The Human MDM2 Oncoprotein Increases the Transcriptional Activity and the Protein Level of the p53 Homolog p63*

Received for publication, July 27, 2001, and in revised form, November 15, 2001
Published, JBC Papers in Press, November 19, 2001, DOI 10.1074/jbc.M107173200

Viola Calabro‡, Gelsomina Mansuetod, Tiziana Parisid, Maria Vivod, Raffaele A. Calogeroe, and Girolama La Mantiad

From the ‡Department of Genetics and General and Molecular Biology, University of Naples “Federico II,” via Mezzocannone 8, 80134 Napoli, Italy and the ¶Department of Clinical and Biological Sciences, S. Luigi Hospital, Orbassano, 10043 Torino, Italy

Genetic alteration of the p53 tumor suppressor gene, which monitors DNA damage and operates cell cycle checkpoints, is a major factor in the development of human malignancies. The p53 protein belongs to a family that also includes two structurally related proteins, p63 and p73. Although all three proteins share similar transcriptional functions and antiproliferative effects, each of them appears to play a distinct role in development and tumor suppression. One of the principal regulators of p53 activity is the MDM2 protein. The interaction of MDM2 with p53 inhibits p53 transcriptional activity and targets p53 for ubiquitin-dependent degradation. The ability of MDM2 to inhibit p53 functions is antagonized by the ARF oncosuppressor protein. We show here that like p53, the p63α and p63γ isoforms are able to associate with human MDM2 (HDM2). Overexpression of HDM2 increased the steady-state level of intracellular p63 and enhanced its transcriptional activity. Both effects appeared to be counteracted by ARF coexpression. These data indicate that p63 can be activated by HDM2 under conditions in which p53 is inhibited. Therefore, HDM2 expression could support p63-specific transcriptional functions on a common set of genes, keeping interference by p53 at a minimum.

The p63 gene, which maps on the 3q27–28 region, is one of the members of the p53 gene family. Unlike p53, it shows a complex pattern of expression due to alternative splicing and promoter usage that results in multiple isoforms with different biological activities (1, 2). Initiation of transcription in exon 1 produces the TA isoforms, containing the evolutionarily conserved transactivation, DNA-binding, and oligomerization domains, whereas initiation in exon 3 gives rise to the AN isoforms that lack the TA domain (3). p63 shows a remarkable structural similarity to p53 and to the related p73 protein: ~60% of the amino acids of the human p63 and p73 proteins in the region corresponding to the DNA-binding domain are identical to those of p53 (4).

In contrast with the ubiquitous expression of p53, p63 exhibits a rather tissue-specific distribution in that it is most detectable in the basal layer of stratified epithelia, including the epidermis, where the ΔNp63α isoform is predominantly expressed (3). However, it is still not known how the expression of different isoforms of p63 is regulated in different tissues and during development.

All three members of the p53 family share similar transcriptional functions, as p63 and p73 can also activate many of the p53 target genes, although with varying efficiency (5). Moreover, like p73, p63 is able to induce apoptosis and growth suppression in a manner similar to p53 (4).

Molecular alterations of p63 or p73 in human cancers appear to be rare; unlike p53-deficient mice, those lacking p73 or p63 show no increased susceptibility to spontaneous tumorigenesis (6, 7). Viral oncoproteins such as SV40 large T antigen, adenovirus E1B, and human papilloma virus E6, which bind and inactivate p53, do not target p73 and p63 (2, 8). Thus, it seems likely that p63 and p73 are not potent suppressors of abnormal proliferation.

Unlike p53, both p73 (6) and p63 appear to contribute to normal development. This is most dramatically illustrated by reports showing that p63-deficient mice have severe defects in limb and skin development (9). Moreover, heterozygous germ-line mutations in the p63 gene are the cause of ectrodactyly-ectodermal dysplasia-clefting (10) and ankyloblepharon-ectodermal dysplasia-clefting (11) syndromes in humans.

p53 is normally a short-lived protein. Regulation of the p53 protein occurs to a large extent through control of protein stability, and the MDM2 (murine double minute 2) protein has been shown to play a key role in targeting p53 for degradation (2). The ARF (alternative reading frame) protein, one of the alternative products of the INK4a locus, binds to the MDM2 protein, preventing MDM2-dependent p53 degradation and transcriptional silencing (12). Concerning the other members of the p53 family, it has recently been demonstrated that p73 also binds MDM2. MDM2 inhibits p73-dependent transcription by masking the p73 transactivation domain and/or disrupting the interaction of p73 with p300/CBP (cAMP-responsive element-binding protein-binding protein), but it is clearly not involved in the degradation of p73 (13). Here, we have investigated whether HDM2 (human homolog of murine double minute 2) and ARF are involved in the control of p63 functions. We have found that p63 is able to physically interact with HDM2. Overexpression of HDM2 increased the steady-state level of intracellular p63 and enhanced its transcriptional activity. Both effects were counteracted by ARF coexpression. Because of its opposite effects on p53 and p63 protein stability and transcriptional activity, MDM2 expression could support p63-specific transcriptional functions on a common set of genes, at the same time reducing interference by p53.
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EXPERIMENTAL PROCEDURES

Plasmids—The p63y and p63a cDNAs were isolated from a human skeletal muscle cDNA library using a PCR-based technique and cloned into the BamHI site of the pcDNA3-His expression vector (Invitrogen) to express them as Xpress epitope-tagged proteins. The amplification sequence consisted of 35 cycles of 98°C for 1 min, 62°C for 1 min, and 72°C for 1 min, after starting with a denaturation step at 95°C and ending with a 10-min extension step at 72°C for 10 min. A common 5′-forward primer (5′-CGGATCCATGGCGAGAAGCACAGACAGAACATGG) and a p63a-specific 5′-GGATATGTTTCCCTCCCTCTCCCTCCTCTCATGGCGAGAAGCACAGACAGAACATGG (and a p63y-specific 5′-GGTTGCGATGCTAGCAGTATGTCATGCATAGG) were employed to obtain p63a and p63y, respectively. Wild-type p53 in pcDNA3 and the L22Q/W23S p53 mutant in the presence of the GFP (originally from Dr. A. J. Levine). The p63a cDNA, previously described (15), was cloned into the pCMV-rImager (Molecular Dynamics, Inc.) and ImageQuant software. The identity of the p63 bands was confirmed using anti-p63 antibodies (N-18, Santa Cruz Biotechnology). The p21WAF1 protein was revealed using mouse anti-human p21 antibody (6B6 (Pierce). Human MMD2 (HDM2) was detected with antibody smp14. The p53 protein was detected with the anti-Xpress antibody. The mouse anti-human p53 antibody Pal240 (Pierce) as indicated. Bands were visualized with an enhanced chemiluminescence system (Pierce). To compare the stability of p63a and p63y, Saos2 cells expressing the indicated cDNAs were treated with cycloheximide (final concentration of 80 μg/ml) and harvested at the indicated time points. Cells were processed for Western blotting as described above. The 26 S proteasome inhibitor ALLN (50 μM) (Sigma) was used. Tubulin was detected with an anti-tubulin antibody (C-20, Santa Cruz Biotechnology). For reverse transcriptase-PCR, 24 h after transfection, cells were collected, and total RNA was isolated using the Trizol LS reagent (Invitrogen) following the manufacturer’s instructions. 500 μg of total RNA were reverse-transcribed using 200 units of Superscript II (Invitrogen) and PCR-amplified as described above. The 600-bp fragment of the human hypoxanthine phosphoribosyltransferase gene was amplified using the following primers: 5′-CTTGAATTCATAGTATTAGTCAAGG

RESULTS

Comparison of Transcriptional Properties of p63y and p63a in Mammalian Cell Lines—The p63y and p63a cDNAs encode proteins of 448 and 641 amino acids, respectively (4). The longer α isoform possesses an extended C-terminal region of 187 residues; but the rest of the protein, with the exception of the last 40 residues of the γ isoform, is shared by the two protein isoforms. The C-terminal region of p63a includes a sterile α-motif that has been described as a putative protein-protein interaction domain (16). The three major domains of p53 (NH2-terminal transactivation, DNA-binding, and oligomerization domains) are conserved in both the γ and α isoforms. We isolated the p63y and p63a cDNAs by reverse transcriptase-PCR from a human skeletal muscle library and cloned them into the pcDNA3.1-His expression vector. Before assessing the effect of HDM2 on the transcriptional activity of both p63 isoforms, it was of interest to compare the transcriptional properties of the two p63 isoforms on two canonical p53-responsive promoters, p21WAF1 and HDM2 (BP100-CAT).

C3a2 (5′-p35) and Saos2 cell lines, which not only lack endogenous p53, but also exhibit low levels of p73 (17), were transfected with equal amounts of p63y or p63a expression vectors together with the CAT reporter plasmids. As a positive control, we also transfected a p53 expression vector. As shown in Fig. 1 (A and B), both p63a and p63y expression vectors activated CAT activity, although a significant difference in efficiency was observed. p63a enhanced CAT expression driven from either promoter less strongly than p63y in both cell lines. Although the ARF promoter does not contain p53-binding elements, it is trans-repressed by p53 (18), suggesting the existence of an autoregulatory feedback loop limiting the effect
of ARF on p53 stabilization. Based on the functional similarity between p53 and p63, we tested whether or not p63 was also able to inhibit transcription driven by the human ARF promoter. C33A cells were cotransfected with the ARF-CAT reporter plasmid and the p53, p63γ, or p63α expression plasmid. Fig. 1C shows that both p63γ and p63α reduced ARF-CAT expression, although less efficiently than p53. These results indicate that p63 also shares the trans-repression ability with p53. Again, p63α appears to be more efficient than the α isoform.

To investigate whether the differences observed in the transcriptional properties of p63γ and p63α were due to different expression levels of the α and γ isoforms, we measured the protein levels of the two isotypes 48 h after transfection in the Saos2 cell line. The stronger transcriptional activity of p63γ cannot be attributed to a higher expression level, as Western blot analysis revealed that p63α levels exceeded those of p63γ (Fig. 2A). Similar results were obtained in COS-7 cells (data not shown). These results suggest that the extended C-terminal region, which distinguishes p63α from p63γ, could influence the level of the p63 protein, perhaps altering its half-life.

We assessed this possibility by introducing expression vectors for p63α and p63γ into Saos2 cells and following their protein levels after treatment with cycloheximide (19). Because cycloheximide inhibits de novo protein synthesis, the half-life of the proteins could be determined by Western blot analysis in cells treated with the drug. As Fig. 2B clearly shows, p63α had a markedly prolonged half-life.

HDM2 Enhances the Transcriptional Activity of p63—Because MDM2 inhibited both p53 and p73 transcriptional activity, we wanted to determine whether it also affected p63-driven transcription. To investigate this point, we cotransfected Saos2 cells with the BP100-CAT reporter plasmid, p63γ (Fig. 3A), or p63α (Fig. 3B) as transactivator and increasing amounts of HDM2. As shown in Fig. 3 (A and B, third and fourth bars), cotransfection of p63γ or p63α and HDM2 expression plasmids in 1:2 and 1:4 molar ratios produced a remarkable enhancement of p63 transcriptional activity. As a control, we performed the same experiment using p53 as transactivator. As expected, coexpression of HDM2 considerably reduced the p53 transcriptional activity.
A 1:1 molar ratio (second bar) of p53 to HDM2 was already sufficient to reduce the p53 transcriptional activity to 46% with respect to that observed without HDM2, and increasing amounts of HDM2 caused no more than an additional 10–16% reduction of p53 transcriptional activity.

In Vitro and in Vivo Association of p63 with HDM2—The interaction between MDM2 and p53 inhibits the p53 transactivation ability and targets p53 for ubiquitin-dependent degradation. Taking into account the effect of HDM2 on p63 transactivation ability, we decided to assess the effect of HDM2 expression on the level of the p63 protein. First, we wanted to test whether p63 may physically interact with HDM2. Extensive mutational analyses of the HDM2-binding domain of p53 (FSDLW) have identified Phe19, Leu22, and Trp23 as the critical residues for transcriptional activation and p53 binding by MDM2. We observed that these amino acid residues, except for a conservative Leu22-to-Ile substitution, are present in the p63 MDM2-binding domain (FQHIW). The p63\(^\gamma\)/H9253 and p63\(^\gamma\)/H9251 proteins were synthesized and \(^{35}\)S-Met-labeled by an in vitro transcription/translation assay. The L22Q/W23S p53 mutant protein, which is unable to interact with MDM2 (20), and wild-type p53 were obtained in a similar way. SDS-PAGE followed by autoradiography revealed that proteins of the expected size and in comparable amounts were obtained in all cases (data not shown). The reticulocyte lysates were then incubated with the GST-agarose-immobilized GST-HDM2 fusion protein. After appropriate washing, the bound proteins were subjected to SDS-PAGE and detected by autoradiography. Fig. 4A shows that the interaction of both p63\(^\gamma\) and p63\(^\alpha\) with the GST-HDM2 protein was comparable to that of wild-type p53. Using the mutant p53 protein, a negligible amount of protein was detected.

To confirm the interaction between p63 and HDM2 in intact cells, we cotransfected Saos2 cells with p63\(^\alpha\) or p63\(^\gamma\) and the HDM2-encoding expression vector. Cellular lysates were immunoprecipitated with polyclonal anti-p63 antibody H-137 and probed with the monoclonal anti-MDM2 antibody. As shown in Fig. 4B, HDM2 was co-immunoprecipitated with the polyclonal anti-p63 antibody when coexpressed with p63\(^\alpha\) or p63\(^\gamma\).
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Fig. 4. HDM2 binds to p63. A, GST-HDM2 fusion proteins immobilized on Sepharose were incubated with 20 μl of in vitro translated, 35S-labeled p53, L22Q/W23S p53, p63γ, or p63α at 4 °C for 1 h as indicated. Bound proteins were analyzed as described under “Experimental Procedures.” A control of binding with GST alone is also shown. B, Saos2 cells were transfected with plasmid encoding p63γ or p63α with or without HDM2 as indicated. At 24 h post-transfection, cells were harvested for immunoprecipitation (IP). p63-HDM2 complexes were analyzed by immunoprecipitation using polyclonal anti-p63 antibodies. Proteins were revealed with anti-Xpress and anti-MDM2 antibodies (as indicated). The positions of molecular mass marker are indicated to the left. WB, Western blot.

degradation (13), indicating that binding to MDM2 is necessary but not sufficient for degradation. Recent findings indicate that a proline-rich sequence (from amino acid 92 to 112) of p53 is a degradation signal (19). This degradation signal is not present in the p73 and p63 proteins. To elucidate the effect of HDM2 on p63 protein levels, we transiently transfected COS-7 cells with equal amounts of p53 (Fig. 5A) and p63 (Fig. 5B) and increasing amounts of HDM2 expression vector. 0.5 μg of plasmid pEGFP-C1 were included as the transfection control. Cellular lysates were subjected to immunoblotting with antibodies against the Xpress epitope (Fig. 5B). The identity of the p63 bands was then confirmed using an antibody raised against the Xpress epitope (Fig. 5B). The positions of molecular mass marker are indicated to the left.

WB with αMDM2

WB with αXpress

To determine whether the elevation in the p63α protein level was due to an increase in transcription or stability of p63α mRNA, we performed reverse transcriptase-PCR experiments. Fig. 6A shows that the relative level of p63α mRNA was similar in the presence and absence of HDM2, suggesting that the effect of MDM2 on p63 may be post-translational. Fig. 6B shows that, after treatment with ALLN, a proteasome inhibitor, more p63α protein was detected, suggesting that the p63α protein may be degraded by a proteasome-dependent pathway. Significantly, ALLN did not further increase the level of p63α in the presence of HDM2, suggesting that HDM2 and ALLN may both act to prevent proteasome-dependent degradation. Similar results were obtained with the p63γ isotype (data not shown). Hence, HDM2 increases p63 protein levels under conditions in which p53 is degraded.

ARF Abolishes p63 Stabilization and Transcriptional Activation Induced by HDM2—Among the growing number of proteins that interact with MDM2, particular interest has recently been focused on ARF, which is encoded by the INK4a locus. Because ARF binds to the MDM2 protein, preventing MDM2-dependent p53 degradation and transcriptional silencing (12, 22), we predicted that ARF could counteract the effect of MDM2 on p63. To test this hypothesis, we cotransfected in Saos2 cells, the BP100-CAT (Fig. 7A) or WAF-CAT (data not shown) reporter with a fixed amount of HDM2 and increasing amounts of ARF. We used a molar ratio (4:1) of HDM2 to p63α.
that we know results in a strong enhancement of p63 transcriptional activation and protein stabilization (Figs. 3B, fourth bar; and 5C, lane 4). As shown in Fig. 7A, when increasing amounts of ARF were added, the increase in p63 transcriptional activity induced by HDM2 was progressively abolished. No effect was observed on the p63-driven transcription of the reporter plasmids when only ARF was expressed (Fig. 7A, sixth bar). We then tested whether or not ARF was also able to reduce the HDM2-induced enhancement of the p63 protein level. The p63α expression plasmid was transfected in the Saos2 cell line with or without a fixed amount of HDM2 expression vector and increasing amounts of ARF expression vector (Fig. 7B). Exogenous expression of HDM2 produced an increase in the p63 level (Fig. 7B, lane 5) that was progressively abolished by the addition of increasing amounts of ARF expression vector (lanes 6–8). Moreover, as the Saos2 cells expressed detectable levels of endogenous HDM2 proteins (Fig. 7B, lane 1), a slight reduction of the p63 protein level was also seen when ARF alone was overexpressed (lanes 2–4). Similar results were obtained using the p63y expression vector (data not shown).

**DISCUSSION**

Although there exists extensive information on the relationship between MDM2 and p53, far less is known about a possible functional interaction of MDM2 with p63. In this study, we report that MDM2 overexpression causes an increase in overall p63 protein levels due, at least in part, to a reduced rate of p63 protein degradation. Moreover, although MDM2 represses p53 transcriptional activity, enforced MDM2 expression causes instead a considerable enhancement of p63-mediated transcription, which can be ascribed to the increase in transcriptionally active p63 protein.

While this paper was in preparation, several studies were published reporting conflicting results on the functional relationship between p63 and the MDM2 protein. For instance, it was reported that exogenously expressed MDM2 represses p63-mediated transcription (23). On the other hand, it was proposed that MDM2 is unable to affect its half-life or its transcriptional function (24), in conflict with the present and above-mentioned papers. It is well documented that transiently transfected p63 is able to strongly induce the endogenous MDM2 protein (25), and we repeatedly observed that only at a low level of p63 exogenous expression is the induction of endogenous MDM2 negligible, so that the stabilization effect by transfected MDM2 becomes apparent. However, compared with our results, both Kadakia et al. (23) and Little and Jochemsen (24) obtained remarkably higher levels of p63α and p63y exogenous expression, already sufficient to induce expression of endogenous MDM2. A possible explanation of the apparent discrepancy with our results is that, under their experimental conditions, p63 exogenous protein had already undergone stabilization, so that addition of exogenous MDM2 caused no further effect.

Moreover, we also demonstrate that p63 proteins are able to form a complex with HDM2 both in vitro and in mammalian cells, suggesting that the mechanism through which HDM2 regulates p63 expression requires a physical interaction between these proteins. Because it is well established that ARF stabilizes p53 by binding and sequestering MDM2, we expected ARF to exert an inhibitory effect on p63 protein stabilization. In fact, ARF coexpression abolishes both MDM2-induced p63 protein stabilization and transcriptional activation, giving further evidence that p63-MDM2 interaction has a functional role.

A recent analysis of the molecular interactions of p63 in a yeast two-hybrid system (26) suggested that p63 does not associate with MDM2 family proteins. However, this analysis was performed using only the N-terminal portion of the p63

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**FIG. 5. HDM2 increases p63 intracellular levels.** A, COS-7 cells were transfected with the pcDNA3 or p53 expression vector (0.2 μg/dish) in combination with increasing amounts of HDM2: 0.2 μg (lanes 2 and 5) and 1.6 μg (lanes 3 and 6). Extracts from COS-7 cells were subjected to immunoblotting with antibodies against p53, MDM2, and GFP as indicated. B, COS-7 cells were transfected with the p63α expression plasmid (0.2 μg/dish; lanes 1–5) in combination with increasing amounts of HDM2: 0.2 μg (lane 2), 0.4 μg (lane 3), 0.8 μg (lane 4), and 1.6 μg (lane 5). Extracts from COS-7 cells were subjected to immunoblotting with antibodies against the Xpress epitope, MDM2, p21, and GFP as indicated. C, Saos2 cells were transfected with expression plasmids (total of 2 μg of DNA) for p63α (0.2 μg/dish; lanes 1–4) and p63y (0.2 μg/dish; lanes 5–8) in combination with increasing amounts of HDM2: 0.2 μg (lanes 2 and 6), 0.4 μg (lanes 3 and 7), and 0.8 μg (lanes 4 and 8). At 48 h post-transfection, the cells were harvested and extracted as described under “Experimental Procedures.” Western blotting was performed with anti-Xpress, anti-MDM2, and anti-GFP antibodies as indicated. The pEGFP-1C vector was included as a control of transfection efficiency.
protein (amino acids 1–111), so it cannot be excluded that other regions of the protein are essential for the p63-MDM2 interaction or that some tertiary structure formed by a more extended region may also be required for the binding. On the other hand, Little and Jochemsen (24) detected a weak interaction by an in vitro assay, but this could not be confirmed by co-immunopre-
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Cipitation in mammalian cells. Because we observed that MDM2 displays a lower affinity for p63 than it does for p53, it is possible that the use of less efficient antibodies or more stringent washing conditions could have hampered the analysis of the protein-protein interaction by immunoprecipitation. Remarkably, MDM2 has been reported to facilitate p63 export from the nucleus (29), but whether a direct MDM2 association with p63 is required for this activity remains to be elucidated.

Until recently, a similarly controversial question has been how MDM2 regulates the stability and transcriptional activity of the third member of the p53 family, the p73 protein (27, 28). Now a clear picture is emerging: MDM2 interacts with p73, stabilizing and enhancing its growth-suppressive function (29). Moreover, in sharp contrast to p53, MDM2 induces p73 to form nuclear aggregates that colocalize with MDM2 (30). Furthermore, p73 levels are increased in MDM2-expressing cells (30). Both p63 and p73 therefore bind to MDM2, but are refractory to MDM2-mediated degradation, indicating that binding is necessary but not sufficient for degradation. These results are not surprising given that, although the N-terminal MDM2-binding motif of p53 is conserved in both p63 and p73 (31), p53 has a unique sequence element (amino acids 92–112) that functions as a signal for a MDM2-mediated degradation (19). How this sequence of p53 functions as a degradation signal remains to be defined.

Although we have not determined the precise mechanism by which MDM2 increases p63 protein levels, our data argue that, like the proteasome inhibitor ALLN, MDM2 may act by preventing p63 proteasome-dependent degradation. In addition to MDM2, the p300/CBP protein has also been shown to play a role in allowing efficient p53 degradation. Surprisingly, loss of p300 activity results in an inability to stabilize p53 in response to DNA damage, indicating that there is a complex relationship between p300 and p53 stability (32). It has been proposed that the reason why p73 is refractory to MDM2 degradation might be related to the observation that, unlike p53, p73 is unable to bind both MDM2 and p300 simultaneously (13). A similar mechanism could also explain the p63 resistance to MDM2 degradation. We are currently investigating the relationship between p63 and the p300/CBP coactivator as well as the exact pathway through which MDM2 induces an increase in p63 intracellular levels.

In conclusion, MDM2 seems to regulate p53 and its homologs through completely opposite mechanisms, suggesting that both p73 and p63 could be involved in specific cellular defense mechanisms against the deregulated expression of MDM2. We can also speculate that, in cells expressing both p63 and p53 proteins, certain stimuli that up-regulate MDM2 can, at the same time, activate p63 functions by keeping p53 activity at a minimum, whereas oncogenic stimuli that induce the ARF protein can cause the opposite. Moreover, once activated, p63 might contribute to its activation by keeping the level of ARF transcription low. These considerations suggest that the role of p63 may not be as central as that of p53 in tumor suppression, although it cannot be excluded that p63 could provide a protection from cancer development in tissues expressing both p53 and p63 proteins.

The role of p73 and p63 during normal development, the identification of differentiation genes specifically activated by p63 and p73 but not by p53 (33), and the difference in the ability to transactivate p53 target genes all strongly support the notion of these proteins, although closely related, have differentiated distinct physiological functions. The difference observed in the mechanisms adopted by MDM2 to control their functions further supports this emerging view.

Acknowledgments—We thank Rosaria Terracciano for technical help and Drs. G. Del Sol, S. Soddu, A. J. Levine, and B. Vogelstein for the generous gift of some of the plasmids used in this study.

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Viola Calabrò, Gelsomina Mansueto, Tiziana Parisi, Maria Vivo, Raffaele A. Calogero and Girolama La Mantia

J. Biol. Chem. 2002, 277:2674-2681.
doi: 10.1074/jbc.M107173200 originally published online November 19, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107173200

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