were grown to ~80% confluency in phenol red-free DMEM with 10% charcoal dextran-stripped fetal bovine serum (PRF-DMEM/CD-FBS) and treated with 4-hydroxytamoxifen (OHT; 250 nM) for 12, 24, and 36 h. Total RNA was prepared and subjected to Northern blot analysis with the cDNA for caveolin-1. The amounts of 18S and 28S rRNA are shown as a control for equal loading. (A) NIH 3T3 cells harboring c-Myc-ER; and (B) NIH 3T3 cells harboring N-Myc-ER.

note that β-tubulin remains unaffected by Myc activation. Caveolin-2 expression levels remained unaffected by Myc activation (not shown), indicating that the effects we observe are specific for caveolin-1. Similarly, oncogenic transformation by H-Ras (G12V) or v-Abl selectively down-regulates caveolin-1 expression, but has little or no effect on the expression of caveolin-2 (*24*).

Subsequently, Northern blot analysis was performed to examine the level at which Myc activation down-regulates caveolin-1 expression. NIH 3T3 cells harboring either c-Myc-ER or N-Myc-ER were subjected to the same conditions as performed above for Western blot analysis. Figure 2 shows that caveolin-1 mRNAs levels are dramatically decreased by >70% at 12 h post-Myc-activation and are virtually absent at 24 and 36 h post-activation. These results indicate that Myc activation down-regulates caveolin-1 expression at the level of the mRNA.

Activation of c-Myc and N-Myc Down-Regulates Caveolin-1 mRNA Levels via Transcriptional Repression. To examine the mechanism by which Myc activation down-regulates caveolin-1 expression, we next performed promoter analysis using a 3 kb portion of the caveolin-1 promoter subcloned into a luciferase reporter construct (*39*). This caveolin-1 promoter construct was used to transiently transfect NIH 3T3 cells harboring c-Myc-ER, N-Myc-ER, or the vector alone control (pBABE).

As shown in Figure 3A, activation of either c-Myc or N-Myc with OHT treatment decreases caveolin-1 promoter activity by greater than 2-fold. No effect is observed with NIH 3T3 cells harboring vector alone (pBABE). In addition, Figure 3B shows that when normal NIH 3T3 cells are cotransfected with caveolin-1 promoter luciferase reporter plus increasing concentrations of c-Myc expression vector (pCMV5-c-Myc), caveolin-1 promoter activity decreases exponentially. In contrast, the empty vector has no effect (pCMV5).

The amino acid sequence of Myc suggests that it contains two major domains critical for its function. The N-terminal 140aa portion contains a region known as the transregulatory domain (TRD), whereas the C-terminal dimerization domain consists of a basic region/helix−loop−helix/leucine zipper motif (bHLH/LZ). Both of these regions have been shown to be critical for Myc-induced transformation.

c-Myc mutants lacking the HLH region (c-MycΔHLH) and TRD (c-MycΔTRD) were next examined for their ability to repress caveolin-1 promoter activity. These mutants are illustrated schematically in Figure 3C. Interestingly, both c-MycΔHLH and c-MycΔTRD mutants lose their ability to repress caveolin-1 promoter activity (Figure 3D). Note the c-MycΔHLH mutant potentiates cav-1 promoter activity over baseline. This result may be explained by the remaining TRD, which may titrate N-terminal binding proteins away from endogenous c-Myc.

To determine whether Myc requires new protein synthesis to down-regulate caveolin-1 protein expression, the protein synthesis inhibitor cycloheximide was utilized. Cycloheximide was added to c-Myc-ER cells 30 min prior to OHT treatment. As shown in Figure 4, the effects of c-Myc activation on caveolin-1 mRNA levels are independent of cycloheximide treatment. These data demonstrate that new protein synthesis is not required for c-Myc-dependent repression of caveolin-1 expression. These results are consistent with the idea that the caveolin-1 promoter is a direct target of Myc repression.

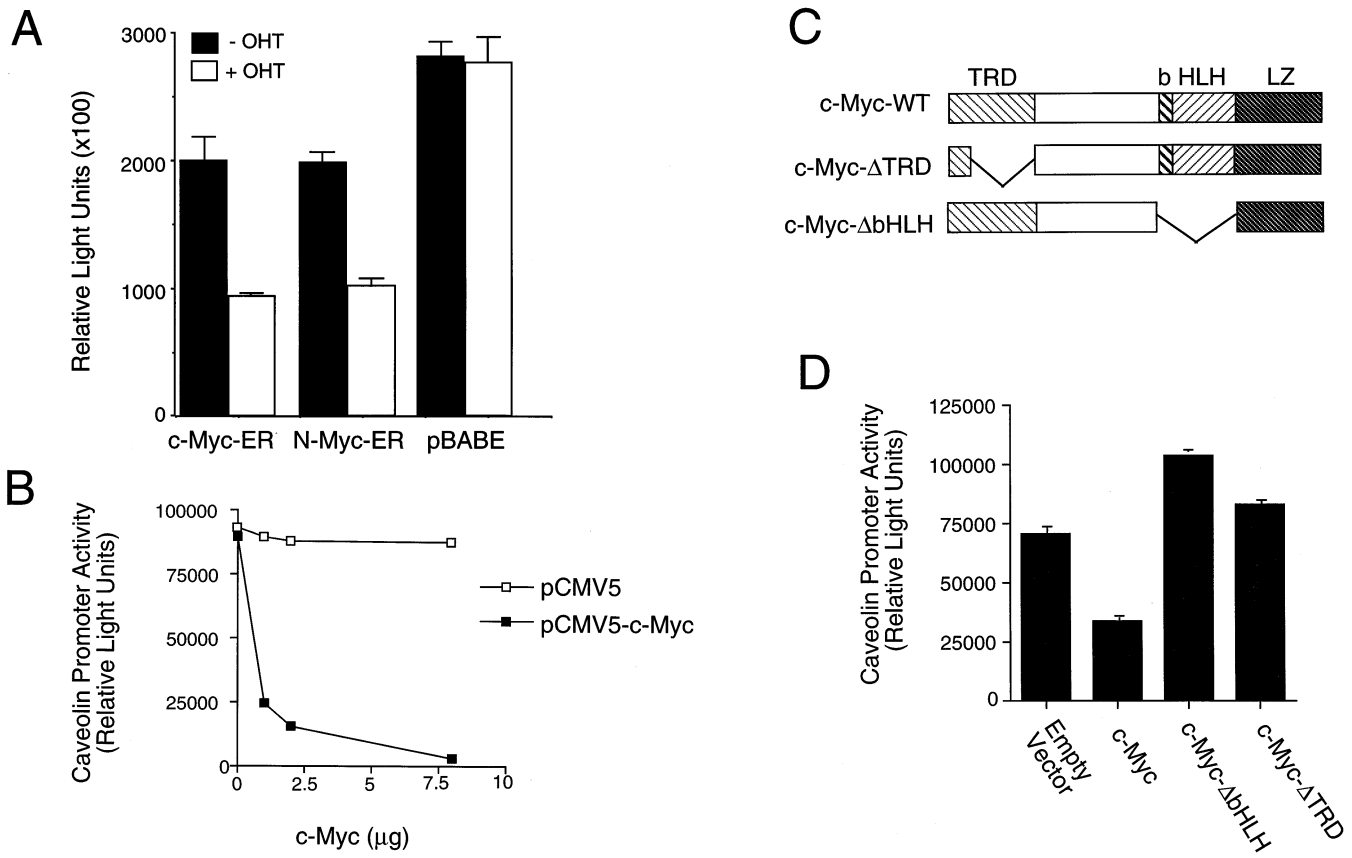

FIGURE 3: Induction of Myc activation represses caveolin-1 promoter activity. (A) NIH 3T3 cell lines [c-Myc-ER, N-Myc-ER, and pBABE (vector alone)] were transiently transfected with the caveolin-1 promoter luciferase reporter and were treated with 4-hydroxytamoxifen (OHT) for 36 h. Note that c-Myc and N-Myc activation with OHT reduces caveolin-1 promoter activity by ~2-fold, while caveolin-1 promoter activity in cells harboring the empty vector (pBABE) remains unaffected by OHT treatment. (B) Note that when normal NIH 3T3 cells are cotransfected with the caveolin-1 promoter luciferase reporter and increasing concentrations of an expression vector encoding c-Myc, the activity of the caveolin-1 promoter falls exponentially. (C) The amino acid sequence of Myc suggests that it contains two major domains critical to its function. The N-terminal 140aa portion contains a region known as the transregulatory domain (TRD), whereas the C-terminal dimerization domain consists of a basic helix−loop−helix/leucine zipper motif (bHLH/LZ). Both of these regions have been shown to be critical to Myc-induced transformation. Two c-Myc mutants lacking the TRD (c-MycΔTRD) or the HLH region (c-MycΔHLH) are shown. c-MycΔTRD contains an internal deletion of residues 41−178 in the transregulatory domain (TRD), while c-MycΔHLH contains an internal deletion of residues 371−412 in the helix−loop−helix (HLH) region. (D) NIH 3T3 cells were transiently transfected with the caveolin-1 promoter luciferase reporter and a vector encoding the c-Myc cDNA (WT or mutant). Note that both c-MycΔHLH and c-MycΔTRD mutants lose their ability to repress caveolin-1 promoter activity, while wild-type c-Myc retains its suppressive activity.

c-Myc Activation Represses the Caveolin-1 Promoter via an INR-Element. Utilizing the TESS search algorithm, an analysis of the mouse and human 3 kb upstream caveolin-1 promoter sequences (TESS search algorithm) revealed a distinct lack of TATA and E-box consensus elements. However, the caveolin-1 promoter does contain a putative pyrimidine-rich (Y), cis-acting INR-element. INR-elements, which have the loose consensus sequence YYCAYYYYY, have been shown to be the site of Myc repression in several TATA-less promoters. c-Myc appears to inhibit at these INR-elements by binding TFII-I, thus abrogating the recruitment of the RNA polymerase II complex [Roy, 1993, #1370 (*6*)].

To delineate the mechanism by which c-Myc activation regulates caveolin-1 gene transcription, several serial deletions were made in the 5′ portion of the caveolin-1 promoter. The deletions were made proximal to putative transcription factor binding sites based on TESS search analysis. As shown in Figure 5, the ability of c-Myc to repress caveolin-1 promoter activity was maintained to within 100 bp of the transcription start site. Because large deletions within the caveolin-1 promoter did not abrogate the ability of c-Myc to negatively regulate caveolin-1 promoter activity, the relationship between c-Myc activation and the caveolin-1 INR-sequence was analyzed.

The caveolin-1 INR-sequence is located 66 bp upstream of the ATG start site within the mouse CAV-1 gene. The caveolin-1 INR-sequence CCTCA(+1)GTTCT closely resembles the INR-elements of HLA-A2 and HLA-C, which are both repressed by Myc (*40*, *41*) (see Table 1).

It has been shown that the ability of c-Myc to repress at the INR-element in other systems is dependent on residues −2, −1, +3, +4, +5, and +6. Considering this information, the caveolin-1 INR-sequence was mutated from CCTCAGTTCT to CCTCAGTATA and then subcloned into the pA3LUC vector as shown in Figure 6A. Note that the ability of c-Myc activation to repress caveolin-1 promoter activity was dramatically reduced when the INR-element was mutated at positions +4, +5, and +6 (Figure 6B). In lieu of the cycloheximide data (Figure 4), it appears that Myc directly interacts with the caveolin-1 INR-sequence and as a consequence inhibits caveolin-1 transcription.

Because the INR is critical for transcriptional initiation in TATA-less promoters, any mutations made within this region can alter transcriptional assembly. Therefore, point mutations

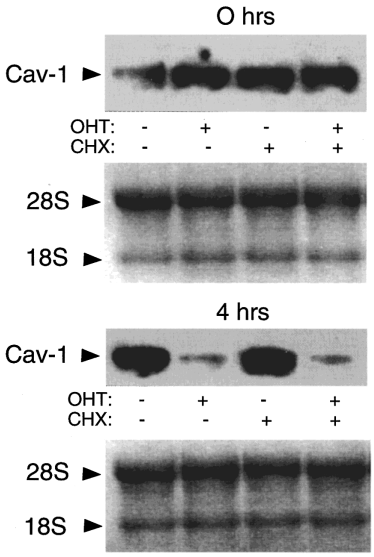

FIGURE 4: Effect of c-Myc activation on caveolin-1 mRNA levels is independent of new protein synthesis. To determine whether Myc requires new protein synthesis to down-regulate caveolin-1 protein expression, cycloheximide was utilized. Cycloheximide (10 μM) was added to NIH 3T3 cells harboring c-Myc-ER 30 min prior to OHT treatment (250 nM). Note that the effect of c-Myc activation on caveolin-1 mRNA levels is independent of cycloheximide treatment. These data demonstrate that new protein synthesis is not required for c-Myc-dependent repression of caveolin-1 expression. We have previously shown that this concentration of cycloheximide (10 μM) is sufficient to abrogate new protein synthesis in these cell lines (*30*).

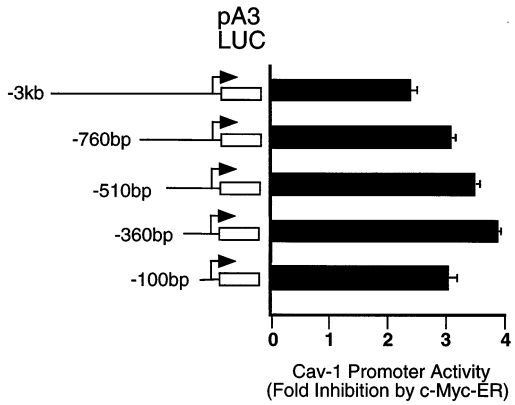

FIGURE 5: Ability of c-Myc to repress the caveolin-1 promoter is maintained to within 100 bp of the caveolin-1 transcription start site. Serial deletions in the caveolin-1 promoter were performed to determine the sites of repression by c-Myc. Deletions were made in the 5′ portion of the 3 kb caveolin-1 promoter and then subcloned into the pA3LUC reporter vector. These luciferase constructs were transiently transfected into NIH 3T3 cells harboring c-Myc-ER and then treated with OHT for 36 h.

were designed which would specifically affect c-Myc binding (see above), without dramatically affecting basal transcriptional activity. Note that the basal transcription of INR mutants stayed well within the repressible range (Figure 6B).

Caveolin-1 Expression Suppresses the Malignant Phenotype Induced by c-Myc Activation. Previous studies have shown that expression of caveolin-1 can abrogate anchorage-independent growth in both H-Ras (G12V) and v-Abl transformed NIH 3T3 cell lines, suggesting that caveolin-1 may function as a transformation suppressor protein (*24*). Thus, we next assessed the effect of caveolin-1 expression on the transformed phenotype induced by c-Myc activation.

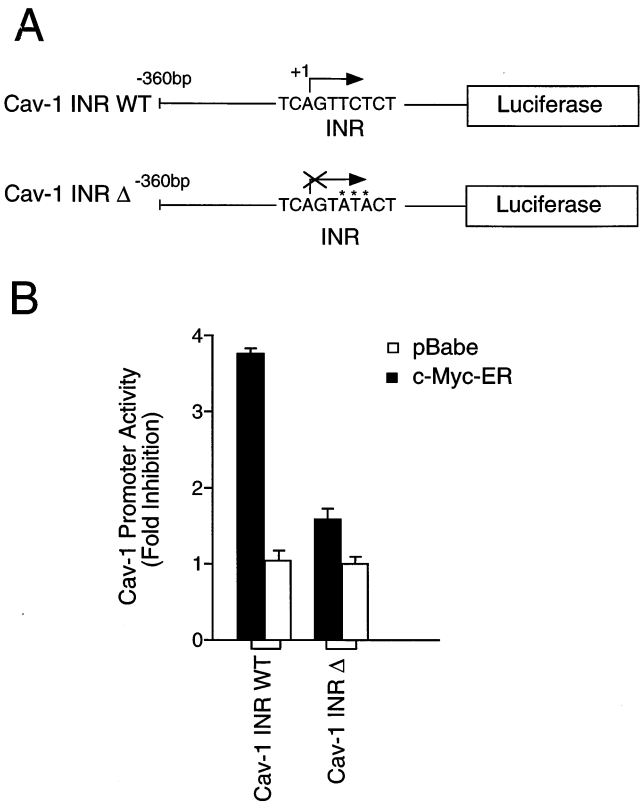

FIGURE 6: c-Myc activation inhibits caveolin-1 promoter activity via an INR-dependent mechanism. (A) The caveolin-1 INR-sequence is located 66 bp upstream of the ATG start site within the mouse CAV-1 gene. It has been shown that the ability of c-Myc to repress at the INR-element in other systems is dependent on residues −2, −1, +3, +4, +5, and +6. Considering this information, the caveolin-1 INR-sequence was mutated from CCTCAGTTCT to CCTCAGTATA and then subcloned into the pA3LUC vector. (B) These luciferase constructs were transiently transfected into NIH 3T3 cells harboring c-Myc-ER or pBABE (empty vector) and then treated with OHT for 36 h. Note that the ability of c-Myc activation to repress caveolin-1 promoter activity was dramatically reduced when the INR-element was mutated at positions +4, +5, and +6.

Table 1: Similarities in the INR-Elements of Myc-Repressed Genes[a]

gene	INR-element	reference
Ad-MLP	CTCACTCTCT	(*49*)
C/EBPα	ATCACCTTTC	(*50*)
MT-1	GTCACCACGA	(*51*)
adhesion		
N-CAM	CTCACTCATT	(*52*)
integrin		
LFA-1a	ATCATTTTCC	(*53*)
MHC class I		
HLA-A2	CTCAGATTCT	(*40*)
HLA-C	CTCAGATTCT	(*54*)
caveolins		
Cav-1	CTCAGTTCTC	this report

[a] Modified from Li et al., 1994 (*9*).

In the presence of tamoxifen, NIH 3T3 cells harboring c-Myc-ER show a transformed phenotype, which includes increased cellular proliferation and anchorage-independent growth. In these studies, we used an adenoviral vector to efficiently deliver the caveolin-1 cDNA (Ad-Cav-1). In addition, this adenoviral vector system is inducible and

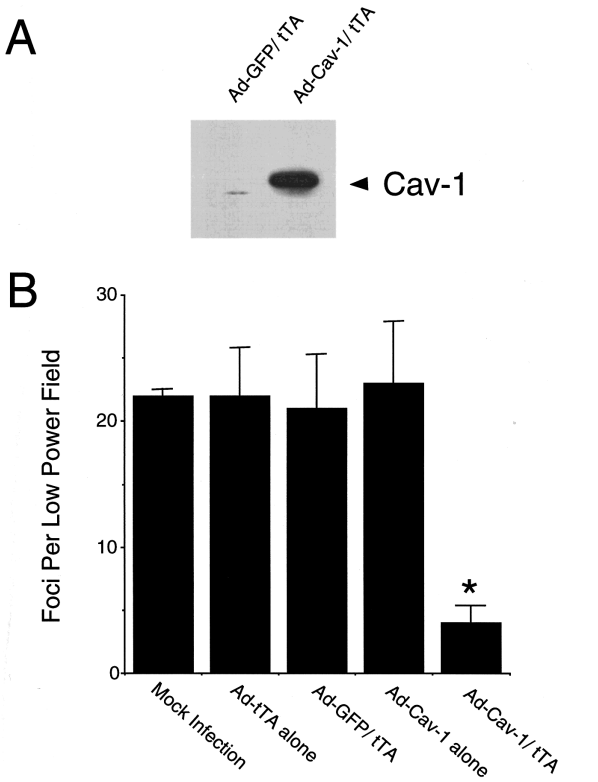

FIGURE 7: Caveolin-1 expression suppresses the malignant phenotype induced by c-Myc-activation. (A) In the presence of tamoxifen, NIH 3T3 cells harboring c-Myc-ER show a transformed phenotype, which includes increased cellular proliferation and anchorage-independent growth. To assess the effect of caveolin-1 expression on the transformed phenotype induced by c-Myc activation, we used an adenoviral vector to efficiently deliver the caveolin-1 cDNA (Ad-Cav-1). In addition, this adenoviral vector system is inducible and requires a co-activator for expression [Ad-tTA, as previously described (*37*)]. Another adenovirus, harboring GFP, was used as a control to rule out the nonspecific effects of protein overexpression (Ad-GFP). After infection, caveolin-1 expression was determined by immunoblot analysis using the caveolin-1-specific mAb 2297. (B) NIH 3T3 cells harboring c-Myc-ER were infected with Ad-Cav-1 alone, Ad-tTA alone, Ad-Cav-1 plus Ad-tTA, or Ad-GFP plus Ad-tTA. Note that only transduction with Ad-Cav-1 plus Ad-tTA inhibited the anchorage-independent growth induced by c-Myc activation. In contrast, the other control adenoviral vectors have no effect.

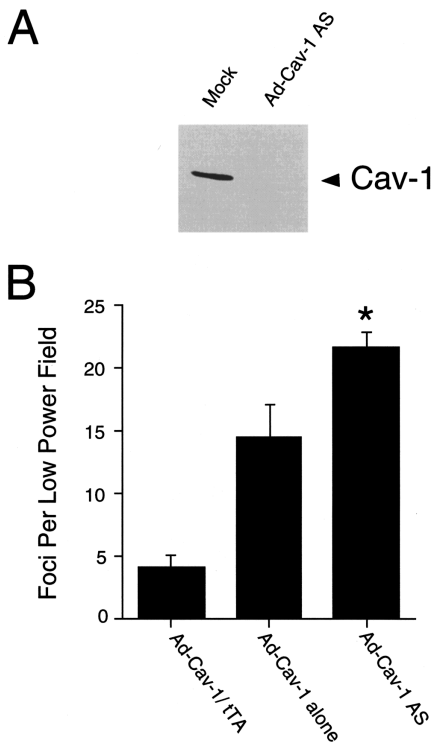

FIGURE 8: Caveolin-1 down-regulation enhances the malignant phenotype induced by c-Myc. (A) In the converse experiment, we analyzed the ability of caveolin-1 down-regulation to potentiate the effect of c-Myc-induced cell transformation. For this purpose, we constructed a constitutive adenoviral vector containing the caveolin-1 cDNA in the anti-sense orientation (Ad-Cav-1-AS) to further down-regulate caveolin-1 levels. Cav-1 protein expression was reduced to negligible levels as determined by immunoblotting. (B) Note that transduction with Ad-Cav-1-AS potentiates the effects of c-Myc-induced cellular transformation.

requires a co-activator for expression [Ad-tTA, as previously described (*37*)]. Another adenovirus, harboring GFP, was used as a control to rule out the nonspecific effects of protein overexpression (Ad-GFP).

Briefly, NIH 3T3 cells harboring c-Myc-ER were infected with Ad-Cav-1 alone, Ad-tTA alone, Ad-Cav-1 plus Ad-tTA, or Ad-GFP plus Ad-tTA. Only Ad-Cav-1 plus Ad-tTA yields caveolin-1 protein expression (Figure 7A). As shown in Figure 7B, only transduction with Ad-Cav-1 plus Ad-tTA inhibited the anchorage-independent growth induced by c-Myc activation. In contrast, the other control adenoviral vectors have no effect. Thus, recombinant expression of the caveolin-1 protein is sufficient to inhibit c-Myc-induced cellular transformation.

In the converse experiment, we next analyzed whether caveolin-1 down-regulation could potentiate the effects of c-Myc-induced cell transformation. For this purpose, we constructed a constitutive adenoviral vector containing the caveolin-1 cDNA in the anti-sense orientation (Ad-Cav-1-AS) in order to further down-regulate caveolin-1 levels (Figure 8A). As shown in Figure 8B, transduction with Ad-Cav-AS potentiated the effects of c-Myc-induced cell transformation. Thus, down-regulation of caveolin-1 appears to be one of the mechanisms by which c-Myc activation leads to a malignant phenotype.

DISCUSSION

Here, we use the well-established Myc-ER system to clearly demonstrate that activation of either c-Myc or N-Myc results in down-regulation of caveolin-1 mRNA and protein expression. In addition, we show that Myc activation represses caveolin-1 expression at the transcriptional level by directly interacting with an INR-element within the caveolin-1 promoter region. Interestingly, we also show that recombinant expression of caveolin-1 can suppress cellular transformation induced by Myc activation. Furthermore, anti-sense-mediated reductions in caveolin-1 potentiated Myc-induced cell transformation.

Since caveolin-1 was first identified as a major v-Src substrate in Rous sarcoma virus-transformed cells (*42*), an increasing body of evidence has accumulated which suggests that caveolin-1 functions as a transformation suppressor protein. For example, caveolin-1 has been shown to be highly up-regulated in fully differentiated tissues and as cultured cells reach confluency (*25*). Conversely, caveolin-1 and caveolae organelles are lost or down-regulated in a variety of tumors and in transformed cell lines (*22, 39*). Recombinant

expression of caveolin-1 in human breast cancer cells results in a ~50% reduction in cellular proliferation and a ~15-fold reduction in anchorage-independent growth (*43*). In addition, down-regulation of caveolin-1 expression in NIH 3T3 cells using an anti-sense approach leads to anchorage-independent growth in soft agar and tumor formation in immunodeficient mice (*24*). Under these conditions, down-regulation of caveolin-1 expression was also associated with hyper-activation of the p42/44 MAP kinase cascade (*25*).

There are several putative mechanisms by which caveolin-1 exerts its transformation suppressor effects. Caveolin-1 expression is known to antagonize the effects of activated Ras by inhibiting its downstream effectors. More specifically, caveolin-1 has been shown to negatively regulate both Ras/MAPK-mediated and basal transcriptional activation of mitogen-sensitive promoters. Also, the caveolin-1 scaffolding domain (residues 82−101) was shown to directly inhibit the kinase activity of both MEK and ERK in vitro (*39*).

Caveolin-1 may also play an important role in integrin signaling. The loss of caveolin-1 expression can alter the formation of focal adhesions and dismantle integrin signaling pathways (*44*, *45*). Aberrations in integrin expression have been implicated in a variety of tumor processes such as cell proliferation, anchorage-independent growth, and metastasis (*46*).

The list of known oncogenes that down-regulate caveolin-1 expression includes H-Ras (G12V), v-Raf, v-Src, v-Abl, activated Neu/HER2, and activated Myc. However, the mechanisms by which oncogenes repress caveolin-1 expression are as varied as the oncogenes themselves. Treatment of Ras (H-, K-, and N-Ras) and v-Raf transformed cells with the well-characterized MEK inhibitor PD98059 restores caveolin-1 expression. However, PD98059 has no effect on caveolin-1 expression in NIH 3T3 cells transformed by nonreceptor tyrosine kinases (NRTKs) or receptor tyrosine kinases (RTK), implicating p42/44 MAPK-independent pathways (*47*). Elucidation of the different mechanisms that lead to caveolin-1 down-regulation has obvious importance for clinical cancer therapy.

The ability of activated Myc to repress the caveolin-1 promoter directly through the INR-element is a novel mechanism by which caveolin-1 is down-regulated during cellular transformation. Genes that are negatively regulated by Myc include cell adhesion molecules, integrins, differentiation markers, as well as caveolin-1 (this report); they possess the consensus sequence TCAYYYYY [Table 1 (*9*)]. In contrast, genes up-regulated by Myc that are integral to DNA synthesis and cell cycle regulation have canonical E-box elements, but distinctly lack INR-sites. The absence or presence of an INR-element in Myc-activated or Myc-repressed genes suggests that Myc may play a pivotal role in the transition from proliferating undifferentiated cells to quiescent fully differentiated cells (*48*).

In order for Myc to mediate cellular transformation, Myc activation represses a repertoire of cell adhesion genes ($\alpha L\beta 2$ and $\alpha 3\beta 1$ integrins), differentiation-specific genes (C/EBPα), and cell cycle/growth arrest genes [growth arrest and DNA damage genes (GADDs) and $p27^{KIPI}$] (*48*). Caveolin-1 represents the newest member of this Myc-repressed gene family.

Caveolin-1 expression has been shown to be down-regulated or absent in a variety of human tumor cell lines, including breast, colon, ovarian, and cervical (*22*, *43*, *55*−*57*). Furthermore, reintroduction of caveolin-1 via transfection or infection with adenoviral vectors reduces the malignant phenotype in various tumor cell lines (*24*, *43*, *56*, *57*). Considering caveolin-1 expression is inversely related to tumorigenicity, recombinant expression of caveolin-1 could be potentially used as an adenoviral-based anti-cancer therapy. Injection of caveolin-1 expressing adenoviral vectors intratumorally or into the tumor arterial supply could serve to attenuate malignant progression. As the intimate relationship between Myc and caveolin-1 becomes further defined, the use of such novel therapeutic modalities may serve to be invaluable in the treatment of various Myc-related malignancies.

REFERENCES

1. Dang, C. V., Resar, L. M., Emison, E., Kim, S., Li, Q., Prescott, J. E., Wonsey, D., and Zeller, K. (1999) *Exp. Cell Res. 253* (1), 63−77.
2. DePinho, R. A., Schreiber-Agus, N., and Alt, F. W. (1991) *Adv. Cancer Res. 57*, 1−46.
3. Schmidt, E. V. (1999) *Oncogene 18*, 2988−2996.
4. Karn, J., Watson, J. V., Lowe, A. D., Green, S. M., and Vedeckis, W. (1989) *Oncogene 4* (6), 773−787.
5. Mateyak, M. K., Obaya, A. J., Adachi, S., and Sedivy, J. M. (1997) *Cell Growth Differ. 8*, 1039−1048.
6. Roy, A. L., Carruthers, C., Gutjahr, T., and Roeder, R. G. (1993) *Nature 365* (6444), 359−361.
7. Roy, A. L., Du, H., Gregor, P. D., Novina, C. D., Martinez, E., and Roeder, R. G. (1997) *EMBO J. 16* (23), 7091−7104.
8. Roy, A., and Roeder, R. (1993) *Nature 365*, 355−358.
9. Li, L. H., Nerlov, C., Prendergast, G., MacGregor, D., and Ziff, E. B. (1994) *EMBO J. 13* (17), 4070−4079.
10. Peukert, K., Staller, P., Schneider, A., Carmichael, G., Hanel, F., and Eilers, M. (1997) *EMBO J. 16* (18), 5672−5686.
11. Schneider, A., Peukert, K., Eilers, M., and Hanel, F. (1997) *Curr. Top. Microbiol. Immunol. 224*, 137−146.
12. FitzGerald, M. J., Arsura, M., Bellas, R. E., Yang, W., Wu, M., Chin, L., Mann, K. K., DePinho, R. A., and Sonenshein, G. E. (1999) *Oncogene 18* (15), 2489−2498.
13. Lee, L. A., Dolde, C., Barrett, J., Wu, C. S., and Dang, C. V. (1996) *J. Clin. Invest. 97* (7), 1687−1695.
14. Sargiacomo, M., Scherer, P. E., Tang, Z.-L., Kubler, E., Song, K. S., Sanders, M. C., and Lisanti, M. P. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9407−9411.
15. Li, S., Okamoto, T., Chun, M., Sargiacomo, M., Casanova, J. E., Hansen, S. H., Nishimoto, I., and Lisanti, M. P. (1995) *J. Biol. Chem. 270*, 15693−15701.
16. Garcia-Cardena, G., Fan, R., Stern, D., Liu, J., and Sessa, W. C. (1996) *J. Biol. Chem. 271*, 27237−27240.
17. Shaul, P. W., Smart, E. J., Robinson, L. J., German, Z., Yuhanna, I. S., Ying, Y., Anderson, R. G. W., and Michel, T. (1996) *J. Biol. Chem. 271*, 6518−6522.
18. Song, K. S., Li, S., Okamoto, T., Quilliam, L., Sargiacomo, M., and Lisanti, M. P. (1996) *J. Biol. Chem. 271*, 9690−9697.
19. Li, S., Couet, J., and Lisanti, M. P. (1996) *J. Biol. Chem. 271*, 29182−29190.
20. Couet, J., Sargiacomo, M., and Lisanti, M. P. (1997) *J. Biol. Chem. 272*, 30429−30438.
21. Oka, N., Yamamoto, M., Schwencke, C., Kawabe, J., Ebina, T., Couet, J., Lisanti, M. P., and Ishikawa, Y. (1997) *J. Biol. Chem. 272*, 33416−33421.
22. Engelman, J. A., Lee, R. J., Karnezis, A., Bearss, D. J., Webster, M., Siegel, P., Muller, W. J., Windle, J. J., Pestell, R. G., and Lisanti, M. P. (1998) *J. Biol. Chem. 273*, 20448−20455.
23. Koleske, A. J., Baltimore, D., and Lisanti, M. P. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1381−1385.
24. Engelman, J. A., Wycoff, C. C., Yasuhara, S., Song, K. S., Okamoto, T., and Lisanti, M. P. (1997) *J. Biol. Chem. 272*, 16374−16381.

25. Galbiati, F., Volonté, D., Engelman, J. A., Watanabe, G., Burk, R., Pestell, R., and Lisanti, M. P. (1998) *EMBO J. 17*, 6633−6648.
26. Engelman, J. A., Zhang, X. L., and Lisanti, M. P. (1998) *FEBS Lett. 436*, 403−410.
27. Engelman, J. A., Zhang, X. L., and Lisanti, M. P. (1999) *FEBS Lett. 448*, 221−230.
28. Scherer, P. E., Tang, Z.-L., Chun, M. C., Sargiacomo, M., Lodish, H. F., and Lisanti, M. P. (1995) *J. Biol. Chem. 270*, 16395−16401.
29. Scherer, P. E., Lewis, R. Y., Volonte, D., Engelman, J. A., Galbiati, F., Couet, J., Kohtz, D. S., van Donselaar, E., Peters, P., and Lisanti, M. P. (1997) *J. Biol. Chem. 272*, 29337−29346.
30. Lasorella, A., Noseda, M., Beyna, M., and Iavarone, A. (2000) *Nature 407* (6804), 592−598.
31. Amin, C., Wagner, A. J., and Hay, N. (1993) *Mol. Cell. Biol. 13*, 383−390.
32. Engelman, J. A., Zhang, X. L., Galbiati, F., and Lisanti, M. P. (1998) *FEBS Lett. 429*, 330−336.
33. Maxwell, I. H., Harrison, G. S., Wood, W. M., and Maxwell, F. (1989) *BioTechniques 7*, 276−280.
34. Wood, W. M., Kao, M. Y., Gordon, D. F., and Ridgway, E. C. (1989) *J. Biol. Chem. 264*, 14840−14847.
35. Pestell, R. G., Albanese, C., Hollenberg, A., and Jameson, J. L. (1994) *J. Biol. Chem. 269*, 31090−31096.
36. Subramaniam, A., Gulick, J., and Robbins, J. (1990) *J. Biol. Chem. 265* (23), 13986−13994.
37. Zhang, W., Razani, B., Altschuler, Y., Bouzahzah, B., Mostov, K. E., Pestell, R. G., and Lisanti, M. P. (2000) *J. Biol. Chem. 275* (27), 20717−20725.
38. Eilers, M., Picard, D., Yamamoto, K. R., and Bishop, J. M. (1989) *Nature 340* (6228), 66−68.
39. Engelman, J. A., Chu, C., Lin, A., Jo, H., Ikezu, T., Okamoto, T., Kohtz, D. S., and Lisanti, M. P. (1998) *FEBS Lett. 428*, 205−211.
40. Koller, B. H., and Orr, H. T. (1985) *J. Immunol. 134* (4), 2727−2733.
41. Tibensky, D., and Delovitch, T. L. (1990) *Immunogenetics 32* (3), 210−213.
42. Glenney, J. R., and Zokas, L. (1989) *J. Cell Biol. 108*, 2401−2408.
43. Lee, S. W., Reimer, C. L., Oh, P., Campbell, D. B., and Schnitzer, J. E. (1998) *Oncogene 16*, 1391−1397.
44. Wary, K. K., Mariott, I. A., Zurzolo, C., and Giancotti, F. G. (1998) *Cell 94*, 625−634.
45. Wei, Y., Yang, X., Liu, Q., Wilkins, J. A., and Chapman, H. A. (1999) *J. Cell Biol. 144*, 1285−1294.
46. Giancotti, F. G., and Ruoslahti, E. (1999) *Science 285* (5430), 1028−1032.
47. Engelman, J. A., Zhang, X. L., Razani, B., Pestell, R. G., and Lisanti, M. P. (1999) *J. Biol. Chem. 274*, 32333−32341.
48. Claassen, G. F., and Hann, S. R. (1999) *Oncogene 18* (19), 2925−2933.
49. Ziff, E. B., and Evans, R. M. (1978) *Cell 15* (4), 1463−1475.
50. Christy, R. J., Kaestner, K. H., Geiman, D. E., and Lane, M. D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88* (6), 2593−2497.
51. Stuart, G. W., Searle, P. F., Chen, H. Y., Brinster, R. L., and Palmiter, R. D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81* (23), 7318−7322.
52. Barton, C. H., Mann, D. A., and Walsh, F. S. (1990) *Biochem. J. 268* (1), 161−168.
53. Cornwell, R. D., Gollahon, K. A., and Hickstein, D. D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90* (9), 4221−4225.
54. Tibensky, D., and Delovitch, T. L. (1990) *Immunogenetics 32* (3), 210−213.
55. Bagnoli, M., Tomassetti, A., Figini, M., Flati, S., Dolo, V., Canevari, S., and Miotti, S. (2000) *Oncogene 19*, 4754−4763.
56. Bender, F. C., Reymond, M. A., Bron, C., and Quest, A. F. (2000) *Cancer Res. 60*, 5870−5878.
57. Razani, B., Altschuler, Y., Zhu, L., Pestell, R. G., Mostov, K. E., and Lisanti, M. P. (2000) *Biochemistry 39*, 13916−13924.

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