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An Induced Ets Repressor Complex Regulates Growth Arrest during Terminal Macrophage Differentiation

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Summary

Defining the molecular mechanisms that coordinately regulate proliferation and differentiation is a central issue in development. Here, we describe a mechanism in which induction of the Ets repressor METS/PE1 links terminal differentiation to cell cycle arrest. Using macrophages as a model, we provide evidence that METS/PE1 blocks Ras-dependent proliferation without inhibiting Ras-dependent expression of cell type-specific genes by selectively replacing Ets activators on the promoters of cell cycle control genes. Antiproliferative effects of METS require its interaction with DP103, a DEAD box-containing protein that assembles a novel corepressor complex. Functional interactions between the METS/DP103 complex and E2F/ pRB family proteins are also necessary for inhibition of cellular proliferation, suggesting a combinatorial code that directs permanent cell cycle exit during terminal differentiation.

Introduction

The linkage between cell cycle arrest and terminal differentiation is a common feature of development in Metazoan organisms. In many instances, the developmental fate of individual cells is established by activating Ras-signaling pathways, with Ets domain transcription factors often acting as crucial transcriptional effectors (Halfon et al., 2000; McCormick, 1999; Olson and Marais, 2000; Tan and Kim, 1999; Wassarman et al., 1995). For example, in *Drosophila*, Ras-dependent phosphorylation of the Ets repressor Yan results in its nuclear export and degradation, while Ras-dependent phosphorylation of the Ets factor PNT-P2 stimulates its transcriptional activity (O'Neill et al., 1994; Rebay and Rubin, 1995). This causes a Ras-dependent switch of activated PNT-P2 for Yan at Ets sites in the promoters of target genes, such as *phyllopod*, that positively regulate photoreceptor differentiation.

In vertebrates, several Ets factors have also been shown to be downstream effectors of Ras-signaling cascades that regulate both cellular proliferation and differentiation (reviewed in Graves and Petersen, 1998; Mavrothalassitis and Ghysdael, 2000; Sharrocks, 2001; Wasylyk et al., 1998). In the hematopoietic system, positively acting Ets factors that include Ets1, Ets2, and PU.1 are thought to play critical roles in mediating both mitogenic and lineage-specific differentiation responses to colony stimulating factors (Bories et al., 1995; McKercher et al., 1996; Muthusamy et al., 1995; Scott et al., 1994; Yamamoto et al., 1999). In regulating the expression of genes that specify cell type-specific functions, Ets factors frequently cooperate with other classes of transcription factors, such as AP-1 proteins, serum response factor, and Pit-1 (Hill and Treisman, 1995; Treier et al., 1995; Wasylyk et al., 1998, 1990). Ras signaling initiated by macrophage colony stimulating factor (M-CSF) leads to transcriptional activation of a large set of target genes by a mechanism that depends on cooperative interactions between Ets factors and members of the AP-1 family of transcription factors (Jin et al., 1995; Reddy et al., 1992). For example, M-CSF-dependent activation of the *urokinase-type plasminogen activator* and *SR-A* genes involves the binding of ternary complexes of AP-1 and Ets proteins to composite AP-1/Ets recognition elements in their respective promoters (Guidez et al., 1998; Stacey et al., 1995; Wu et al., 1994).

With respect to mitogenic signaling, Ets1 and Ets2 transactivate the *c-Myc* and *c-Myb* genes (Roussel et al., 1994; Sullivan et al., 1997), and a dominant-negative form of Ets2 blocks proliferative responses to M-CSF (Langer et al., 1992), indicating that Ets2 itself and/or additional Ets factors with overlapping DNA binding specificity activate cell cycle-regulatory genes. The involvement of positively acting Ets factors in both Ras-dependent differentiation and Ras-dependent proliferation events raises an apparent paradox; maintenance of Ras signaling is required for expression of cell type-specific genes during terminal macrophage differentiation, while the proliferative response to Ras signaling is inhibited.

Here, we present evidence that induction of the Ets repressor METS during macrophage differentiation contributes to terminal cell cycle arrest by repressing the transcription of cell cycle control genes that include *c-Myc*, *c-Myb*, and *Cdc2*. While METS exhibits an overlapping DNA binding specificity with Ets2 and other Ets activators, it does not inhibit transcription of macrophage-specific genes that are activated by AP-1/Ets ternary complexes. METS thus selectively represses Ets target genes involved in Ras-dependent proliferation while sparing genes that are targets of Ras-dependent differentiation. The antiproliferative actions of METS require

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its direct interaction with DP103, a protein recently identified to interact with the transforming Epstein-Barr nuclear antigens (EBNAs) 2 and 3c (Grundhoff et al., 1999). Intriguingly, members of the pRb family proteins are also required for METS-mediated growth arrest, suggesting a combinatorial mechanism by which permanent exit from the cell cycle can be achieved.

Results

METS Is an Ets Repressor that Is Induced during Macrophage Differentiation

To identify members of the Ets family that might potentially play roles in the coordination of myeloid proliferation and maturation, we applied a degenerate PCR approach to amplify the DNA binding domains (DBDs) of Ets family members in THP-1 monocytic leukemia cells that had been treated with the phorbol ester TPA to induce macrophage differentiation. These studies identified an Ets family member that was markedly upregulated in TPA-treated cells. By subsequently screening a murine macrophage cDNA library with the PCR product as the probe, we obtained two independent clones encoding a 513 amino acid protein. Because this protein blocks mitogenic responses mediated by Ets factors such as Ets2, we refer to it as *m*itogenic *E*ts *t*ranscriptional *s*uppressor (METS). It is highly related to Ets2 repressor factor (ERF) (Sgouras et al., 1995) throughout the Ets DNA binding domain, the adjacent conserved region 1 (CR1), and three additional regions, identified as CR2, CR3, and CR4, and is identical to mPE1 reported in murine osteoblasts while this work was in progress (Bidder et al., 2000; Figure 1A). Electrophoretic mobility shift assays using competitor oligonucleotides indicated that METS bound with high affinity to a consensus DNA site recognized by Ets2, but not to the Ets site contained within the serum response element (SRE) of the *c-Fos* promoter (Figure 1B). When expressed in CV1 cells, METS strongly inhibited the activity of the *thymidine kinase* (*TK*) promoter when linked to the consensus Ets2 site, but did not inhibit the *TK* promoter itself or the β-*actin* promoter (Figure 1C). Fusion of C-terminal fragments of METS to the DNA binding domain of GAL4 revealed the presence of two independent repression domains, referred to as RD-N and RD-C (Figures 1A and 1D).

RNase protection assays failed to detect METS mRNA in freshly isolated immature bone marrow progenitor cells (Figure 1E). However, treatment of these cells with macrophage colony stimulating factor (M-CSF) to induce differentiation of adherent macrophages resulted in a marked upregulation of METS mRNA. Western blotting experiments detected low levels of METS protein up to day 4 of M-CSF-dependent differentiation, with a marked increase in expression observed by day 7 of culture (Figure 1F). High levels of METS mRNA and protein were also observed in elicited peritoneal macrophages that are terminally differentiated (Figures 1E and 1F). Quantification of METS protein levels in elicited peritoneal macrophages indicated more than 80,000 molecules per cell, while terminally differentiated bone marrow-derived macrophages expressed greater than 600,000 molecules of METS protein per cell. A similar pattern evolved in Hoxa9 HF-1 cells, a Hoxa9-immortalized promyelocytic cell line that further differentiates into granulocytes or macrophages when treated with the granulocyte colony stimulating factor (G-CSF) or M-CSF, respectively (Calvo et al., 2000; Figure 2A). In these cells, M-CSF induced expression of *METS* together with that of *Ets2*, *c-Fos*, and *SR-A*. In contrast, expression of the cell cycle-regulatory genes *c-Myb* and *c-Myc* was suppressed (Figure 2A).

METS Selectively Represses Cell Cycle Control Genes and Blocks Proliferation

Based on the coordinate upregulation of METS and downregulation of *c-Myb* and *c-Myc* during macrophage differentiation and previous studies indicating that *c-Myb* and *c-Myc* are targets of positive Ets factors (Roussel et al., 1994; Sullivan et al., 1997), we evaluated the possibility that they might represent direct target genes of METS. Consistent with this, forced expression of METS repressed *c-Myb* and *c-Myc* promoter activity (Figure 2B). Evaluation of other promoters directing the expression of cell cycle control genes indicated that that the *Cdc2* promoter was also strongly repressed by METS (Figure 2B). In contrast, METS had relatively little effect on the activities of the *SR-A*, *Gelatinase B*, or *Macrosialin* promoters (Figure 2B), which are activated by AP-1/Ets ternary complexes in response to Ras signaling (Guidez et al., 1998; Li et al., 1998; Saarialho-Kere et al., 1993). Although the repression function of the related Ets repressor ERF has been demonstrated to be inactivated by Ras signaling (Sgouras et al., 1995), GAL4-METS retained repressor activity in cells expressing a constitutively active Val^{12}-Ras mutant and in cells stimulated with phorbol 12-myristate 13-acetate (PMA; Figure 2C).

These observations raised the possibility that METS might negatively regulate myeloid proliferation. To test this hypothesis, MSCV retroviral vectors were generated directing expression of green fluorescent protein (GFP) and either wild-type METS or a fusion protein of the METS DBD linked to the transactivation domain of VP16 that does not repress transcription. A downstream internal ribosomal binding site was used for initiation of GFP translation (Figure 2D). Following infection, ECoM1-G cells, which are promyelocytic cells that are transformed with a conditional E2A-Pbx1 fusion protein (Sykes and Kamps, 2001), GFP expression was used to select cells by FACS for further analysis. Cells infected with the control MSCV-GFP vector lacking METS, or the MSCV vector directing expression of an activating form of METS (VP-16-METS), proliferated equivalently in GM-CSF-containing growth media, while cells infected with MSCV-METS remained viable but did not proliferate (Figure 2D).

To further examine the ability of METS to block growth factor-dependent proliferation, calibrated amounts of GST-METS fusion proteins were microinjected into the nuclei of Rat 1 cells treated with epidermal growth factor (EGF), which stimulates DNA synthesis and cell division in a Ras-dependent manner (Stacey et al., 1988). GST-METS potently inhibited EGF-dependent incorporation of BrdU, with half-maximal inhibition observed with injection of approximately 7500 molecules of METS protein per cell (Figures 2E and 2F). Rat 1 cells were also infected with either the control MSCV-GFP retroviral

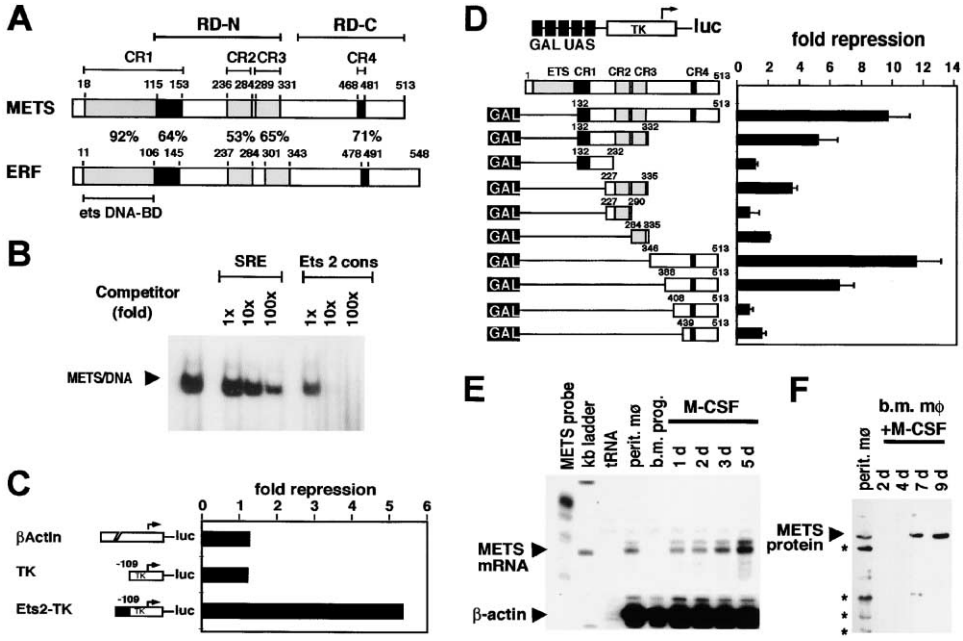

Figure 1. METS Is a Transcriptional Repressor that Is Upregulated during Terminal Macrophage Differentiation

(A) Sequence alignment between METS and ERF. The bar graphs show percent homologies between the conserved regions (CR) of METS and ERF and indicate the N-terminal repressor domain (RD-N) and C-terminal repressor domain (RD-C).

(B) Binding of recombinant GST-METS to a ^{32}P-labeled probe corresponding to a consensus Ets2 recognition element and competition by unlabeled oligonucleotides containing the *c-fos* promoter serum response element (SRE) or a consensus Ets2 site. The numbers on top of the panel indicate the ratio of unlabeled competitor oligonucleotides to labeled probe.

(C) Transient transfection of METS into CV-1 cells represses transcription from the TK promoter when linked to the Ets2 consensus site, but not the TK promoter itself. Basal promoter activities were as follows: β-actin; 220 relative light unit (RLU)/U β-gal activity, TK promoter; 14 RLU/U β-gal, Ets-TK promoter · 82 RLU/U β-gal.

(D) METS contains two transferable repressor domains. The indicated regions of METS were linked to the DNA binding domain of GAL4 and tested for their ability to repress activity of a TK promoter under control of five repeats of the GAL1 upstream activating sequence (GAL UAS) in CV-1 cells.

(E) RNase protection assay demonstrating high expression of METS mRNA in peritoneal macrophages (perit. mφ) and its upregulation in bone marrow progenitor cells (b.m. prog.) induced to differentiate into macrophages by treatment with M-CSF for 1 to 5 days.

(F) Western blotting of METS protein in peritoneal macrophages and bone marrow progenitor cells induced to differentiate into macrophages by 2 to 9 days of M-CSF treatment. (Asterisks denote METS degradation products.)

vector or the MSCV-METS-GFP vector, and the infected populations of cells were sorted based on GFP expression 72 hr later. The GFP-positive cells infected with the MSCV-METS-GFP vector expressed METS at levels comparable to primary macrophages and exhibited markedly reduced expression of endogenous c-Myc protein compared to GFP-negative cells or GFP-positive cells infected with control virus (Figure 2G). Intriguingly, recombinant METS truncated at amino acid 332 was as effective as the full-length protein (Figure 2E). In contrast, the DBD tethered to C-terminal residues 391–513 (GST-METSΔNRD) did not block EGF-mediated cell proliferation. Likewise, a deletion mutant lacking the Ets domain residues 1–131 (GST-METSΔDBD) and a short splicing variant found to be expressed in a human adenocarcinoma (METS$^{1-143}$) did not inhibit BrdU incorporation. Together, these experiments indicated that METS exerted antiproliferative effects through the N-terminal repression domain and that the intact DBD was required for this function.

To begin to investigate the mechanisms responsible for repression of a subset of Ets target genes by METS, the *c-Myc* promoter was subjected to further analysis. Repression of the *c-Myc* promoter by METS required the Ets DNA binding domain (Figure 3A). Evaluation of the *c-Myc* promoter sequence revealed the presence of multiple potential binding sites containing the core Ets recognition sequence GGAA. Double-stranded oligonucleotides corresponding to each of these sites were tested for their ability to compete for the binding of full-length METS to the radiolabeled consensus Ets2 site in electrophoretic mobility shift assays (EMSAs). A representative experiment is illustrated in Figure 3B. Several high-affinity binding sites were identified by this approach, including sites at +350, –153, −336, –853, and −1153, with respect to the upstream promoter P1. A composite site previously found to bind both E2F proteins and Ets2 (Myc E2F/Ets2; Roussel et al., 1994) was not required for METS-mediated repression of the c-Myc promoter because repression was still observed when this site was mutated (Figure 3A). By arranging these binding sites in order of decreasing affinity, a marked preference for G/A at position 5 and T/G at position 6 of the highest affinity binding sites was observed, whereas no clear sequence specificity was discernable upstream of position 1 (Figure 3C). The sequence requirements at positions 4 and 5 for high-affinity binding were confirmed by a systematic analysis of oligonucleo-

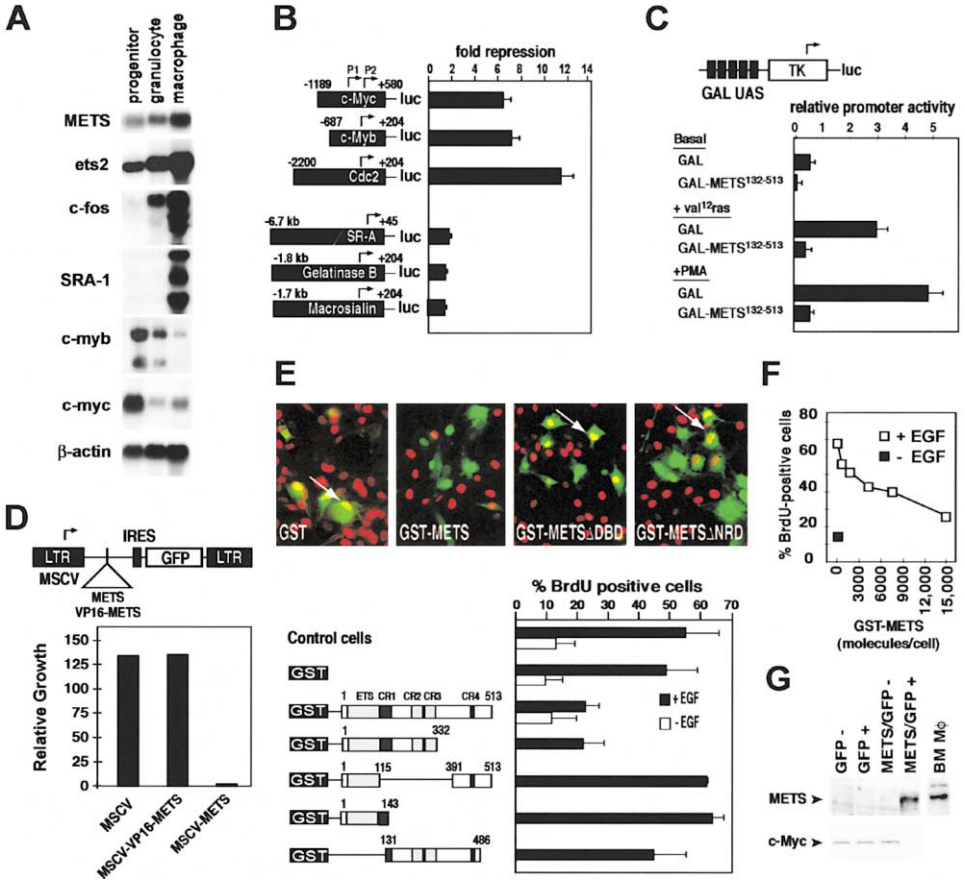

Figure 2. METS Represses Cell Cycle Control Genes and Inhibits Growth Factor-Dependent DNA Synthesis

(A) Northern blot analysis of mRNA derived from Hoxa9-immortalized bone marrow-derived cells in the progenitor stage and after granulocyte differentiation stimulated by G-CSF or macrophage differentiation stimulated by M-CSF.

(B) Forced expression of METS results in significant repression of the *c-Myc*, *c-Myb*, and *Cdc2* promoters, but not the *SR-A*, *Gelatinase B*, or *Macrosialin* promoters.

(C) Gal-METS$^{132-513}$ retains repressor activity in CV-1 cells transfected with Val^{12}Ras or treated with PMA.

(D) Retroviral expression of METS blocks proliferation of E2A-Pbx1-transformed myeloid progenitor cells in response to GM-CSF.

(E) Antiproliferative effects of METS require the N-terminal repression domain. Cells were microinjected with GST or the indicated GST-METS fusion proteins. The upper panels indicate representative fields showing, from left to right, GST control, GST-METS (aa 1–513), GST-METSΔDBD (aa 131–486), and GST-METSΔNRD (aa 1–115 plus 391–513). Injected cells appear green; cells with incorporated BrdU are red. Injected cells demonstrating BrdU incorporation are indicated by arrows and have a yellow nucleus. Quantification of the percent of injected cells incorporating BrdU is indicated in the lower panel.

(F) Microinjection of physiologic levels of METS block EGF-dependent DNA synthesis in Rat 1 cells. Cells were microinjected with the indicated amounts of GST-METS protein (molecules/cell), treated with EGF, and scored for incorporation of BrdU.

(G) Expression of METS in Rat1 cells inhibits endogenous c-Myc expression. Cells were infected with the control or METS retroviral vectors described in (D). GFP+ and GFP− cells were sorted, and 10^6 cells were analyzed by Western blotting for METS and c-Myc expression 72 hr later. METS expression in 10^6 bone marrow-derived Mϕ is shown for comparison.

tides containing all possible base pair combinations introduced into the context of the Myc-153 sequence (data not shown). The consensus METS DNA binding site defined by these experiments is nearly identical to the consensus binding sites previously determined for the Ets activators Ets1, Ets2, and PEA3 (Graves and Petersen, 1998). However, in contrast to the Ras-response elements recognized by Ets1, Ets2, and PEA3 in cell type-specific genes such as SR-A, which consist of AP-1/Ets composite elements, none of the high-affinity METS binding sites in the *c-Myc* promoter contained adjacent AP-1 recognition motifs. Thus, these sites appear to represent recognition elements for Ets monomers. Using the consensus recognition site for METS of GGAA(G/A)(T/G), we searched sequence databases for these sites in other promoters. Potential METS sites were identified in several additional cell cycle control genes, including *Cdc2* and the *p54* subunit of *DNA primase*. Intriguingly, in each case at least one METS site could be identified within 150 bp of an E2F binding site.

To determine whether METS discriminated between monomeric binding sites present in cell cycle-regulatory genes such as *c-Myc, Cdc2*, and the *p54* subunit of *DNA primase* versus composite elements in cell type-specific genes such as *SR-A* in cells, chromatin immunoprecipitation (ChIP) experiments were performed. Specific primers were designed to amplify promoter regions containing putative Ets and E2F binding sites in each

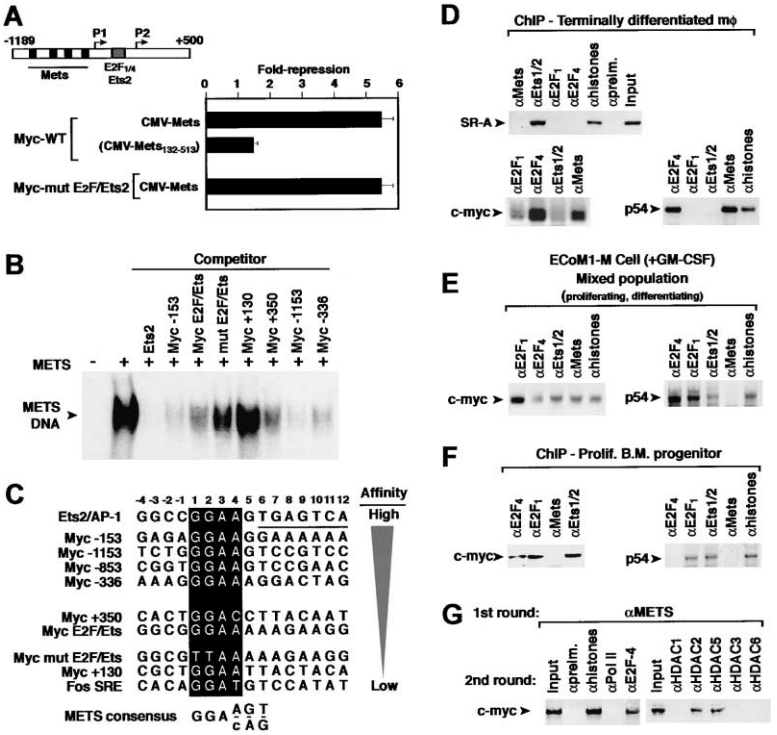

Figure 3. METS Discriminates between Monomeric and Composite Ets Binding Sites in Cell Cycle-Regulatory and Cell Type-Specific Promoters

(A) METS represses the *c-Myc* promoter in a DNA binding domain-dependent manner. CV-1 cells were transfected with a *c-Myc* promoter-luciferase reporter gene or a *c-Myc* promoter containing a mutation in the composite E2F/Ets2 element (Myc-mut E2F/Ets2). Cells were cotransfected with a CMV vector directing the expression of wild-type METS or METS132-513 lacking the Ets DNA binding domain.

(B) METS binds to a subset of Ets recognition elements with high affinity. Full-length METS was incubated with a ^{32}P-labeled DNA probe containing an Ets2 consensus recognition site, and protein/DNA complexes were resolved by an electrophoretic mobility shift assay. Relative binding to other DNA sequences was assessed by competition with a 100-fold excess of the indicated, unlabeled oligonucleotides.

(C) Alignment and relative binding affinities of METS for potential Ets binding sites within the *c-Myc* promoter.

(D) ChIP assays in terminally differentiated mouse peritoneal macrophages. Following formaldehyde crosslinking, genomic sequences corresponding to *c-Myc* +114/+215, the *p54* subunit of *DNA primase* −213/−66, and *SR-A* −486/−96 regions were subjected to PCR amplification following immunoprecipitation of protein-DNA products with the antibodies listed on top of each panel.

(E) ChIP assays for the *c-Myc* and *p54* subunit of *DNA primase*—promoters in EcoM1-M cells representing a mixed population of proliferating promyelocytes and differentiated F4/80-positive macrophages.

(F) ChIP assays for the *c-Myc* and *p54* subunit of *DNA primase* promoters in primary bone marrow progenitor cells.

(G) ChIP assays in terminally differentiated macrophages after primary immunoprecipitation with anti-METS IgG. In the second round, the antibodies listed at the top of the panel were used followed by PCR amplification of *c-Myc* +114/+215.

promoter (see Experimental Procedures). In addition, primers were designed to amplify a 3′ coding region of the c-Myc gene, which served as a negative control. ChIP experiments were initially carried out in thioglycolate-elicited mouse peritoneal macrophages, which are terminally differentiated and express high levels of *SR-A* but virtually no *c-Myc* (Guidez et al., 1998). Specific anti-METS IgG, but not anti-Ets1/2 IgG, was able to immunoprecipitate the *c-Myc* promoter region from terminally differentiated macrophages (Figure 3D). In contrast, the anti-Ets1/2 IgG, but not the anti-METS IgG, was capable of precipitating the *SR-A* promoter from the same population of sheared DNA fragments (Figure 3D). No enrichment of the 3′ region of the *c-Myc* gene was observed with either antibody, demonstrating the specific nature of protein/promoter DNA interactions (data not shown and Figure 6E). These experiments indicated that METS binds to Ets recognition elements in the *c-Myc* gene, but not to the composite AP-1/Ets site in the *SR-A* gene. In contrast, Ets2, and perhaps other Ets activators, remain bound to composite AP-1/Ets sites in terminally differentiated macrophages despite high levels of METS expression (Figure 3D). ChIP experiments were also used to evaluate the relative binding of METS, Ets1/2, and E2F transcription factors to the *c-Myc* and *p54* promoters in terminally differentiated macrophages, EcoM1-M cells, and proliferating bone marrow progenitor cells (Figures 3D–3F). EcoM1-M cells represent a mixed population in which the majority of cells proliferate continuously in the presence of GM-CSF as undifferentiated promyelocytes, with 20%–30% of the cells undergoing spontaneous differentiation to F4/80-positive macrophages. Anti-E2F$_4$ IgG was more effective than anti-E2F$_1$ IgG in enriching the *c-Myc* and *p54* promoters from terminally differentiated macrophages (Figure 3D), while the converse pattern was observed in EcoM1-M cells and proliferating bone marrow progenitor cells (Figures 3E and 3F). Similar results were obtained for the *Cdc2* promoter (data not shown and Figure 6E). In contrast to the results obtained in terminally differentiated macrophages, the anti-Ets1/2 antibody was capable of precipitating the *c-Myc* and *p54* promoters from both of these proliferating populations of cells (Figures 3E and 3F). These findings are consistent with previous studies reporting that Ets2 activates the *c-Myc* promoter in proliferating cells (Langer et al., 1992; Roussel et al., 1994) and suggest that Ets2 may also play roles in activation of other genes required for proliferation, such as *p54* and *Cdc2*.

To determine whether METS and E2F$_4$ bind simultaneously to the *c-Myc* promoter, ChIP experiments were performed in which sheared DNA was first immunoprecipitated with anti-METS IgG and then subjected to a second round of immunoprecipitation with anti-E2F$_4$ IgG (Figure 3G). These experiments indicated that E2F$_4$ and METS were bound to the same region of the *c-Myc*

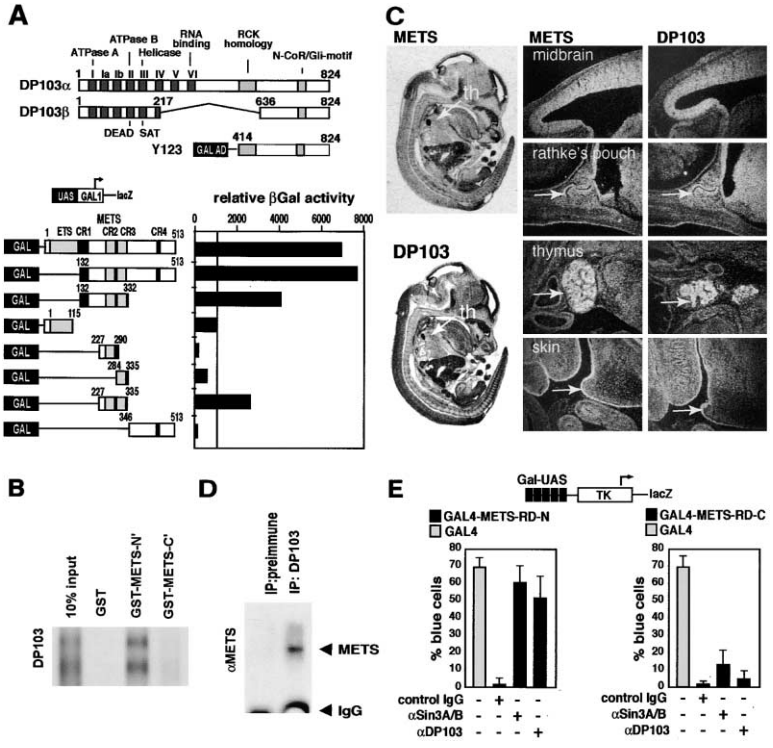

Figure 4. DP103 Interacts with METS and Is Required for RD-N Repression Activity

(A) Schematic representation of DP103α and β, indicating conserved domains. Y123 represents one of the initial METS-interacting clones obtained by two-hybrid screening and was used for quantitative interaction assays with the indicated Gal4-METS fusion proteins. The vertical line indicates the background activity of the GAL1-lacZ reporter gene.

(B) Interaction of ^{35}S-labeled DP103-414-824 with GST alone and GST fused to N- and C-terminal fragments of METS.

(C) Comparison of METS and DP103 expression patterns in whole-body sections of mouse embryos at E13.5.

(D) Western blot indicating coimmunoprecipitation of METS from whole-cell lysates of E2A-Pbx1-immortalized bone marrow precursors immunoprecipitated with anti-DP103 antiserum.

(E) DP103 is required for repression by RD-N of METS. A 3x-UAS-TK-lacZ reporter was injected into the nuclei of Rat 1 cells along with the indicated antibodies and plasmids directing expression of the GAL4 DNA binding domain, GAL4-METS-RD-N, or GAL4-METS-RD-C. Promoter activity was quantified by the percentage of injected cells that stained blue (X-gal staining).

promoter. In contrast, anti-RNA polymerase II IgG failed to precipitate the *c-Myc* promoter following initial precipitation with anti-METS IgG, consistent with the role of METS as a transcriptional repressor.

The ability of METS to repress transcription in transient transfection assays was sensitive to trichostatin A (TSA), suggesting the involvement of histone deacetylases (HDACs; data not shown). ChIP experiments were therefore carried out to determine whether specific HDACs were associated with *c-Myc* promoter sequences occupied by METS in terminally differentiated macrophages. Following initial immunoprecipitation with anti-METS IgG, the *c-Myc* promoter region could be secondarily immunoprecipitated with anti-HDAC2 and anti-HDAC5 IgGs, but not HDAC1, HDAC3, or HDAC6 IgGs (Figure 3G). Experiments testing the ability of METS to directly interact with HDAC2 and HDAC5 in vitro revealed little or no binding (data not shown), raising the possibility that they were recruited to the *c-Myc* promoter through an intermediary factor.

Identification of DP103 as a Corepressor of METS

To identify potential intermediary factors that might assemble a histone deacetylase complex at the N-terminal repression domain of METS and also mediate its antiproliferative actions, we searched for interacting proteins using a GAL4-based yeast two-hybrid screen. Among 1×10^6 cotransformants, three independent clones were isolated encoding the C terminus of a protein that bound METS with high affinity and specificity. A quantitative yeast two-hybrid assay using one of these clones (Y-123) indicated specific interactions with regions of METS encompassing RD-N, but not with RD-C (Figure 4A). The specificity of these interactions was confirmed in vitro by GST pull-down experiments (Figure 4B). Sequence analysis of full-length clones obtained by secondary screening of a human liver cDNA λ phage library revealed the encoded protein to be a member of the DEAD box family of RNA helicases independently isolated as DP103/Gemin3 (Charroux et al., 1999; Grundhoff et al., 1999), hereafter referred to as DP103α. Murine cDNAs and a previously unknown shorter splicing variant that we refer to as DP103β were obtained by analyzing expressed sequence tag (EST) clones of the IMAGE project. As shown in Figure 4A, the N-terminal half of DP103α contains eight conserved regions for ATP binding and hydrolysis, helicase activity, and RNA binding. In contrast, the C-terminal stretch of DP103α (amino acids 400–834) is unique to this molecule with the exception of amino acids 599–611 that can also be found in two other human DEAD box genes, i.e., RCK and the nuclear RNA helicase U90426. At its very C-terminal end, DP103 harbors the motif WYDCHRE found in Gli proteins and the SANT domain of N-CoR (Aasland et al., 1996). In situ hybridization analysis of METS and DP103 mRNA in mouse embryos indicated coexpression in several tissues, including midbrain, Rathke's pouch, thymus, and skin (Figure 4C). DP103 was also expressed in E2A-Pbx1-immortalized myeloid progenitor cells in which METS expression is induced during differentiation. METS and DP103 could be coimmunoprecipitated from these cells using an anti-METS antibody (Figure 4D).

To determine whether DP103 was a corepressor required for function of METS RD-N, nuclear microinjection experiments were performed in Rat 1 cells. Consistent with the specific binding of DP103 to RD-N of METS, microinjection of purified anti-DP103 IgG abolished the repressor activity of this METS domain, but had no effect on repression by the C-terminal domain (Figure 4E). Microinjection of anti-Sin 3A/B IgG also selectively blocked

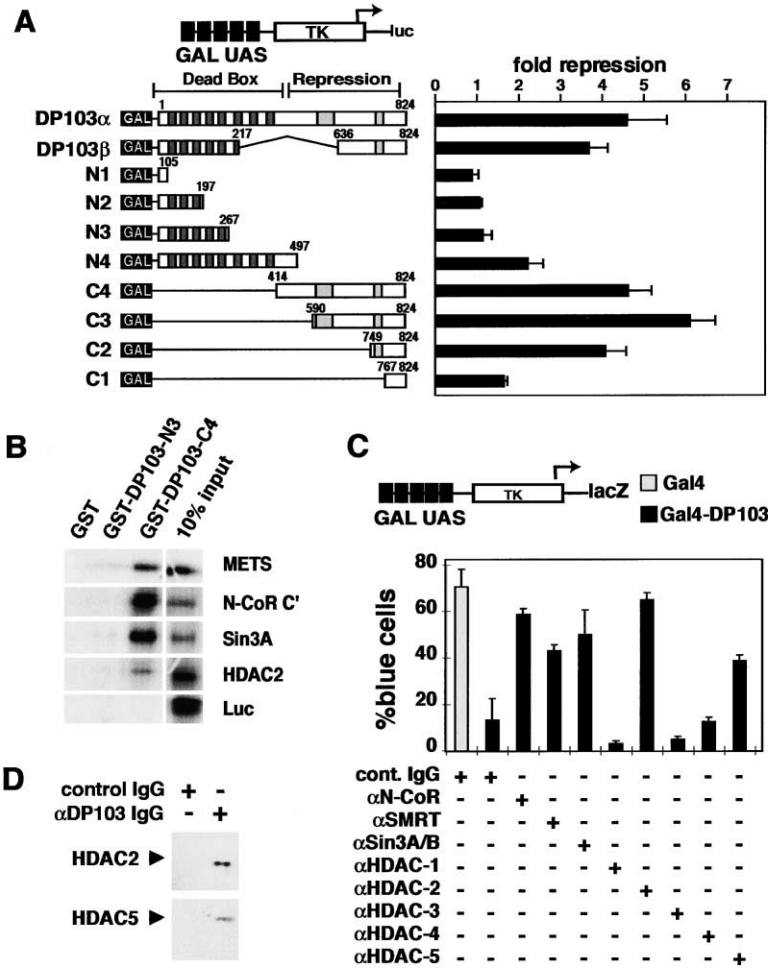

Figure 5. DP103 Exhibits Intrinsic Repressor Activity Dependent on Interactions with Sin3A/B, NCoR, and HDACs 2 and 5

(A) Mapping of the repressor activity of DP103 to the C terminus in a GAL-based transient transfection assay in CV-1 cells.

(B) In vitro translated SMRT, NCoR, Sin3A, and HDAC2 interact directly with GST fused to the C-terminal repression domain of DP103, but not the N-terminal DEAD box domain.

(C) DP103 repressor activity is abolished by microinjection of antibodies specific for N-CoR, SMRT, Sin3A/B, and histone deacetylases (HDACs) 2 and 5. Expression plasmids for GAL4 DNA binding domain alone or GAL4-DP103 were microinjected into the nuclei of quiescent Rat 1 fibroblasts along with the indicated antibodies and tested for their effects on the activity of the 5x-Tk-LacZ reporter.

(D) DP103 interacts with HDACs 2 and 5 in cells. Whole-cell extracts of E2A-Pbx1-immortalized bone marrow precursors overexpressing METS were immunoprecipitated with preimmune guinea pig serum or anti-DP103 antibodies. The immunoprecipitates were subsequently analyzed for HDAC2 and HDAC5 by Western blot using specific antibodies.

repression by RD-N, suggesting an additional involvement of Sin3 corepressor complexes.

When linked to the GAL4 DNA binding domain, DP103 exhibited intrinsic repressor activity on the UAS-TK (Figure 5A). This activity was mediated by the unique C-terminal domain of DP103, which was found to interact with METS, N-CoR, Sin3A, and histone deacetylase 2 (HDAC-2) in GST pull-down assays (Figure 5B). To test the functional importance of these interactions, the effects of nuclear microinjection of specific antibodies on the transferable repression activity of DP103 were evaluated in Rat 1 cells using the GAL4 system. Microinjection of affinity-purified antibodies specific for N-CoR, SMRT, Sin3A/B, HDAC-2, and HDAC-5 abolished or significantly reduced repression activity (Figure 5C). In agreement with these findings, HDAC-2 and HDAC-5 were coimmunoprecipitated from whole-cell extracts of E2A-Pbx1-immortalized bone marrow progenitor cells using a DP103-specific antibody (Figure 5D). These findings are also consistent with the ability to detect METS, HDAC2, and HDAC5 bound to the *c-Myc* promoter in terminally differentiated macrophages (Figure 3G). We have as yet been unable to localize DP103 itself to the *c-Myc* promoter by ChIP assay. However, available anti-DP103 antibodies are directed against the identical domains required for interaction with other corepressor components (data not shown). Therefore, these epitopes do not appear to be available for antibody recognition in ChIP experiments. In concert, these findings suggest that DP103 serves as an adaptor protein that nucleates the assembly of a novel corepressor complex required for function of METS RD-N.

To determine whether DP103 was required for METS-mediated inhibition of DNA synthesis, microinjection experiments were performed in Rat 1 fibroblasts using recombinant GST-METS and affinity-purified anti-DP103 IgG. Microinjection of this IgG reversed the inhibitory effects of METS on EGF-dependent incorporation of BrdU (Figure 6A). In the presence of anti-DP103 antibody, the antiproliferative effect of METS could be restored by injecting recombinant GST-DP103β that contains the C-terminal repressor domain. In contrast, an N-terminal fragment of DP103 containing only the RNA helicase domains was ineffective in rescuing the METS-mediated cell cycle arrest (Figure 6A). Hence, the DP103 repressor domain was required for the METS-dependent block of cell proliferation.

Antiproliferative Effects of METS Require pRb Family Proteins

To investigate whether METS can induce cell cycle arrest independently of pRb family proteins and E2F transcription factors, microinjection experiments were performed in murine embryonic fibroblasts (MEFs) lacking pRb, p107, and p130 (TKO MEFs; Sage et al., 2000). Wild-type and mutant MEFs were cultured in serum-free

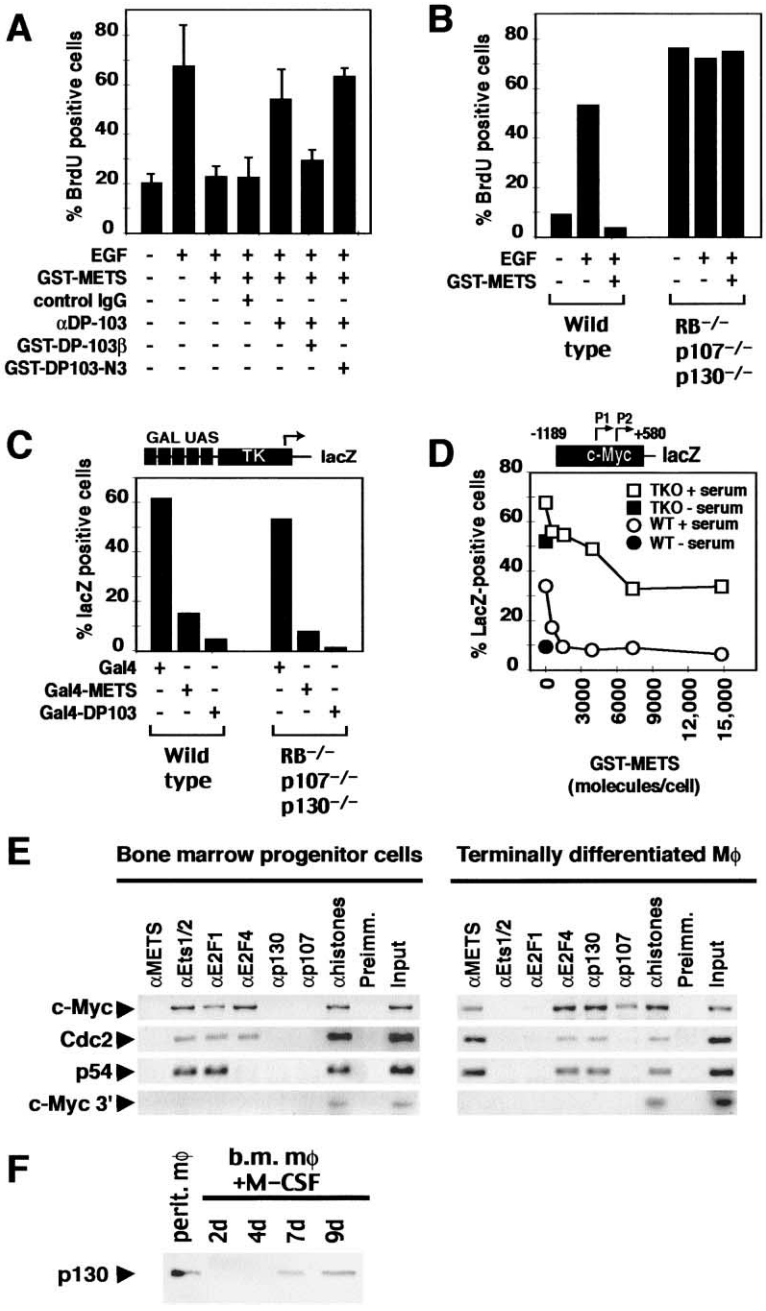

Figure 6. DP103 and the pRb Family Proteins Are Required for METS-Dependent Inhibition of DNA Synthesis

(A) Nuclei of Rat 1 cells were injected with GST-METS and affinity-purified IgGs directed against DP103, NcoR, and SMRT as indicated. Cells were treated with EGF to stimulate DNA synthesis as shown in Figure 2E. Anti- DP103 IgG abolished METS-mediated inhibition of EGF-stimulated cell proliferation. Effects of anti-DP103 IgG could be reversed by coinjection for GST-DP103β, but not by coinjection of GST-DP103-N3.

(B) MEFs with the indicated genotypes were injected with GST-METS and treated with EGF to stimulate DNA synthesis. MEFs lacking pRb, p107, and p130 exhibited a high rate of BrdU incorporation regardless of the presence of EGF that was not sensitive to inhibition by METS.

(C) MEFs with the indicated genotypes were injected with the UAS-TK-lacZ reporter gene and expression plasmids for GAL4, GAL4-METS, or GAL4-DP103.

(D) Effect of microinjection of calibrated amounts of GST-METS on expression of a *c-Myc*-lacZ reporter gene in wild-type MEFS (WT-MEFs) and MEFs lacking Rb, p107, and p105 (TKO cells).

(E) Chromatin immunoprecipitation assays assessing occupancy of the *c-Myc*, *Cdc2*, and *p54* promoters by Ets 1 and/or Ets2 (Ets1/2), E2F1, E2F4, p107, or p130 in asynchronous bone marrow progenitor cells (left) or terminally differentiated peritoneal macrophages. C-Myc 3′ represents a region 3′ of the *c-Myc* coding exons.

(F) Western blot analysis of p130 expression in peritoneal macrophages and macrophages derived from bone marrow progenitor cells in the presence of M-CSF; 100 μg of total cellular protein was run in each lane.

conditions for 24 hr, microinjected with GST or GST-METS, and treated with EGF as illustrated in Figure 6B. Wild-type MEFs exhibited EGF-dependent DNA synthesis that was blocked by METS, similar to results obtained in Rat 1 fibroblasts. In contrast, TKO MEFs lacking all three proteins of the Rb gene family exhibited a high rate of BrdU incorporation in the presence or absence of EGF, and this incorporation was not inhibited by METS (Figure 6B). These findings indicate that METS cannot inhibit DNA synthesis independently of the pRb family members. One possible explanation is that pRb, p107, and/or p130 are required for the intrinsic repression activity of METS. This does not appear to be the case, however, as GAL-METS and GAL-DP103 retained full repressor activity on the UAS-TK-lacZ promoter in TKO MEFs (Figure 6C). An alternative explanation is that the METS/DP103 complex functions in a combinatorial manner with E2Fs and the pRb-related proteins to repress cell cycle control genes. To investigate this possibility, the ability of calibrated amounts of microinjected METS protein to repress transcription of the *c-Myc* promoter in wild-type MEFs and TKO MEFs was evaluated (Figure 6D). In wild-type MEFs, *c-Myc* promoter activity was markedly induced by serum treatment, and this activity was completely inhibited by coinjection of physiologic concentrations of GST-METS protein. In contrast, the *c-Myc* promoter was highly expressed in TKO MEFs in the presence or absence of serum, and concentrations

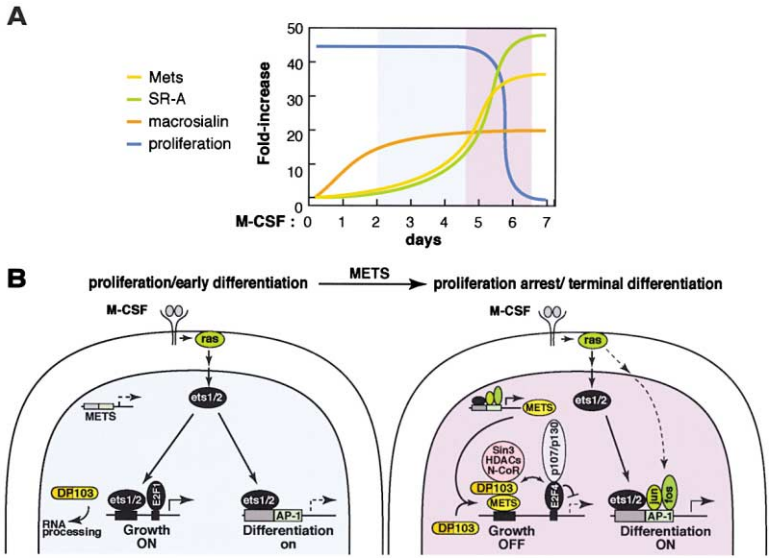

Figure 7. Model for Mechanism by which Induction of METS and Its Association with DP103 Functions to Selectively Inhibit Ras-Dependent Cell Proliferation

(A) Temporal sequence of events in macrophage progenitor cells treated with M-CSF. Induction of METS correlates with maximal induction of SR-A and cell cycle arrest.

(B) During terminal differentiation, METS selectively replaces Ets activators on cell cycle control genes and cooperates with E2F/p130 complexes to permanently silence transcription.

of GST-METS that fully repressed the *c-Myc* promoter in wild-type MEFs were nearly inactive in TKO MEFs.

To further investigate cooperation between METS and the Rb-related pocket proteins, ChIP experiments were performed in bone marrow progenitor cells and terminally differentiated macrophages (Figure 6E). In unsynchronized bone marrow progenitor cells, Ets1/2 and $E2F_1$ were observed to occupy the *c-Myc*, *Cdc2*, and *p54* promoters, with $E2F_4$ also being detected on the *c-Myc* and *Cdc2* promoters. While the pattern of $E2F_1$ and $E2F_4$ binding was similar to that reported in unsynchronized T98G cells (Takahashi, et al., 2000), p107 and p130 were not identified on E2F target genes in the progenitor cell population. METS was also not detected, consistent with its lack of expression in these cells. In terminally differentiated macrophages, neither Ets1/2 nor $E2F_1$ were detected on the *c-Myc*, *Cdc2*, and *p54* promoters. In contrast, METS, p107, and p130 were now detected on the c-Myc promoter in terminally differentiated macrophages, and METS and p130 were detected on the Cdc2 and p54 promoters. Intriguingly, Western blotting experiments indicated that p130 was not detectable in bone marrow progenitor cells, but was induced following 7–9 days of M-CSF-dependent macrophage differentiation (Figure 6F), coincident with induction of METS (Figure 1E).

Discussion

Dissociation of Ras-Dependent Proliferation and Differentiation Programs by METS

In this manuscript, we present evidence for a novel mechanism linking terminal differentiation to cell cycle arrest that is based on the induction of the Ets repressor METS/PE1. METS is expressed at low levels in macrophage progenitor cells and is maximally induced in terminally differentiated macrophages (Figure 7A). Retroviral-mediated expression or microinjection of physiologic levels of METS protein blocked growth factor-dependent DNA synthesis and inhibited expression of the endogenous c-Myc gene. Identification of METS target genes and analysis of its DNA binding specificity in vitro and in vivo suggest a general mechanism for how METS inhibits Ras signals required for proliferation, but not differentiation (Figure 7B). We propose that a set of Ets activators (e.g., Ets1, Ets2, and PEA3) that are responsive to Ras signaling and promote both proliferation and differentiation programs bind to two distinct classes of DNA recognition elements in target genes. One class of DNA binding sites, found in cell type-specific genes such as *SR-A,* consists of composite elements that are recognized by AP-1/Ets ternary complexes. The second class of recognition elements, present in target genes such as *c-Myc*, *c-Myb*, *Cdc2*, and the *p54* subunit of *DNA primase* that control cell cycle progression, are proposed to be recognized by Ets monomers. While METS is capable of binding to the same monomer consensus sequence recognized by Ets activators, it appears to be unable to form high-affinity ternary complexes required for activation of many cell type-specific genes. Thus, induction of METS expression results in selective displacement of Ets activators from monomeric binding sites on cell cycle control genes, thereby inducing cell cycle arrest without inhibiting Ras-dependent expression of cell type-specific genes (Figure 7B). A critical test of the ability of METS to discriminate between monomeric sites in cell cycle-regulatory genes and composite elements in cell type-specific genes was provided by ChIP assays, which clearly established selective binding of METS to Ets sites in the *c-Myc*, *Cdc2*, and *p54* promoters, but not to the composite AP-1/Ets site in the *SR-A* promoter in terminally differentiated macrophages. In contrast, Ets2 binding was observed on the *SR-A* promoter, but not the *c-Myc*, *Cdc2*, or *p54* promoters in these same cells. A recent report presented evidence that METS/PE1 can inhibit expression of the AP-1/Ets responsive *MMP1* gene by a DNA binding-independent mechanism in an osteoblast cell line (Bidder et al., 2000). This activity of METS/PE1 is not apparent in the primary macrophages or macrophage cell lines used in these studies, as AP-1/Ets-responsive genes such as SR-A, MMP9, and macrosialin were maximally induced

coincident with maximum expression of METS/PE1 (Figures 1 and 2).

Although the mechanisms that regulate *METS* expression remain to be defined, it is interesting to note that the sequence of the *METS* promoter itself contains two composite AP-1/Ets elements within 150 bp of the transcriptional start site (D.S.-V. and C.K.G., unpublished data). The progressive increase in the expression and activities of Ets2, c-Jun, and c-Fos that occurs during terminal macrophage differentiation (e.g., Figure 2A) may therefore not only drive the expression of genes such as *SR-A*, but may also serve as a timing mechanism for permanent exit from the cell cycle (Figure 7B) by inducing the expression of METS.

METS Recruits a Novel Corepressor Complex Required for Inhibition of Cell Proliferation

While factors required for function of the C-terminal repression domain of METS remain to be defined, the N-terminal repressor domain of METS that conferred antiproliferative effects required interaction with DP103. DP103 appears to nucleate the assembly of a complex consisting of components that are widely used by many other transcriptional repressors, although the specific composition of a complex involving both HDAC2 and HDAC5 has not been reported previously. DP103 has been independently identified as Gemin3, a component of the survival of motor neurons (SMN) complex, in which the putative RNA helicase activity is implicated in the building and recycling of spliceosomes (Charroux et al., 1999, 2000). It has also been described as a regulator of the steroidogenic factor-1 (Ou et al., 2001). The present studies indicate that DP103 is a bifunctional protein, containing independent RNA processing and corepressor functions. The observation that the C-terminal repression domain of DP103 has been found to interact with EBNA2 and EBNA3C is of particular interest with respect to the putative function of the METS/DP103 complex in inhibition of cellular proliferation. EBNA2 and EBNA3C are synthesized by the Epstein-Barr virus during infection of B-lymphocytes and are essential for viral transformation, but the mechanisms responsible for their transforming properties remain poorly understood. The present studies suggest the possibility that interactions of EBNA2 and EBNA3C with DP103 interfere with the formation and/or function of the METS/DP103 complex, contributing to the transforming activities of the Epstein-Barr virus.

A Combinatorial Code for Cell Cycle Arrest Involving Ets and E2F Corepressor Complexes

The observation that METS is unable to block DNA synthesis or completely repress the c-Myc promoter in cells lacking Rb, p107, and p130, but that it retains intrinsic repressive activity points to a combinatorial code of METS/DP103 and E2F/Rb-related signals for physiologic repression of cell cycle control genes (Figure 7B). Combinatorial interactions between multiple repressors have been shown to be required for appropriate cell-specific restriction of *zen* expression in the *Drosophila* embryo (Jiang et al., 1993; Kirov et al., 1993) and growth hormone gene expression in the anterior pituitary of mice (Scully et al., 2000). The present studies suggest a model in which functional interactions between cell type-specific transcriptional repressors, such as METS, and E2F/pRB family protein complexes can provide a mechanism for terminating proliferative responses to growth signals during development and thus ensure permanent exit from the cell cycle. In view of the critical importance of coordinating cell growth and differentiation and the redundancy of function of Ets activators, E2F transcription factors, and Rb-related pocket proteins, it is likely that additional Ets repressors will prove to contribute to this combinatorial mechanism of growth arrest.

Experimental Procedures

Cells and Transient Transfection Assays

CV-1 and 293T cells, Rat 1 fibroblasts, and pRB family TKO MEFs were maintained in DMEM with 10% fetal calf serum (FCS) and 1× penicillin-streptomycin (Pen/Strep). Primary murine progenitors were isolated from the femurs and tibias of BALB/c mice and separated from mature phagocytotic and lymphocytic cells on Ficoll-Paque gradients. Differentiation was induced by culture in RPMI with 20% FCS and 1× Pen/Strep supplemented with M-CSF (10 ng/ml). Culture and differentiation of Hoxa9-immortalized myeloid progenitor cells, EcoM1-G, and EcoM1-M myeloid progenitor cells was performed as described in Calvo et al. (2000) and Sykes and Kamps (2001). CV-1 and 293T cells were transfected with lipofectamine (Life Technologies) and analyzed as previously described (Moulton et al., 1994).

Antibodies

Anti-METS and anti-DP103 IgGs were raised in guinea pigs using purified, recombinant proteins corresponding to METS amino acids 391–513 and DP103 amino acids 590–824, respectively. The following antibodies were supplied by Santa Cruz Biotechnologies: anti-Pol II (#sc-900), anti-Ets2 (#sc-351), anti-HDAC-1 (#sc-7872), anti-HDAC2 (#sc-6296), anti-HDAC-3 (#sc-8138), anti-HDAC-5 (#sc-5252), anti-HDAC-6 (#sc-5258), anti-mSin3A (#sc-767), anti-mSin3B (#sc-768), anti-$E2F_1$ (#sc-193), anti-$E2F_4$ (#sc-866), anti-p107 (#sc-193), anti-p130 (#sc-317), and anti-RbAp46 (#sc-8273).

Cloning of METS cDNAs

Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using total RNA from THP-1 monocytic leukemia cells as the template along with the degenerate primers 5′-[ATC] [AC] [GATC] [CT] T [GATC] TGG [GC] A [GA] TT [TC] [TC] T [GATC] [CT] T-3′ (sense) and 5′-[CG] [GATC] [GATC] A [GAT] [GATC] [GT] T [GATC] [GC] [GA] TA [GATC] [GT] TCAT-3′ (antisense) corresponding to highly conserved regions of the ets DBD. The PCR product obtained for METS was used to screen a murine macrophage cDNA library, resulting in isolation of two independent full-length clones. A splicing variant of METS, encoding the N-terminal 135 amino acids and 8 unique C-terminal amino acids, was derived from two clones of a human adenocarcinoma cDNA library (IMAGE #1473929 and 1560232).

Cloning of DP103 cDNAs

A GAL4-based yeast two-hybrid screen of a human HeLaS3 cDNA library (Clontech) was performed by cotransforming expression vectors encoding METS_131-513 tethered to the GAL4-DBD (bait) and library cDNAs linked to the GAL4-AD coding sequence (prey) into the yeast strain Y190. Three independent clones were obtained that encoded the C-terminal end of DP103, whose full-length sequence was obtained by secondary screening of a human liver cDNA λ phage library.

DNA Binding Assays

EMSA assays were performed as described previously (Moulton et al., 1994). The sequence of the double-stranded oligonucleotides with core Ets binding motifs underlined was as follows: 5′-GATCCTA CACAGGATGTCCATATTAGGACAGATC-3′ (c-fos SRE), 5′-GATCAA

GAAAGGAAATGACACATTCCTGGATC-3′ (SRA promoter AP-1/Ets), 5′-GATCTTGGCCGGAAGTGAGTCATTCGTATTTTGGATC-3′ (consensus Ets2).

Chromatin Immunoprecipitation Assays

Cells were fixed with 1% formaldehyde and treated as previously described (Hecht and Grunstein, 1999). Recovered cross linked adducts were sheared by sonication, resulting in an average length of DNA postsonication of 400 bp. The supernatants were subjected to immunoprecipitation by using tosylactivated Dynabeads M-280 (Dynal, Oslo) coated with antibodies. Precipitated protein-DNA complexes were washed three times using binding buffer supplemented with 3% Empigene BB and then three times with the same buffer containing 1% Empigene BB. Protein bound, immunoprecipitated DNA was dissolved in TE buffer and incubated at 65°C overnight to reverse cross links. Following proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation, the samples were subjected to 35 cycles of PCR amplification using the following primers: for the *c-myc* promoter (+114/+215), forward 5′-CTC GCTGTAGTAATTCCAGCG-3′, reverse 5′-AGAGCTGCCTTCTTAG GTCG-3′; for the *c-Myc* 3′-coding region, forward 5′-CATCATCCAG GACTGTATGTGG-3′, reverse 5′-GGAGGCCAGCTTCTCCG-3′; for the *SR-A* promoter (−486/−96), forward 5′-CAGCCTCCTTTAGTCC ACATGG-3′, reverse 5′-CTCATTCCCTTTCCTAAAGGACA-3′; for the *Cdc2* promoter (+40/+214), forward 5′-GGTTGTTGTAGCTCGC TGC-3′, reverse 5′-CTAGCCGGCTTCTTAGAGG-3′; for the *p54* promoter (−213/−66), forward 5′-GCCACCAATTCCAAGTTAGC-3′, reverse 5′-TAGCGACTGAAGGTAGTGCG-3′.

Retroviral Expression of METS

cDNAs encoding full-length METS or METSΔDBD fused to the activation domain of VP16 were subcloned into the polylinker of the proviral vector MSCV-irGFP which contains an internal ribosomal entry site allowing for the bicistronic expression of GFP and the gene of interest (Persons et al., 1999). Helper-free virus was produced by cotransfection of 293T cells with MCSV vectors and an ecotropic packaging construct. Following infection of EcoM1-G or Rat 1 cells, fluorescence-activated cell sorting was performed to select GFP-positive cells.

RNA Analysis

RNase protection analysis of total RNA from primary bone marrow progenitor cells was performed as described (Guidez et al., 1998). Northern blots of Hoxa9-immortalized progenitor cells were performed as previously described (Calvo et al., 2000). The Hoxa9 cell line was maintained in the progenitor stage by culture in RPMI with 10% fetal calf serum and murine GM-CSF at 16 U/ml. To induce differentiation, the cytokine was switched to either 0.5 ng of G-CSF per ml or 10 ng of M-CSF per ml. In situ hybridization of sagittal sections of mouse embryos was carried out as previously described (Jepsen et al., 2000).

Nuclear Microinjection, Staining, and Fluorescence Microscopy

Microinjection experiments were carried out as previously described (Rose et al., 1999). Each experiment was performed on three independent coverslips totaling approximately 1000 cells. Where no experimental antibody was used, preimmune rabbit or guinea pig IgG was coinjected, allowing the unambiguous identification of injected cells in addition to serving as a preimmune control. BrdU incorporation experiments were conducted in serum-starved Rat 1 cells (Kolch et al., 1996). GST fusion proteins purified from bacterial extracts were microinjected at a concentration 10 to 100 μg/ml into the nuclei of cells with fluorescein-conjugated dextran as a carrier, resulting in the introduction of on the order of 1,000 to 10,000 molecules of fusion protein/cell. Cells were then treated with/without 100 ng/ml recombinant EGF in the presence of BrdU-labeling solution (Amersham) for 14 hr. After fixation, the cells were stained with a rat monoclonal anti-BrdU antibody (Accurate Scientific) and counterstained with a rhodamine-conjugated secondary antibody.

Protein-Protein Interaction Assays

Coimmunoprecipitations were carried out in whole-cell extracts from extracts E2A-Pbx1-immortalized bone marrow progenitor cells using specific guinea pig antisera raised against DP103_590-824 and METS_391-513. GST protein-protein interaction assays were carried out as described previously (Jepsen et al., 2000); DP103_590-824, full-length METS, N-CoR_1586-2453, full-length Sin3A, and full-length HDAC2 were translated in vitro using ^{35}S-labeled methionine and rabbit reticulocyte lysates (Promega).

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Accession Numbers

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