Role of MDM2 In Cell Growth Regulation

Rebecca Anne Frum
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ROLE OF MDM2 IN CELL GROWTH REGULATION.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

By

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Dedication.

I would like to dedicate this work to my family and friends. Thank you for your patience and support. This work is also dedicated to anyone who looks beneath the surface and gives marginalized ideas a chance.

Acknowledgments.

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List of abbreviations.

7AAD 7-amino actinomycin D
ARF Alternate reading frame
BrdU Bromodeoxyuridine
CDK Cyclin-dependent kinase
DAPI 4”,6-Diamidino-2-phenylindole, dihydrochloride
FITC Fluorescein isothiocyanate
HDAC Histone deacetylase
ID1 Inhibitory domain 1
ID2 Inhibitory domain 2
IP Immunoprecipitation
MDM2 Murine double minute-2
MEF Mouse embryo fibroblast
PE Phycoerythrin
PI Propidium iodide
Rb Retinoblastoma susceptibility protein
ROLE OF MDM2 IN CELL GROWTH REGULATION.

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Virginia Commonwealth University, 2006.

Director: Swati Palit Deb, Ph.D., Associate Professor, Biochemistry

MDM2 has been shown to induce G0-G1/S phase arrest. To determine the cell cycle step targeted by MDM2, flow cytometry was employed to detect induction of events during the G1-S phase transition in MDM2-arrested cells. MDM2 overexpression does not prevent expression of cyclin D, cyclin D-CDK mediated phosphorylation of Rb or cyclin E in normal, immortal or tumor-derived cells. However, MDM2 down-regulates cyclin A expression specifically in normal cells, which is associated with G1 arrest. The domain of MDM2 capable of this function is located within its N-terminal 58-109 amino acids. To down-regulate cyclin A, MDM2 requires a functional p16/Brg1 pathway, as silencing of either of these proteins disables this function of MDM2. Bromodeoxyuridine incorporation studies suggest that another inhibitory domain, ID2, inhibits DNA replication, while an MDM2 deletion mutant containing the N-terminal 1-220 amino acids including inhibitory domain ID1 does not effectively prevent BrdU incorporation in an immortal cell line that is non-responsive to growth arrest by the cyclin A inhibitory
domain. This suggests that induction of MDM2 leads to G1 arrest by at least two independent mechanisms, and multiple genetic damages are necessary to overcome MDM2-mediated growth arrest. To determine novel interacting partners of MDM2, proteomic analysis of MDM2 overexpressed in tumor-derived H1299 cells was carried out. This analysis revealed interaction of MDM2 with the translation elongation factor ef1-α, and was validated by immunoprecipitation and Western blotting and shown to colocalize with MDM2 in the cytoplasm. To interact with ef1-α, MDM2 was determined to require two domains, one of which is located within amino acids 221-325 and another within the N-terminal 58 amino acids of MDM2.
H. Chapter 1. Introduction

**MDM2 is potentially oncogenic:** The human homologue of the murine double minute-2 gene (MDM2) is frequently overexpressed in many human carcinomas, soft tissue sarcomas and other cancers (1-17). Amplification of the mdm2 gene enhances the tumorigenic potential of murine cells (18,22). Overexpression of mdm2 messenger RNA from the amplified gene (5,11,19-20) and enhanced translation of mRNA have been proposed as mechanisms of MDM2 overexpression (12,21,23). These findings suggest that MDM2 has oncogenic properties. Targeted overexpression of MDM2 in transgenic mice causes polyplody. Overexpression of MDM2 in breast epithelial cells inhibits normal development of the mammary gland. (24). Targeted overexpression of MDM2 in the basal layer of the epidermis increases papilloma formation by chemical carcinogens (25). These findings suggest the requirement of added genetic damage for MDM2-induced tumorigenesis.

**MDM2 interacts with the tumor suppressor p53:** MDM2 recognizes the transactivation domain of p53 and this interaction physically blocks p53-mediated transcriptional activation (19,26-31). Work from our laboratory showed that the interaction of MDM2 with p53 is needed for inhibition of p53-mediated transactivation (27,29). Consistent with its ability to inhibit p53-mediated transactivation, MDM2 inhibits p53-mediated growth suppression and apoptosis in tumor-derived cells (32-34). The p53-regulatory function of MDM2 is required for embryonic development (35,36).

Wild-type p53 induces MDM2 expression through the P2 promoter by recognizing a response element situated downstream of the first exon of the oncogene (37-39). Thus, presence of an autoregulatory feedback loop has been suggested, in which a
Figure 1. Domains of MDM2. Domains of MDM2 identified for various protein interactions and other functions are shown.

A higher level of p53 expression will arrest the cell cycle at the G1 phase and, at the same time, will induce MDM2 expression, which in turn will inactivate p53 (40). However, recent studies suggest that DNA damaging agents induce phosphorylation of p53 and ATM-dependent phosphorylation of MDM2. The phosphorylated p53 and MDM2 are incapable of mutual interaction (41-43).

**MDM2 is an E3 ubiquitin ligase and can degrade p53**: MDM2 degrades p53 by targeting p53 to ubiquitination (44-47). However, mutants of MDM2 lacking the E3 ubiquitin ligase activity can efficiently bind with wild-type p53 and inhibit p53-mediated transcriptional activation (29). This could be a cellular mechanism for turnover of p53-MDM2 complexes. This view is also consistent with the fact that MDM2 can bind and degrade many other proteins. MDM2 promotes its own degradation (48), degradation of mutant p53 (49) and the growth suppressor p14/p19.
MDM2 binds and degrades the cell-fate protein “numb” (51). Also the ubiquitin ligase activity is not required for MDM2-mediated tumorigenesis.

MDM2 interacts with several growth suppressors and other proteins: MDM2 interacts with several growth suppressors, including the tumor suppressor p53, the retinoblastoma susceptibility gene product Rb and the growth suppressor p14 ARF. These interactions are perceived as possible mechanisms for MDM2’s oncogenic function (reviewed in 52-58; 26-31, 59-62).

MDM2 is induced by oncogenic challenges: Several oncogenic challenges induce MDM2 in a p53-dependent or independent manner. Ionizing irradiation, including γ- and UV-irradiation and DNA damaging agents induce MDM2 expression in a p53-dependent manner (33,62-64). UV irradiation also induces p53-nonbinding forms of MDM2 (65). DNA-damaging aryl hydrocarbons induce MDM2 expression in a p53-independent manner (66). Oncogenic Ras also induces MDM2 through the Raf/MEK/MAP kinase pathway in a p53-independent manner (67). These findings suggest that normal cells induce MDM2 in response to oncogenic challenges.

MDM2 induces G1 arrest in non-transformed cells: Considering all the evidence for MDM2’s oncogenic function, one would expect that overexpression of MDM2 would confer growth advantage in cultured cells. However, work from our laboratory demonstrated that overexpression of MDM2 from its cDNA efficiently arrests the growth of NIH3T3 cells (68). MDM2 accomplishes this in NIH3T3 cells by utilizing two inhibitory domains, ID1 and ID2, that can independently arrest these cells at the G0-G1/S phase transition (68). Recently, two different laboratories have also reported growth inhibitory functions of MDM2 (69,70). Consistent with these observations,
multiple laboratories have reported that overexpression of MDM2 is a favorable prognostic marker in cancer (3,71-73).

The question arises therefore how to reconcile the growth-promoting and growth-arresting properties of MDM2. Although overexpression of full-length MDM2 with its functional inhibitory domains does not lead to development of antibiotic resistant colonies expressing the protein in NIH3T3 cells, injection of nude mice with NIH3T3 cells stably expressing an MDM2 deletion mutant containing the N-terminal 1-130 amino acids, thus deleting the inhibitory domains, led to development of tumors at 6/6 sites injected. This suggests that MDM2 contains a tumorigenic domain located within the N-terminal 1-130 amino acids. Interestingly, this growth-promoting domain overlaps with the p53-interacting domain, enforcing the hypothesis that MDM2 enhances tumorigenesis by inactivating p53. Since MDM2 can only be stably expressed in cells harboring genetic defects such as H1299, this leads to the suggestion that MDM2’s normal function in the cell is to induce growth arrest, and inactivation of the function of its inhibitory domains unleashes its tumorigenic potential.

**Mechanisms of G1-S progression provide a control on cell growth.** If MDM2 arrests cells at the G0-G1/S phase transition, then cellular defects in one or more of these mechanisms in cancer cells may create the cellular context for abrogation of MDM2’s normal growth suppressing functions and may promote the unmasking of its tumorigenic ability. The pathway controlling progression from G1 into S phase involves the retinoblastoma susceptibility protein Rb and the activity of cyclin-dependent kinases. Cyclins are induced sequentially in the cell division cycle and associate with their partner kinases to promote progress through the cell cycle. Cyclin
D is first induced and associates with CDK4/6 to phosphorylate Rb at the restriction point. Prior to this phosphorylation, the activity of cyclin D/CDK4/6 is restrained by

**Figure 2. G1-S phase transition.** Components in the Rb pathway to regulate progression through G1 into S phase are shown. In panel 1, Rb is shown to bind at the cyclin E promoter along with HDAC and BrG1 and E2F to repress expression of cyclin E. p16 inhibits cyclin D-CDK4/6 from phosphorylating Rb before the restriction point. In panel 2, to enable cells to cross the restriction point thus committing the cell to a round of DNA replication, cyclin D-CDK4/6 phosphorylates Rb, causing HDAC to disassociate from Rb and enabling cyclin E to expressed from its promoter. In panel 3, the induced cyclin E then associates to its kinase, CDK2, and this complex then further phosphorylates Rb and BrG1 as well, causing BrG1 to leave and leading to induction of cyclin A. Induction of cyclin A helps to initiate entry into S phase.
the cyclin-dependent kinase inhibitor p16, which provides a control to deregulated progression through G1. Phosphorylation of Rb by cyclin D/CDK4/6 causes HDAC, which was in association with Rb and Brg1 to repress the cyclin E promoter, to dissociate, leading to expression of cyclin E protein. Cyclin E/CDK2 then further phosphorylates Rb and Brg1, causing Brg1 to leave Rb at the cyclin A promoter, thus inducing expression of cyclin A whose activation of CDK2 is required to advance the cell into DNA replication in S phase.

Cancer is the result of multiple genetic defects that lead to loss of control on cell growth. Deregulation of G1-S progression and the process of DNA replication may provide the cellular context that converts MDM2 from a growth suppressor to an oncogene.
I. Chapter 2. Hypothesis

a. Major hypothesis.

The major hypothesis of this work involves deciphering the ways in which the human oncoprotein MDM2 regulates cell growth. The objective was to (a) decipher the ways in which MDM2 induces cell cycle arrest at the G0-G1/S phase transition and (b) determine novel interactions of MDM2 to further elucidate MDM2’s complex cellular functions. The hypothesis in (a) was based on the laboratory’s previous observation that MDM2 induces growth arrest at the G0-G1/S phase transition in NIH3T3 cells. This is accomplished in NIH3T3 through two inhibitory domains of MDM2, ID1 and ID2, which are independently capable of inducing arrest. Since MDM2 can only be stably expressed in transformed cells such as H1299, and the function of MDM2 in normal cells is to induce G0-G1/S phase arrest when overexpressed, the hypothesis in (a) was that to induce growth arrest MDM2 may be targeting one step in the G0-G1/S progression, while this function is disabled in cells harboring corresponding genetic defects. This would suggest an explanation for the ability of some cancer cells to tolerate MDM2 overexpression. In part (b) the hypothesis was that since MDM2 has been shown to interact with numerous proteins, proteomic analysis would reveal novel interacting partners of MDM2 which could be validated by other methods.

b. Specific Aims.

The following were the specific aims: (1) To determine how MDM2 induces growth arrest specifically in normal cells and suggest components of the pathway required for this arrest; (2) To determine whether ID1 and ID2 arrest in G1 or inhibit DNA
replication; (3) To determine novel interacting partners of MDM2 through proteomic analysis.
J. Chapter 3
MDM2 induces G1 arrest by down-regulating cyclin A through a mechanism requiring the p16/Brg1 pathway.

The manuscript for the work presented in this chapter is currently in preparation.

a. Introduction.

Despite MDM2's well-established oncogenic potential, our laboratory has previously shown that MDM2 overexpression is capable of inducing G0-G1/S phase arrest. This is accomplished in the immortalized cell line NIH3T3 through two inhibitory domains, ID1 and ID2. However, although the previous data showed cell cycle arrest using flow cytometry, staining DNA to determine cell cycle phase using this method does not allow for distinguishing whether the growth arrest is in G0, G1 or initiation into S, as the DNA content in these phases should be similar. Therefore, we set out to determine the mechanism of MDM2-mediated growth arrest by analyzing MDM2's effect on the sequential induction of G1-S proteins as markers for the cell cycle step inhibited by MDM2.

b. Experimental Results.

Expression of cyclin D, cyclin-D mediated phosphorylated Rb and cyclin E is not affected by MDM2. Cyclins are induced in a sequential order as cells progress through the cell cycle, and their expression is required to activate cyclin-dependent kinases whose activity advances cells through the next step of the cell division cycle. If MDM2 arrests the cell cycle at a point after a cyclin is induced, the induction of that cyclin and the preceding cell cycle steps should be detected. Failure to detect expression of an inducible cell cycle protein at the next step in the process would suggest the point at which MDM2 overexpression is arresting cell cycle progression. Since the transfection
protocols available led to more cells being untransfected than transfected and extract made from this population for Western blotting would reflect the average expression of the endogenous protein levels of both MDM2 expressing and non-expressing cells, to reliably detect the inhibited step we used flow cytometry. This method has the advantage of separating transfected from untransfected cells and quantitating the level of the endogenous protein being studied in each individual cell. To determine the growth-arrested step, we looked for down-regulation of endogenous G1-S cyclins D, E and A as well as phosphorylation of Rb at the restriction point by cyclin D-specific kinase in response to MDM2 overexpression. We compared normal human lung fibroblast WI38 cells capable of senescence, immortal murine fibroblast NIH3T3 and lung carcinoma H1299 cells after transfection with MDM2 to determine any effect of added genetic damage on MDM2's ability to arrest the cell cycle.

Since cyclin D is the first cyclin to be induced as cells enter G1, we first looked for any change in level of expression of this cyclin in MDM2 expressing cells compared to untransfected cells. MDM2 was detected using a phycoerythrin (PE)-coupled antibody, while cyclin D was stained with a flourescein (FITC)-coupled antibody. Transfected MDM2-expressing cells were then separated from untransfected cells in a flow cytometer, and a curve was generated to compare cyclin D levels between the two populations based on FITC intensity. Figure 3 shows a representative detection of transfected cells in WI38 cells. Mock-transfected cells were stained with PE-anti-MDM2 and gated so that background staining in the MDM2-transfected sample using the same gate would be represented at 0.1%. This PE staining of the mock sample encompasses both endogenous MDM2 levels as well as non-specific staining. The mock cells were
also stained with a FITC isotype matched to the cyclin D antibody to show non-specific binding so that staining of endogenous cyclin D in the transfected sample using the specific antibody could be detected over background. Cells in the MDM2-transfected sample that stained higher than background on the PE scale were taken as transfected, while staining of endogenous cyclin D can be seen as a shift to the right on the FITC scale. This method was used for all of the following analyses.

**Figure 3. Detection of transfected cells by flow cytometry.** (A) Dot plot of mock-transfected WI38 cells stained with PE-coupled anti-MDM2 antibody and FITC-coupled IgG isotype of cyclin D antibody. The boundary of the mock-transfected cells was drawn to achieve 0.1% background staining. (B) Detection of WI38 cells successfully transfected with MDM2 expression plasmid visualized by flow cytometry. The figure shows a dot plot of WI38 cells transfected with MDM2 expression plasmid and immunostained with PE-coupled anti-MDM2 antibody and FITC-coupled cyclin D antibody. MDM2-expressing cells are PE-labeled, and are indicated as “transfected”. PE-unlabeled cells from the same sample are shown as “untransfected”. Transfected cells
overexpressed MDM2 and therefore are more intensely labeled with PE-coupled MDM2 antibody.

Comparison of the FITC-cyclin D curves in WI38, NIH3T3 and H1299 cells generated in the above manner is shown in Figure 4. The figure plots cell count versus FITC-cyclin D intensity, so the peak of each curve represents the maximum level of cyclin D in the main cyclin D-expressing population, which should represent a synchrony of cells at the same point in G1. Since transfected cells are shown to have stained higher than isotype as seen in the middle panel, this suggests that cyclin D can be detected in the transfected cells. Transfected and untransfected cells were taken from the same plate and thus were stained in one tube under the same conditions; therefore, any decrease in level of cyclin D in the transfected population in comparison to the untransfected cells should indicate that MDM2 inhibits the cell cycle at the step of cyclin D induction. However, comparison between MDM2-transfected and untransfected cells shows that MDM2 does not alter the level of cyclin D in any of the three cell lines tested.
Figure 4. Cyclin D levels are not altered by MDM2 overexpression. MDM2-transfected and untransfected W138 cells were gated and separated after staining with a PE-coupled anti-MDM2 antibody, and the comparative FITC intensities of the cyclin D-specific antibody transfected or untransfected cells are shown by a single parameter histogram. The left panels show comparative FITC intensity of untransfected cells stained with FITC-coupled cyclin D antibody and FITC-coupled IgG isotype. As expected, the FITC-coupled anti-cyclin D antibody successfully detected cyclin D expressing cells and stained cyclin D expressing cells more intensely than the FITC-coupled isotype. The middle panels show comparative staining of MDM2 transfected cells stained with the cyclin D-coupled FITC antibody compared with cells stained with FITC-coupled IgG isotype. In the case of both transfected and untransfected cells, which were stained in the same population, cyclin D stained higher with the specific antibody than the isotype antibody. The right panels show comparative FITC intensity of transfected and untransfected cells stained with FITC-coupled cyclin D antibody. In the case of WI38 and H1299 cells (and NIH3T3 cells, not shown), there was no difference in level of cyclin D between MDM2 and non-MDM2 overexpressing cells. (NIH3T3 not shown), as there is no significant change in the position of the peaks. Since cyclin D is expressed as cells enter G1, this suggests that the MDM2-mediated cell cycle arrest shown previously by our laboratory (68) is not in G0 and that MDM2 arrests the cell cycle at a point after cyclin D induction.
Following cyclin D induction, cyclin D activates its associated kinase CDK4/6 which then phosphorylates Rb at the restriction point. We therefore next checked for any change in phosphorylation status of Rb by this specific cyclin/kinase complex using the method described above. In Figure 5, comparison of the peak intensity between transfected and untransfected cells showed no change in status of cyclin D/kinase-mediated phosphorylation of Rb by MDM2 overexpression in any of the three cell lines. This suggests that MDM2 enables cells to cross the restriction point before exerting any change on the cell cycle.

Figure 5. Cyclin D-mediated phosphorylation of Rb levels are not latered by MDM2 overexpression. Following the same analysis described for Figure 4, WI38, NIH3T3 and H1299 cells were gated for MDM2-expressing and non-expressing cells, and intensity of FITC was compared for staining with a FITC-coupled antibody specific for cyclin D-mediated phosphorylated Rb. Transfected and untransfected cells
from the same population both stained more intensely than FITC-coupled isotype antibody; however, there was no significant change in level of cyclin D-specific phosphorylated Rb between these cells in response to MDM2 overexpression.

Figure 6. Cyclin E levels are not altered by MDM2 overexpression. Following the same analysis as in Figure 4, WI38, NIH3T3 and H1299 cells were gated to separate the MDM2 and non-MDM2 overexpressing populations, and FITC intensity of the anti-cyclin E antibody between these cells was compared. Transfected and untransfected cells both stained higher for cyclin E-FITC levels than the matched FITC isotype antibody; however, there was no significant decrease in level of cyclin E mediated by MDM2 overexpression.

After phosphorylation of Rb at the restriction point, cyclin E is induced, so we next looked for any effect of MDM2 on the cell cycle at the level of cyclin E induction.
However, MDM2 overexpression was found to cause no significant change in cyclin E level in either WI38, NIH3T3 or H1299 cells (Figure 6). Therefore, MDM2 affects the cell cycle at a point after cyclin E induction.

**MDM2 overexpression down-regulates cyclin A in normal WI38 cells.** After induction of cyclin E, the next cyclin to be expressed in the sequence is cyclin A which enables cells to enter S phase, so we analyzed the three cell lines for any reduction in cyclin A level mediated by overexpression of MDM2. Using the same method described above, we stained MDM2 with a PE-coupled antibody and cyclin A with a FITC-coupled antibody. The results in Figure 7 show that MDM2 overexpression in normal WI38 cells causes a significant (8)-fold reduction in cyclin A levels in comparison to untransfected cells, suggesting that MDM2 acts on the cell cycle before S phase begins in these cells. Down-regulation of cyclin A level by MDM2 in WI38 cells was confirmed by confocal microscopy Figure 8. Using this method, we analyzed 50 MDM2-transfected WI38 cells, and all MDM2-overexpressing cells were found to lack expression of cyclin A. By comparison, some untransfected cells in the same field were shown to express

![Figure 7. Cyclin A levels are down-regulated by MDM2 in WI38 cells.](image_url)

MDM2 overexpressing and non-overexpressing cells were gated after staining with a PE-coupled anti-MDM2 antibody. The left panel shows immunostaining of untransfected...
WI38 cells with a FITC-coupled anti-cyclin A antibody over background staining with FITC-coupled IgG isotype of cyclin A antibody. The middle panel shows comparative staining of the transfected cells from the population with a FITC-coupled anti-cyclin A antibody. The right panel shows expression of cyclin A in transfected (PE-labeled) and untransfected (PE-unlabeled) WI38 cells, demonstrating that MDM2 overexpression leads to downregulation of cyclin A expression in WI38 cells.

cyclin A because they are in S phase, while other untransfected cells do not show cyclin A expression, as expected in an asynchronous population.

Figure 8. Confocal imaging of cyclin A down-regulation by MDM2 in WI38 cells.

Confocal imaging analysis shows cells expressing MDM2 do not express cyclin A. Cyclin A expression was detected by a Rhodamine-coupled anti-cyclin A antibody and MDM2 expression was detected by a FITC coupled anti-MDM2 antibody. Cells
expressing MDM2 (FITC-stained) are shown by arrows, and cells expressing cyclin A (rhodamine-stained) are shown by block arrows. Nuclei were stained with DAPI. All cells expressing MDM2 lacked cyclin A expression.

**Absence of endogenous MDM2 derepresses cyclin A expression.** Our data suggests that overexpression of MDM2 is capable of down-regulating cyclin A. We therefore wanted to determine whether endogenous levels of MDM2 have a role in regulating the expression of cyclin A. Since absence of MDM2 has been shown to stabilize p53, and p53 can down-regulate cyclin A, instead of knocking down MDM2 with siRNA we compared endogenous cyclin A levels in normal, p53 knockout, and p53/MDM2 double knockout MEFs to show an effect of MDM2 over that of p53. The results in Figure 9 show that absence of p53 alone increases cyclin A level approximately 2-fold over normal MEFs, while knockout of MDM2 along with p53 increases endogenous level of cyclin A 5-fold over normal MEFs. This suggests that endogenous MDM2 levels contribute to repression of expression of cyclin A.
Figure 9. Absence of MDM2 derepresses expression of cyclin A. To compare level of cyclin A expression in normal, p53-/−, and p53-/−/MDM2-/− MEFs, the cells were harvested and the pellet was boiled in equal volume of lammeli loading buffer. Equal amounts of the extracts were subjected to SDS-PAGE and cyclin A detected with an antibody purchased from Santa Cruz (SC-751).

MDM2 is unable to down-regulate cyclin A in NIH3T3 and H1299 cells. In contrast to WI38, the cyclin A level in NIH3T3 or H1299 cells is not altered by overexpression of MDM2. Figure 10 shows no significant difference in mean FITC-cyclin A intensity between transfected and untransfected cells. Since NIH3T3 cells are immortal and H1299 are tumor-derived, this suggests that the cellular defects present in these cells allow them to overcome this cell-cycle regulatory step mediated by MDM2.

Figure 10. Cyclin A levels are unaffected by MDM2 overexpression in NIH3T3 and H1299 cells. The figure compares level of FITC intensity of a cyclin A-specific antibody in MDM2 transfected and untransfected cells, or each population compared with a matched FITC-coupled IgG isotype. The left and middle panels show
staining of cyclin A expressing cells over nonspecific background binding to FITC-coupled IgG isotype of cyclin A antibody in untransfected and transfected cells, respectively. The right panels show comparative expression of cyclin A in transfected (MDM2-expressing, PE-labeled) and untransfected cells.

The N-terminal 59-109 amino acids of MDM2 down-regulate cyclin A in WI38 cells. The results above demonstrate that full-length MDM2 down-regulates cyclin A in normal WI38 cells while the same protein is incapable of this function in NIH3T3 and H1299, allowing them to advance past the point of cyclin A induction. Since full-length MDM2 was previously shown by our laboratory to inhibit the cell cycle in G0-G1/S in NIH3T3 cells through the ID1 and ID2 domains (68) but our data shows that MDM2 allows NIH3T3 and H1299 to progress further through G1 than WI38 before inducing arrest, this suggests that MDM2 contains a third inhibitory domain that is disabled in the defective cells. To locate this domain, we separately transfected a series of N- and C-terminal deletion mutants of MDM2 into WI38, and stained the fixed cells with both PE-MDM2 and FITC-cyclin A antibodies to determine MDM2’s effects on cyclin A levels as described above. The results in Figure 11 show that a deletion mutant of MDM2, MDM2 1-109 containing the N-terminal 109 amino acids, is capable of down-regulating cyclin A levels in WI38 to an extent similar to the wild-type protein. To further define the required domain, we used another MDM2 deletion mutant that contains amino acids 59-220 and found that on deletion of the N-terminal 58 amino acids, MDM2 retains its ability to down-regulate cyclin A. This further defines the cyclin A-inhibitory domain
Figure 11. The N-terminal 1-109 amino acids of MDM2 down-regulate cyclin A. The left panels shows immunostaining of untransfected WI38 cells with FITC-coupled anti-cyclin A antibody over background staining with FITC-coupled IgG isotype of cyclin A antibody. Comparative expression of cyclin A in MDM2 del 491-110 transfected (curve 3) and untransfected (curve 2) WI38 cells is also shown.

to amino acids 59-109 of MDM2. Since our laboratory had previously demonstrated that deletion of the N-terminal 58 amino acids, which is in the p53-binding domain of MDM2, eliminates MDM2’s ability to interact with p53 (29), the data in Figure 12 also suggests that the MDM2-p53 interaction is not required for MDM2 to down-regulate cyclin A.

The cyclin A inhibitory domain of MDM2 induces G1 arrest in normal WI38 cells but not tumor-derived H1299. Since MDM2 inhibits expression of cyclin A in normal but not tumor-derived cells, and induction of cyclin A is required for entry into S phase, we hypothesized that transfection of the MDM2 1-109 deletion mutant into WI38 should lead to G1 arrest, while having no effect in H1299. To test this, we transfected
Figure 12. Deletion of the N-terminal 1-58 amino acids of MDM2 does not abrogate cyclin A down-regulation by MDM2. The top panel shows the domains of MDM2 overlapping with the cyclin A inhibitory domain. The left lower and middle panels compare anti-cyclin A-PE staining of untransfected and MDM2 59-220 transfected WI38 cells with a matched PE-coupled isotype, respectively. The lower right panel compares cyclin A PE intensity of MDM2 transfected and untransfected cells. The boxed middle panel shows further definition of the cyclin A inhibitory domain of MDM2 to amino acids 58-109.
Figure 13. The N-terminal 1-109 amino acids of MDM2 induce G1 arrest in normal WI38 cells but not tumor-derived H1299 cells. WI38 and H1299 cells were transfected with MDM2 del 491-110 and MDM2 was stained in the fixed cells with an FITC-coupled antibody and DNA was stained with either PI or 7AAD. Panel A shows mock-transfected WI38 cells stained with the MDM2-FITC antibody with a gate drawn to achieve 0.1% background staining. Panel B shows detection of MDM2 del 491-110-transfected WI38 that stain in this gate. Panel C and D show DNA histograms of untransfected and MDM2 del 491-110 transfected WI38 and H1299 cells, respectively.
MDM2 1-109 into both WI38 and H1299 cells and stained the fixed cells with a FITC-MDM2 antibody and DNA with either propidium iodide (PI) or 7-aminoactinomycin D (7AAD) to obtain a DNA histogram. A representative gating of MDM2-expressing FITC-labeled cells over mock-transfected background in WI38 cells is shown in Figure 13 panels A and B. Generation of a DNA histogram overlay of the transfected and untransfected populations showed that MDM2 1-109 led to G1 arrest in WI38 but not H1299, consistent with the previous finding that MDM2 down-regulates cyclin A in normal but not tumor-derived cells, Figure 11 panels C and D. This suggests that the domain situated within the N-terminal 1-109 amino acids of MDM2 that is able to down-regulate cyclin A is also capable of inducing G1 arrest in normal cells.

The N-terminal 1-109 amino acids of MDM2 induce G1 arrest by down-regulating cyclin A. If the G1 arrest induced by the MDM2 1-109 domain is a result of MDM2 down-regulating cyclin A, forced expression of cyclin A in the presence of the MDM2 1-109 expression product should permit cells to bypass the inhibited step. To test this, we co-transfected MDM2 1-109 and cyclin A expression plasmids into WI38 cells in which MDM2 normally down-regulates cyclin A, and compared the DNA histograms of the untransfected cells with those positive for both MDM2 and cyclin A expression. In this experiment, MDM2 was labeled with a PE-coupled antibody, while cyclin A was stained with FITC and DNA was labeled with 7AAD. Figure 14 panel A shows down-regulation of cyclin A by 1-109 expression alone, while panel C shows that the level of cyclin A in the co-transfected cells is similar to endogenous cyclin A levels in the untransfected population. Comparison of the histograms of untransfected and MDM2 1-109/cyclin A co-expressing cells in panel D demonstrates that when cyclin A is co-
Figure 14. MDM2 induces G1 arrest by down-regulating cyclin A. Comparison of cyclin A levels (A, C) and DNA histograms (B, D) of untransfected cells with that of MDM2 Del 491-110 expression plasmid transfected (A, B), and MDM2 Del 491-110 and cyclin A expression plasmids cotransfected (C, D) WI38 cells.

expressed with MDM2 1-109, the growth inhibition caused by the 1-109 domain alone (panel B) is overcome and cells no longer arrest in G1. This data links the G1 arrest function of the MDM2 1-109 domain with this domain’s ability to down-regulate cyclin A.

MDM2 requires cyclin-dependent kinase inhibitor p16 to down-regulate cyclin A.

We wished to investigate the mechanism for down-regulation of cyclin A by MDM2. One common cellular defect in cancer is lack of expression of the cyclin-dependent kinase inhibitor p16, whose expression is deregulated in greater than 30% of human
**Figure 15. Specific silencing of p16 by siRNA.** Silencing of p16 in WI38 extracts after transfection with 80 pmol of specific siRNA is compared by Western blot with equal loading of WI38 extracts transfected with 80 pmol of scrambled siRNA.

cancers. It is known that NIH3T3 cells contain deleted p16 gene, while in H1299 the p16 gene is methylated, preventing expression of the protein. Since absence of expression of p16 is common to both of these cell lines in which MDM2 cannot down-regulate cyclin A, and p16 regulates the pathway leading to cyclin A expression, we wanted to determine whether MDM2 requires p16 to down-regulate cyclin A. To accomplish this, we turned to a p16 siRNA system. Figure 15 shows knockdown of p16 protein expression in WI38 cells by Western blot. We then plated WI38 cells onto coverslips after transfection with MDM2 and either scrambled or p16 siRNA, and stained MDM2 and cyclin A for analysis by confocal microscopy. Figure 16 shows that transfection of MDM2 with scrambled siRNA does not alter MDM2’s ability to down-regulate cyclin A. However, when MDM2 is co-transfected with p16 siRNA, MDM2 loses its ability to down-regulate
cyclin A (Figure 17). This suggests that MDM2 requires the cyclin-dependent kinase inhibitor p16 to prevent expression of cyclin A.

Figure 16. Transfection with scrambled siRNA does not alter MDM2's ability to down-regulate cyclin A by confocal microscopy. Confocal imaging analysis of WI38 cells transfected with scrambled siRNA shows cells expressing MDM2 do not express cyclin A. Cyclin A expression was detected by a Rhodamine-coupled anti-cyclin A antibody and MDM2 expression was detected by a FITC coupled anti-MDM2 antibody. Cells expressing MDM2 (FITC-stained) are shown by arrows, and absence of expression of cyclin A (rhodamine-stained) in the MDM2-expressing cells is shown by corresponding arrows. Nuclei were stained with DAPI. All cells expressing MDM2 lacked cyclin A expression.
Figure 17. Transfection with p16 siRNA abrogates MDM2's ability to down-regulate cyclin A by confocal microscopy. Confocal imaging analysis of WI38 cells transfected with p16 siRNA shows cells expressing MDM2 express cyclin A. Cyclin A expression was detected by a Rhodamine-coupled anti-cyclin A antibody and MDM2 expression was detected by a FITC coupled anti-MDM2 antibody. Cells expressing MDM2 (FITC-stained) are shown by arrows, and expression of cyclin A (rhodamine-stained) in the MDM2-expressing cells is shown by corresponding arrows. Nuclei were stained with DAPI.

Treatment of H1299 cells with azacytidine partially restores the ability of the N-terminal 109 amino acids of MDM2 to down-regulate cyclin A. The expression of the p16 protein is silenced in H1299 cells because of methylation of the p16 gene. Treatment of cells with azacytidine has been shown to reestablish expression of the p16 gene through demethylation of its promoter and leads to reexpression of proteins from
other genes whose promoters have been methylated as well. Since we have demonstrated that silencing of p16 prevents MDM2 from down-regulating cyclin A in WI38 cells, and MDM2 cannot down-regulate cyclin A in H1299, we hypothesized that treatment of H1299 cells with azacytidine should re-enable this function of MDM2 in this cell line.

To test this hypothesis, we analyzed MDM2’s ability to decrease the percent of cyclin A expressing cells using flow cytometry or lower luciferase activity from the cyclin A promoter in H1299 cells treated with azacytidine compared to buffer treatment alone.

The results in Figure 18 part A show that azacytidine treatment enables MDM2
promoter activity can be reinforced in H1299 cells by re-expressing endogenous p16 after 5-aza-2'-deoxycytidine treatment. Reinduction of p16 after treatment with 5-aza-2'-deoxycytidine is shown by Western. Expression of MDM2 Del 491-110 is also shown. (A) Percent of cyclin A expressing cells in MDM2 Del 491-110 expression plasmid transfected and untransfected population after buffer- or 5-aza-2'-deoxycytidine-treatment are shown by a bar graph. Cyclin A expression in H1299 cells was determined by a FITC-coupled anti-cyclin A antibody, and MDM2 Del 491-110 expression was detected by a PE-coupled anti-MDM2 antibody. (B) Cyclin A promoter activity was determined by assaying expression of luciferase from a luciferase reporter plasmid under the control of cyclin A promoter. For transfection of buffer-treated and 5-aza-2'-deoxycytidine-treated-cells, 200 ng of a cyclin A luciferase reporter plasmid and 2 μg of pCMVMDM2 expression plasmids were used.

Del 491-110 to reduce the percent of cyclin A expressing cells to a greater extent than azacytidine treatment or MDM2 transfection alone. Similarly in Figure 18 part B, this trend was evidenced by decreased luciferase activity from the cyclin A promoter in MDM2 transfected cells treated with azacytidine in comparison to azacytidine treatment or MDM2 expression alone. These data suggest that treatment of H1299 cells with azacytidine partially restores the ability of MDM2 to down-regulate cyclin A.

**MDM2 reduces expression from a cyclin A reporter gene when cotransfected with p16 in H1299 cells.** Since azacytidine treatment is not specific for demethylation of the p16 gene, we wished to confirm that the effects on cyclin A expression by MDM2 in the preceding data was due to reinduction of p16 and not global demethylation.
Therefore, we cotransfected H1299 cells with a cyclin A-luciferase reporter construct and MDM2 and p16 plasmids, or with either vector, MDM2 or p16 plasmids alone, and compared the luciferase activity of the extracts. Figure 19 shows that MDM2/p16 cotransfection reduces luciferase activity from the cyclin A promoter approximately 4-fold over p16 transfection alone, and 6-fold over MDM2 transfection and 3-fold over vector alone. This suggests that re-expression of p16 partially restores MDM2-mediated down-regulation of cyclin A in H1299 cells.

**Figure 19. Cotransfection of p16 with MDM2 enables MDM2 to inhibit expression from a cyclin A reporter plasmid.** MDM2 inhibits the cyclin A promoter after re-expressing p16 in H1299 cells by using a p16 expression plasmid. H1299 cells were transfected with 400 ng of a cyclin A luciferase reporter plasmid in the presence or absence of 100 ng pCMV p16 or 400 ng pCMV MDM2 expression plasmids. Luciferase activity of cell extracts containing equal amounts of proteins was determined 24 hours after transfection, shown in the left panel. The right panel shows expression of p16 and MDM2 determined by Western blot analysis. Additions of expression plasmids during transfection are shown in the figure. Migration of p16, MDM2 and β-actin are indicated by arrows.
**MDM2 requires Brgl to down-regulate cyclin A.** Another component in the p16-dependent pathway to control cyclin A expression is Brgl, part of the chromatin remodeling SWI/SNF complex, which remains bound to Rb until its phosphorylation by cyclin E/cdk2 causes it to leave, thus leading to derepression of cyclin A expression. Since phosphorylation of Brgl is accomplished by cyclin E/cdk2, and MDM2 inhibits the cell cycle at a point after cyclin E induction, we checked the requirement of Brgl in the mechanism for MDM2 to down-regulate cyclin A. After transfection of WI38 cells with MDM2 and either scrambled or Brgl siRNA, we stained MDM2 with a FITC-coupled antibody and cyclin A with an antibody coupled to rhodamine and analyzed the cells by confocal microscopy. Staining of Brgl in Figure 20 part B shows that Brgl levels were down-regulated by the specific siRNA in comparison to scrambled siRNA (Figure 20
Figure 20. MDM2 requires Brg1 to down-regulate cyclin A. Panel A shows detection of Brg1 in the nucleus after transfection with scrambled siRNA, while Panel B shows that transfection with Brg1 siRNA downregulates this protein in WI38 cells. Panel C shows the ability of MDM2 to down-regulate cyclin A after transfection with scrambled siRNA in WI38 cells (shown by arrows). Panel D shows that after transfection of WI38 cells with Brg1 siRNA, MDM2 loses its ability to down-regulate cyclin A. MDM2 was stained with a FITC-coupled antibody (N20, Santa Cruz), and cyclin A was stained with an antibody purchased from Santa Cruz (SC751 TRITC). The antibody used to detect Brg1 was purchased from Santa Cruz (SC-17796) followed by staining with a rhodamine goat anti-mouse secondary antibody from Jackson Immunoresearch (115-295-146). DAPI was used to stain nuclei.

part A) when visualized using the same settings by confocal microscopy. The data in Figure 20 part D demonstrate that in the MDM2/Brg1 siRNA transfected cells, MDM2 loses its ability to down-regulate cyclin A, while MDM2 in the scrambled siRNA cotransfected cells retains this function (Figure 20 part C). This data in conjunction with the preceding p16 data suggests that MDM2 functions to down-regulate cyclin A dependent on the p16/Brg1 pathway.

c. Chapter Summary.

To determine one pathway by which MDM2 induces growth arrest, we analyzed progression of the cell cycle in MDM2-arrested cells to find the inhibited step by detecting the sequential induction of proteins involved in transition from G1 into S phase.
Flow cytometric analysis in WI38, NIH3T3 and H1299 cells showed no change to cyclin D, cyclin D-mediated phosphorylated Rb, or cyclin E levels in response to MDM2 overexpression, suggesting that the point of inhibition is at a point later than these cellular events. However, MDM2 overexpression was found to down-regulate cyclin A in normal WI38 cells while affecting no change to this cyclin in immortal NIH3T3 or tumor-derived H1299 cells. The domain responsible for cyclin A down-regulation is located within amino acids 59-109 of MDM2. Absence of endogenous MDM2 derepresses cyclin A levels in MEFs, suggesting that endogenous MDM2 levels as well as MDM2 overexpression assist in down-regulating cyclin A expression. MDM2 induces G1 arrest by down-regulating cyclin A, which is expected since cyclin A is required for entry into S phase. Silencing of p16 prevents MDM2 from inhibiting cyclin A expression in WI38, while reinduction of p16 partially restores this function of MDM2 in H1299 cells. Down-regulation of cyclin A is also eliminated by absence of Brg1 in WI38. These data suggest that MDM2 functions to inhibit expression of cyclin A through the p16/Brg1 pathway. Since absence of p16 expression is a common defect in human cancer, this also suggests one mechanistic pathway by which MDM2 is converted from a growth inhibitor to a potentially oncogenic protein.
K. Chapter 4.

Inhibitory domain 2 (ID2) of MDM2 arrests the cell cycle by inhibiting DNA replication.

The manuscript for the work presented in this chapter is currently in preparation.

a. Introduction.

Previous work from our laboratory using flow cytometry showed that MDM2 arrests the cell cycle in NIH3T3 cells through two inhibitory domains ID1 and ID2 that function in this cell line to arrest the cell cycle at the G0/G1-S phase transition (68). The data presented in the preceding chapter demonstrates that a third inhibitory domain located between amino acids 59-109 of MDM2 induces G1 arrest by down-regulating cyclin A in cells that have a functional p16/Brg1 pathway. Since induction of cyclin A is one of the events that initiates entry into S phase but ID1 and ID2 inhibit cell cycle progression at a step downstream of cyclin A induction, we hypothesized that these inhibitory domains may target cells for arrest at a point prior or later than replication initiation and wanted to determine the significance of MDM2 overexpression in the absence of cyclin A down-regulation in an immortal or tumor-derived cellular context.

b. Experimental results.

A deletion mutant containing the N-terminal 1-109 amino acids of MDM2 prevents DNA replication in cells that respond to the cyclin A down-regulatory function of this domain. Replication of DNA can be detected using a bromodeoxyuridine (BrdU) assay. BrdU is a thymidine analog that is incorporated into DNA as replication proceeds. Detection of incorporated BrdU using a flourescently-labeled antibody therefore provides a way to determine whether MDM2 overexpression enables cells to enter S phase. To determine the ability of MDM2's inhibitory domains to prevent entry into S phase, we transfected normal WI38, immortal NIH3T3, and tumor-
derived H1299 cells with MDM2 or its deletion mutants. Forty-eight hours after transfection, cells were labeled with BrdU to detect replication status and the cells were harvested and fixed. MDM2 was stained with a PE-coupled antibody while BrdU was labeled with a FITC-coupled antibody, and DNA was stained with 7-AAD. Transfected and untransfected cells were gated and analyzed by flow cytometry. The difference between the percent of transfected or untransfected cells from the same population that incorporated BrdU indicated the ability of the inhibitory domains of MDM2 to inhibit DNA replication. The results in Figure 21 show a representative experiment using MDM2 del 491-110 transfected into W138 cells. The gates were drawn so that background staining from the FITC-coupled anti-BrdU antibody in cells that were not subjected to BrdU treatment was 0.1% or less. Cells from the untransfected population that labeled as BrdU positive on the FITC scale are shown in the M gate in Figure 21 part A to be 29.0%, while BrdU negative cells in the L gate are 67.5%. In comparison, only 7.7% of MDM2 del 491-110 transfected cells in the M gate in Figure 21 part B are labeled as BrdU positive, while 91.1% of the cells in the L gate are BrdU negative. This
Figure 21. Detection of BrdU positive cells by flow cytometry. MDM2 del 491-110 transfected and untransfected cells from the same population were labeled with BrdU and were harvested and fixed. Panel A shows detection of cells that were actively incorporating BrdU in the untransfected population in the M gate (29%), versus cells that did not incorporate BrdU in the L gate (67.5%). MDM2 del 491-110 cells separated from the untransfected population are shown in Panel B to have only 7.7% of the transfected cells actively incorporating BrdU using the same gate.

suggests that MDM2 del 491-110 prevents entry into S phase in WI38 cells as expected since this deletion mutant harbors the inhibitory domain that down-regulates cyclin A. Similarly, we transfected NIH3T3 and H1299 cells with this deletion mutant and analyzed the cells using the same method. The results for all three cell lines are summarized in Figure 22. The data shows that in both NIH3T3 and H1299, MDM2 del 491-110 does not have the ability to inhibit DNA replication, as there is no significant difference between BrdU incorporation in transfected and untransfected cells. This corresponds with the finding in the preceding chapter that the inhibitory domain contained within this region of MDM2 down-regulates the S-phase initiating factor cyclin A in WI38 cells while losing this function in the context of NIH3T3 and H1299 cells.

<table>
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Figure 22. Effects of the N-terminal 1-109 amino acids of MDM2 on BrdU incorporation. The cyclin A inhibitory domain inhibits DNA replication only in W138 cells but not in NIH3T3 or H1299 cells. The chart shows percentage of untransfected cells or transfected cells expressing MDM2 or MDM2 Del 491-110 actively incorporating BrdU. Cells expressing MDM2 or MDM2 Del 491-110 were detected by PE-coupled anti-MDM2 antibody. FITC-coupled anti-BrdU antibody was used to detect BrdU incorporating cells.

The N-terminal 1-154 amino acids of MDM2 inhibit replication in W138 but not NIH3T3 or H1299 cells. We next tested the ability of MDM2 del 491-155 to induce a block to replication in W138, NIH3T3 and H1299 cells. Since this deletion mutant harbors the cyclin A inhibitory domain but not ID1 or ID2 we expected similar results in the three cell lines as was obtained with MDM2 del 491-110. As expected, MDM2 del 491-155 was capable of efficiently preventing BrdU incorporation in W138 but not NIH3T3 or H1299, as shown in Figure 23.

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Percent BrdU positive cells

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**Figure 23. Effects of the N-terminal 1-154 amino acids of MDM2 on BrdU incorporation.** The deletion mutant MDM2 del 491-155 does not alter the effects on BrdU incorporation compared to MDM2 del 491-110. BrdU and MDM2 were detected as described in Figure 22.

The N-terminal 1-220 amino acids of MDM2 containing the cyclin A inhibitory domain and ID1 inhibit BrdU incorporation efficiently in WI38 but not NIH3T3 or H1299 cells. Previous data from our laboratory suggested that the growth of NIH3T3 cells is arrested by MDM2 overexpression through two inhibitory domains that are functional in these cells, ID1 and ID2, which can independently arrest the cell cycle at the G0-G1/S phase transition. We therefore wanted to determine whether the deletion mutant MDM2 del 491-221 containing functional ID1 could inhibit BrdU incorporation in NIH3T3 and thus entry into S phase. We transfected this deletion mutant into WI38 and H1299 cells as well to determine its effect in these two cell lines. The results in Figure 24 show that this deletion mutant harboring ID1 along with the cyclin A inhibitory domain prevents BrdU incorporation in WI38 to a similar extent in these cells as the MDM2 del 491-110 deletion mutant containing the cyclin A inhibitory domain alone. However, in NIH3T3 and H1299 cells MDM2 del 491-221 does not significantly decrease the percent of BrdU positive cells in response to MDM2 overexpression compared to untransfected cells, suggesting that this deletion mutant does not inhibit initiation to DNA replication in NIH3T3 or H1299. Prior data from the laboratory demonstrated that MDM2 del 491-221 expression leads to G0-G1/S arrest in NIH3T3 through ID1, although inefficiently, while elimination of the N-terminal 58 amino acids
from this deletion mutant increases the efficiency similar to the extent seen with the full-length protein in this cell line. To determine whether the N-terminal 58 amino acids interfere with the ability of ID1 to inhibit BrdU incorporation requires further BrdU incorporation experiments using MDM2 59-221. Previous data from the laboratory suggests that MDM2 del 491-221 cannot be expressed stably in NIH3T3 (68), suggesting that inclusive of the contribution from the N-terminal 58 amino acids, ID1 is inhibitory to the growth of NIH3T3 cells.

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Percent BrdU positive cells

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<td>H1299   --</td>
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*Figure 24. Effects of the N-terminal 1-220 amino acids of MDM2 on BrdU incorporation.* A deletion mutant MDM2 del 491-221 including ID1 along with the cyclin A inhibitory domain does not significantly inhibit BrdU incorporation in NIH3T3 or H1299 cells but maintains an inhibition to BrdU incorporation in WI38 similar to MDM2 del 491-110 or MDM2 del 491-154. BrdU and MDM2 were detected as described in Figure 22.
Overexpression of full-length MDM2 inhibits BrdU incorporation in WI38, NIH3T3 and H1299 cells. We finally tested full-length MDM2 in WI38, NIH3T3 and H1299 cells for whether presence of ID2 would lead to an inhibition of replication in H1299 or NIH3T3 which incorporate BrdU to the same extent as untransfected cells when overexpressing deletion mutants of MDM2 containing ID1 or the cyclin A inhibitory domain. The results summarized in Figure 25 show that full-length MDM2 overexpression in NIH3T3 and H1299 cells inhibits BrdU incorporation as efficiently as it does in WI38. Since ID1 and ID2 both arrest the G0-G1-S phase transition by DNA histogram in NIH3T3 but ID2 prevents BrdU incorporation in this cell line with apparently minimal effect to this process contibuted by ID1, this suggests that ID2 functions as the replication inhibitory domain, while ID1 may function after replication initiation in the full-length protein, possibly by inhibiting elongation of the DNA strand. Further experiments need to be done to confirm this hypothesis.

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Percent BrdU positive cells

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</table>
Figure 25. Effects of full-length MDM2 on BrdU incorporation. The percent of BrdU positive cells is shown in MDM2 overexpressing and non-overexpressing WI38, NIH3T3 and H1299 cells. Inclusion of ID2 enables NIH3T3 and H1299 cells to prevent BrdU incorporation to a similar extent as WI38 cells. BrdU and MDM2 were detected as described in Figure 22.

Accumulation of cellular mutations desensitizes cells to growth arrest mediated by MDM2. The preceding data demonstrates that the N-terminal 1-109 amino acids of MDM2 prevent BrdU incorporation in normal WI38 cells but not immortal NIH3T3 or tumor-derived H1299, which is expected since this domain is capable of inhibiting expression of cyclin A in WI38 but not NIH3T3 or H1299. However the BrdU incorporation experiments in this chapter in conjunction with the data in Chapter 3 suggest that ID2 functions to inhibit DNA replication downstream of cyclin A induction while ID1 apparently functions downstream of replication initiation. The data presented in Chapter 3 shows that cellular defects such as p16, Brg1 or p53 deficiency abrogate the ability of the N-terminal 59-109 amino acids of MDM2 to downregulate cyclin A and lead to G1 arrest, and suggests that further cellular damages may contribute to the inactivation of the other inhibitory domains ID1 and ID2. We therefore wanted to assay the significance of MDM2 overexpression in the three different cell types to determine whether a normal, immortal or tumor-derived cellular context has any consequence for the ability of MDM2's inhibitory domains to arrest cell growth. The results in Figure 26 compare the ability of full-length MDM2 containing the three functional inhibitory domains or a deletion mutant MDM2 del 1-120 that contains only ID1 and ID2 to induce
G1 arrest in WI38 cells after transfection with 500 ng of either DNA. Both MDM2 and
MDM2 del 1-120 led to a similar percent increase in the number of cells in G1, 18.9%
and 15.7%, respectively, from the number of untransfected cells from the same
population. MDM2 overexpression from 500 ng of plasmid DNA resulted in growth
arrest with 77.5% of transfected cells in G1, while MDM2 del 1-120 expression from 500
ng of plasmid DNA led to 81% of transfected cells in G1. This suggests that the cyclin A
inhibitory domain in full-length MDM2 and ID1 and ID2 present in the MDM2 del 1-120
deletion mutant can induce growth arrest in normal WI38 cells to a similar extent when
MDM2 is expressed from 500 ng of plasmid DNA, suggesting that dysfunction of the
pathway utilized by the cyclin A inhibitory domain does not have consequence for the
ability of MDM2 to induce growth arrest through ID1 and ID2 in the absence of other
cellular defects at this level of expression.
Figure 26. Comparison of the ability of full-length MDM2 and a deletion mutant of MDM2 harboring ID1 and ID2 to induce G1 arrest in WI38 cells. Full-length MDM2 and MDM2 del 1-120 were transfected using 500 ng of either DNA into WI38 cells. Forty-eight hours after transfection the cells were harvested and fixed. MDM2 was stained with 2A10 (courtesy of Arnold Levine) followed by staining with an anti-mouse IgG (goat) fluorescein conjugated secondary antibody (Calbiochem DC13L). DNA was stained with PI and DNA histograms generated after analysis by flow cytometry. Percent increase in G1 cells by MDM2 represents the difference in the percent of MDM2-expressing cells in G1 in comparison to the percent of cells in G1 in the untransfected population from the same experiment. For the full-length MDM2 experiment the standard deviation for transfected cells in G1 was 2.52% while for untransfected cells the standard deviation was 0.234%. For the MDM2 del 1-120 experiment the standard deviation for transfected cells in G1 was 2.0% while for untransfected cells it was 0.167%.

In NIH3T3 and H1299 cells, the cyclin A inhibitory domain is not functional, but NIH3T3 is known to induce growth arrest through ID1 and ID2 (68) while we have shown in this chapter that H1299 cells do not incorporate BrdU in the presence of ID2 when full-length MDM2 is expressed from 5 ug of plasmid DNA. We therefore wanted to determine whether the defects present in immortal NIH3T3 would desensitize these cells to MDM2-mediated G1/S arrest and whether tumor-derived H1299 cells would be further desensitized in the context of their more extensive mutations. To test this, we transfected these cells with 500 ng of MDM2 and compared the ability of MDM2 to increase the number of G1 cells in the different cell types. Figure 27 shows that MDM2
expressed from 500 ng of plasmid has a decreasing ability to arrest at G1/S in cells with increasing genetic damage. While MDM2 expressed at this level increases the percent of G1 cells by 18.9% in normal WI38 cells, this number is decreased to 15.3% in immortal NIH3T3 and is decreased to 6.7% in tumor-derived H1299. To show this difference

![Bar graph showing percent increase in G1 cells by MDM2 overexpression in WI38, NIH3T3, and H1299 cells with different concentrations of MDM2 DNA.]

**Figure 27.** Increasing amounts of cellular mutations lead to desensitivity for the growth-arrest function of MDM2. WI38, NIH3T3 and H1299 cells were transfected with different concentrations of MDM2 DNA shown in the bar graph. Forty-eight hours after transfection the cells were harvested and fixed, and MDM2 was stained with 2A10 (provided by Arnold Levine) followed by a anti-mouse IgG (goat) fluorescein conjugated secondary antibody (Calbiochem DC13L). DNA was stained with PI and DNA histograms were generated by flow cytometry. The percent of cells in G1 was estimated by using a modfit program. Percent increase in G1 cells by MDM2 represents the difference in the percent of MDM2-expressing cells in G1 in
comparison to the percent of cells in G1 in the untransfected population from the same experiment. For the 250 ng experiment in WI38 the standard deviation for transfected cells in G1 was 1.74% while for untransfected cells the standard deviation was 0.132%. For the 500 ng experiment in WI38 the standard deviation for transfected cells in G1 was 2.52% while for untransfected cells the standard deviation was 0.234%. For the 1 μg experiment in WI38 the standard deviation for transfected cells in G1 was 1.12% while for untransfected cells it was 0.232%. For the 250 ng NIH3T3 experiment the standard deviation for transfected cells in G1 was 1.23% while for untransfected cells it was 0.229%. For the 500 ng NIH3T3 experiment the standard deviation for transfected cells in G1 was 1.33% while for untransfected cells it was 0.309%. For the 500 ng H1299 experiment the standard deviation for transfected cells in G1 was 1.23% while for untransfected cells it was 0.332%. For the 1 μg H1299 experiment the standard deviation for transfected cells in G1 was 2.46% while for untransfected cells it was 0.342% For the 2 μg H1299 experiment the standard deviation for transfected cells in G1 was 1.16% while for untransfected cells it was 0.252%

Further between WI38 and NIH3T3, we transfected both with 250 ng of MDM2 and found that while MDM2 retains the ability to increase the number of G1 cells by 15.4%, this is dramatically lowered in NIH3T3 to 8.5%. Even more MDM2 was required to achieve efficient G1/S arrest in H1299 similar to WI38, as transfection with 1 μg of MDM2 increased the number of G1/S cells in H1299 by 20% while a comparable effect could be obtained in WI38 (18.9%) using only 500 ng of plasmid. Transfection with 2 μg of MDM2 increased the efficiency of G1/S arrest in H1299, consistent with this trend.
These data demonstrate a cell-type variation in response to MDM2 overexpression, suggesting that increasing amounts of cellular mutations lead to desensitivities for the growth-arrest function of MDM2.

**Chapter summary.**

To determine whether ID1 and ID2 arrest the cell cycle in G1 or S phase, we employed BrdU incorporation assays in WI38, NIH3T3, and H1299 cells. Experiments using overexpression of the N-terminal 1-109 amino acids of MDM2 in the three cell lines supported the data in Chapter 3 that MDM2 induces G1 arrest by down-regulating cyclin A only in normal cells, as this deletion mutant harboring the cyclin A inhibitory domain was capable of preventing BrdU incorporation only in WI38 cells, and not NIH3T3 or H1299. A deletion mutant containing the N-terminal 1-154 amino acids of MDM2 did not alter this effect. MDM2 del 491-221 containing the cyclin A inhibitory domain and ID1 did not significantly decrease the percent of BrdU positive cells in NIH3T3 or H1299 cells, suggesting that this deletion mutant does not inhibit initiation to DNA replication in NIH3T3 or H1299 cells. However, overexpression of full-length MDM2 was capable of inhibiting BrdU incorporation in NIH3T3 and H1299 to a similar extent as in WI38 cells. This suggests that ID2 is the DNA replication inhibitory domain. Flow cytometric analysis of different concentrations of MDM2 transfected into the three cell lines showed that MDM2 is capable of inducing G1 arrest at lower levels of expression in normal cells, and increasingly higher levels of expression are required for similar efficiency in G1 arrest in cells with increasing levels of genetic damage. This suggests that added genetic damage leads to toleration of MDM2 overexpression in cancer cells.
L. Chapter 5.
MDM2 interacting partners: MDM2 interacts with the translation elongation factor efl-α.

The manuscript for the work presented in this chapter is currently in preparation.

a. Introduction.

Although MDM2's interaction with the tumor suppressor p53 is considered to be the most important, MDM2 interacts with several other proteins involved in cell growth regulation. These interactions include tumor suppressors Rb and p14ARF, as well as E2F1/DP1, TATA-binding protein, sp1, DNA polymerase epsilon, and SV40 T antigen. MDM2 also interacts with its related protein MDMX and ribosomal proteins L5, L11 and L23. To determine novel interacting partners of MDM2, we performed proteomic analysis of MDM2 overexpressed in the tumor-derived cell line H1299 in an attempt to gain more insight into the diversity of MDM2's cellular functions.

b. Experimental Results.

Proteomic analysis of MDM2 expressed in H1299 cells. The tumor-derived human lung carcinoma cell line H1299 was chosen for proteomic analysis due to its high transfection efficiency using the methods available in the laboratory, which would facilitate detecting MDM2-associated protein complexes. For this purpose, H1299 cells were transfected with either vector or MDM2 plasmids, and the cells were fractionated into cytoplasmic and nuclear fractions. After immunoprecipitation with an anti-MDM2 antibody to pull down MDM2-associated proteins, the beads were eluted and subsequently sent for proteomic analysis. The results in Table 1 show the proteins found to be in complex with MDM2 in the nuclear and cytoplasmic fractions. This analysis confirms MDM2 interactions already identified in the literature, such as ribosomal proteins L5, L23 and L11. Among the other proteins identified in Table 1,
Table 1. MDM2 interacting proteins in H1299 cells.

<table>
<thead>
<tr>
<th>Nuclear fraction</th>
<th>Cytoplasmic fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>ribosomal protein L5</td>
<td>ribosomal protein L5</td>
</tr>
<tr>
<td>histone H2A</td>
<td>beta tubulin</td>
</tr>
<tr>
<td>ribosomal protein L23</td>
<td>elongation factor 1-alpha1</td>
</tr>
<tr>
<td>histone H2a.z variant isoform 2</td>
<td>actin depolymerizing factor</td>
</tr>
<tr>
<td>ribosomal protein L11</td>
<td>ribosomal protein S20</td>
</tr>
<tr>
<td>histone H2B family member A</td>
<td>GAPDH</td>
</tr>
<tr>
<td>ribosomal protein S23 40S</td>
<td>alpha tubulin</td>
</tr>
<tr>
<td>histone 3.3B</td>
<td>ribosomal protein L11 cytosolic</td>
</tr>
<tr>
<td>elongation factor 1-alpha</td>
<td></td>
</tr>
<tr>
<td>p14</td>
<td></td>
</tr>
<tr>
<td>protein similar to helix destabilizing protein</td>
<td>human ATP synthase alpha chain mitochondrial precursor</td>
</tr>
<tr>
<td>actin gamma cytoskeleton</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. H1299 cells were transfected with MDM2 and prepared for proteomic analysis as described in Experimental Designs. The proteins found to interact with MDM2 in the cytoplasmic and nuclear fractions are shown in the table.

we found a novel association of MDM2 in H1299 cells with the translation elongation factor ef1-α. Ef1-α is involved in catalyzing binding of aminoacyl tRNAs to the A site of the ribosome during translation and has also been shown to act with the 26S protease complex to degrade ubiquitin conjugated proteins, thus providing a possible link between protein synthesis and degradation. Since expression of ef1-α decreases toward the end of the life span of mouse and human cells but forced expression of this protein has been shown to prolong the lifespan of drosophila and may function to make cells competent for growth, we wanted to validate its interaction with MDM2 because of its possible implications for MDM2 in regulation of cell growth or other yet-unknown functions.
**MDM2 interacts with ef1-α.** To confirm MDM2's interaction with ef1-α, H1299 cells were transfected with either vector or MDM2 and the cell lysates were immunoprecipitated (IP) using an antibody against ef1-α or IgG as a control. Western blotting of the proteins bound to the beads showed that the ef1-α antibody pulled down ef1-α specifically in comparison to IgG, and MDM2 was detected in the MDM2 transfected sample and not in the vector or IgG controls (Figure 28). This suggests that MDM2 and ef1-α specifically interact in H1299 cells, and confirms this interaction from our proteomic analysis.

**Figure 28. Validation of the MDM2-ef1-α interaction by Western blot.** H1299 cells were transfected with MDM2 and immunoprecipitated using an anti-ef1-α antibody (Upstate). MDM2 transfected cells immunoprecipitated using IgG were used as a control. Detection of MDM2 and ef1-α in the Western blot are shown with arrows.
**Ef1-α levels are elevated in cancer cells.** It had previously been demonstrated that ef1-α levels are elevated in tumor-derived cells. We therefore compared the endogenous level of ef1-α in normal WI38 cells and the tumor-derived cell lines H1299, MCF7 and OSACL. Cell extract was made, and equal amounts of the lysate was analyzed by Western blotting. The results in Figure 29 show that ef1-α levels are elevated in the tumor-derived cell lines approximately 2-fold in comparison to normal WI38 cells, correlating with the previously reported data.

![Figure 29. Ef1-α levels are elevated in tumor-derived cells.](image)

**Figure 29. Ef1-α levels are elevated in tumor-derived cells.** Extracts were made from normal WI38 and tumor-derived H1299, MCF7 and OSACL cells and were separated by SDS-PAGE. Ef1-α and actin expression is shown by arrows.

**MDM2 overexpression does not alter the level of ef1-α expression.** We next wanted to determine whether MDM2 overexpression could affect cellular ef1-α levels, and whether this function would be altered in normal versus tumor-derived cells. To address this issue, we transfected normal WI38 and tumor-derived H1299 cells with
vector or MDM2 and analyzed the lysates by Western blotting. The results in Figure 30 show that transfection with MDM2 did not appreciably alter the level of ef1-α in WI38 or H1299 cells, suggesting that the function of the interaction is at a level other than regulation of expression.

**Figure 30. MDM2 does not affect levels of ef1-α protein.** WI38 and H1299 cells were transfected with either vector pcmv or pcmv MDM2. Forty-eight hours after transfection the cells were harvested and lysed, and the extracts separated by SDS-PAGE. Expression of MDM2, ef1-α and actin are shown by arrows.

**MDM2 and ef1-α colocalize in the cytoplasm of WI38 cells.** Ef1-α has been shown to be mainly localized in the cytoplasm but has also been detected at lower levels in the nucleus. Since MDM2 is mainly a nuclear protein but can also be shuttled into the cytoplasm, we wanted to determine where MDM2 and ef1-α co-localize in the cell. To
achieve this, we transfected WI38 cells with MDM2 and plated them onto sterile coverslips. Forty-eight hours after transfection, the cells were fixed. To detect MDM2, efl-\(\alpha\) and location of the nucleus, MDM2 was subsequently directly stained with a FITC-coupled antibody, efl-\(\alpha\) was stained indirectly with rhodamine and the nucleus was stained with DAPI. The cells were then analyzed by confocal microscopy. The results in Figure 31 show that efl-\(\alpha\) is localized mainly in the cytoplasm, while MDM2 is found both in the nucleus and cytoplasm. Co-localization of the two proteins was found to occur in the cytoplasm, as demonstrated by the yellow color.

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**Figure 31. MDM2 and efl-\(\alpha\) colocalize in the cytoplasm.** WI38 cells were transfected with MDM2 and plated on sterile coverslips. Forty-eight hours after transfection the cells were fixed with formaldehyde. MDM2 was stained with a FITC-
coupled antibody (Santa Cruz, N20), ef1-α was stained with an antibody purchased from Upstate, followed by staining with a rhodamine goat anti-mouse secondary antibody from Jackson Immunoresearch. Nuclei were stained with DAPI. Colocalization of MDM2 and ef1-α is visualized as a yellow color.

**Two domains of MDM2 are required for interaction with ef1-α.** To determine the domains of MDM2 that the protein utilizes to contact ef1-α, we employed a series of MDM2 deletion mutants by immunoprecipitation to find the required interaction domains. To show expression of MDM2 deletion mutants and ef1-α, we chose to see the interaction from radiolabeled proteins and autoradiography rather than Western blotting in which detection of the interacting proteins would be complicated by presence of IgG bands. For this purpose, WI38 cells were transfected with MDM2 or its deletion mutants. Eighteen to twenty-four hours after transfection the cells were labeled with 35S methionine, and the extract from these cells was precleared followed by immunoprecipitation with an anti-MDM2 antibody and the gel exposed to film. The results in Figure 32 demonstrate that while full-length MDM2 interacts with ef1-α (shown by an arrow), a deletion mutant MDM2 del 491-221 loses its ability to interact, suggesting that a domain located between amino acids 221-491 is required for the interaction. Following this determination, we tested the ability of MDM2 del 491-325 to associate with ef1-α. Figure 33 shows that similar to the full-length protein, this deletion mutant of MDM2 is capable of interaction. This further defines the ef1-α interaction domain to within amino acids 221-325 of MDM2.
Figure 32. MDM2 del 491-221 does not interact with efl-α. WI38 cells were transfected with vector pcmv, pcmv MDM2 or pcmv MDM2 del 491-221. Sixteen to 24 hours later the cells were translabeled with $^{35}$S methionine, harvested and equal counts were immunoprecipitated using an anti-MDM2 antibody-agarose conjugate (Santa Cruz, SMP14). Proteins that bound to the beads were washed and subjected to polyacrylamide gel electrophoresis, and the dried gel was exposed to film, as described in Experimental Designs. The efl-α band (identified using extract IPed with an anti-efl-α antibody) is designated by an arrow. Expression of MDM2 or its deletion mutant is shown as the darkest band in their respective lanes.
Figure 33. MDM2 del 491-325 interacts with ef1-α. Vector pcmv, pcmv MDM2 or pcmv MDM2 del 491-325 were transfected and identified as described in Figure 33 and Experimental Designs. The ef1-α band (identified using extract IPed with an anti-ef1-α antibody) is designated by an arrow. Expression of MDM2 or its deletion mutant is shown as the darkest band in their respective lanes.

We next tested an N-terminal deletion mutant MDM2 del 1-58 for its ability to form the interaction. The results in Figure 34 show that loss of the N-terminal 58 amino acids causes MDM2 to lose its ability to associate with ef1-α. These data suggest that MDM2 requires two domains for interaction with ef1-α, one which is located between amino acids 1-58 and another located between 221-325. Loss of either of these domains is enough to prevent MDM2's association with ef1-α.
**Figure 34. MDM2 del 1-58 does not interact with ef1-α.** Vector pcmv, pcmv

MDM2 or pcmv MDM2 del 1-58 were transfected and identified as described in Figure 33 and Experimental Designs. The ef1-α band (identified using extract IPed with an anti-ef1-α antibody) is designated by an arrow. Expression of MDM2 or its deletion mutant is shown as the darkest band in their respective lanes.

c. Chapter Summary

To determine novel interactions of the human oncoprotein MDM2, we performed proteomic analysis of H1299 cells transfected with MDM2. The results of this analysis confirmed some previously reported interactions and showed a novel interaction of MDM2 with the translation elongation factor ef1-α. To validate this interaction, MDM2 co-IPed with ef1-α in H1299 cells, as presence of these two proteins was specifically detected in the MDM2-transfected sample by Western blot. Although ef1-α levels were shown to be higher in tumor-derived cells such as H1299 in comparison to normal WI38 cells, MDM2 overexpression was shown to have no effect on expression level of ef1-α in either cell type. MDM2 and ef1-α were found to co-localize in the cytoplasm of WI38 cells, supporting the finding that these two proteins interact. Finally, for interaction of MDM2 with ef1-α, MDM2 was determined to require two domains, one of which is located between amino acids 221-325 and another within the first 58 amino acids of MDM2.
M. Discussion.

Previous data from our laboratory suggested that in spite of its oncogenic potential, MDM2 has the ability to induce growth arrest in immortal NIH3T3 cells at the G0-G1/S phase transition (68). These cells respond to the growth inhibitory property of MDM2 through two domains, ID1 and ID2, located on the MDM2 protein within amino acids 155-220 and 270-324, respectively. A tumorigenic domain found to be located within amino acids 1-130 of MDM2 induced tumors in nude mice when injected as stable transfectants of NIH3T3 cells. This led to the hypothesis that the normal function of MDM2 is to induce growth arrest on overexpression, and inactivation of the growth inhibitory domains would turn the overexpressed MDM2 into an active growth-promoting protein through the function of its tumorigenic domain. The work in this dissertation was centered around deciphering the mechanisms of MDM2-mediated cell cycle arrest. Data published previously by our laboratory suggested that MDM2 arrests cells at the G0-G1/S phase transition as determined in DNA histograms by flow cytometry, but this method does not provide a clear distinction between arrest in G0, G1, or early S, since cells in these phases should stain with similar DNA content. Therefore, to make this determination we looked for induction of G1-S cell cycle events in MDM2 arrested cells as a marker to determine the inhibited step, and compared levels of these proteins in the MDM2 overexpressing cells with untransfected cells in the same population as controls for normal endogenous levels. Flow cytometry was used in these experiments instead of Western, as a method to compare levels of endogenous proteins in individual cells in the sorted transfected and untransfected populations. Normal W138 lung fibroblast, immortal mouse fibroblast NIH3T3 and lung carcinoma H1299 cells were
used to see the effects of MDM2 in cells with varying degrees of genetic damage. Our analyses show that MDM2 does not significantly alter the level of endogenous cyclin D, cyclin D/CDK specific phosphorylation of Rb or cyclin E in all three cell lines, suggesting that MDM2 does not prevent cells from crossing the restriction point. However, MDM2 was shown to down-regulate cyclin A specifically in WI38 cells. The domain of MDM2 involved in this down-regulation was shown to be located within the N-terminal 59-109 amino acids. Since NIH3T3 cells only respond to arrest by ID1 and ID2 and stable transfectants expressing the N-terminal 1-130 amino acids can be developed in NIH3T3 cells, this suggests that the cyclin A downregulatory function of MDM2 represents a third inhibitory domain whose function is inactivated by the cellular defects present in NIH3T3. Consistent with this, our analyses show that in response to MDM2 overexpression, NIH3T3 and H1299 cells show no significant decrease in cyclin A levels. Since previous data from our laboratory demonstrated that loss of MDM2’s N-terminal 58 amino acids eliminates its ability to bind p53, the location of the cyclin A inhibitory domain between amino acids 59-109 suggests that the MDM2-p53 interaction is not required for MDM2 to down-regulate cyclin A. Cyclin A is required for progression into S phase, and in agreement with the ability of MDM2 to down-regulate cyclin A in normal but not tumor-derived cells, MDM2 del 491-110 induced G1 arrest in WI38 but not H1299. The G1 arrest function was linked to cyclin A down-regulation by the release of G1 arrest after cotransfection of WI38 cells with MDM2 and cyclin A expression plasmids.

The ability of MDM2 to down-regulate cyclin A in WI38 but not NIH3T3 or H1299 cells suggests that there is a common genetic defect in these cells that prevents MDM2’s
normal function. One common alteration in cancer cells is loss of expression of the cyclin-dependent kinase inhibitor p16. In NIH3T3 cells the p16 gene is deleted, while in H1299 cells it is methylated, thus preventing its expression. p16 is a component of the Rb pathway, and provides an important regulatory control on the G1-S phase transition leading to expression of cyclin A (Figure 2). We therefore wanted to determine whether MDM2 requires expression of p16 to down-regulate cyclin A. Silencing of p16 in W138 cells was shown to abrogate the ability of MDM2 to down-regulate cyclin A, visualized by confocal microscopy, suggesting it is required for this function of MDM2. Re-expression of p16 in H1299 cells either by treatment of cells with 5-aza-deoxycytidine or cotransfection with a p16 expression plasmid enabled partial restoration of MDM2's ability to decrease the percent of cyclin A expressing cells or inhibit expression from the cyclin A promoter, respectively. This suggests the possibility that by restoring function of p16 in cancer cells that overexpress MDM2, this could turn potentially oncogenic MDM2 into a growth suppressor. Another component of the Rb pathway is Brg1, which assembles in complex with Rb at the cyclin A promoter to repress its expression. Using Brg1 siRNA, confocal imaging shows that although MDM2 can down-regulate cyclin A in W138 cells when cotransfected with scrambled siRNA, silencing of Brg1 disables this process. Thus, another component of the Rb pathway, Brg1, is required for MDM2 to down-regulate cyclin A.

The finding that MDM2 requires the p16/Brg1 pathway to down-regulate cyclin A leads to the question of which step in this pathway MDM2 is directly targeting. MDM2 affects the cell cycle at a point after cyclin E induction and before cyclin A is expressed. The known steps in this process that MDM2 could potentially target are the kinase
activity of cyclin E/CDK2 or phosphorylation of Rb or Brg1 by this cyclin/kinase complex. Experiments done to immunoprecipitate cyclin E-associated kinase and analyze its ability to phosphorylate histone H1 suggests that MDM2 does not significantly affect this kinase activity (data not shown). However, the percent of MDM2 transfection in these assays was not determined, and since the transfection efficiency may be low, an inhibition of cyclin E/kinase activity by MDM2 may be obscured by a high percent of contaminating kinase activity from the untransfected cells. Additional experiments need to be done to test this hypothesis. An alternative to MDM2 functioning by inhibiting cyclin E/kinase activity is the possibility that MDM2 binds to either Rb or Brg1, preventing their phosphorylation or preventing Brg1 from disassociating from Rb, thus enabling repression of the cyclin A promoter. However, preliminary data suggests that H1299 cells do not have detectable levels of Brg1. Since MDM2’s ability to regulate cyclin A expression in H1299 cells can be partially restored by re-expressing p16 in the absence of detectable Brg1, while silencing of either p16 or Brg1 prevents MDM2 from down-regulating cyclin A in WI38 cells, this suggests the possibility that MDM2 does not directly target either of these proteins but instead requires their contribution toward providing the normal cellular framework that prevents deregulation of MDM2’s growth regulating function. This is supported by MDM2’s differential ability to down-regulate cyclin A in normal WI38 cells, while the unmutated protein loses this function in NIH3T3 and H1299 cells which harbor genetic/cellular defects.

The data presented in Chapter 4 shows that an MDM2 deletion mutant, MDM2 del 491-110 possesses the ability the inhibit BrdU incorporation in WI38 but not NIH3T3 or H1299 cells. Since this deletion mutant contains the cyclin A inhibitory domain which is
functional only in the normal cells, the BrdU data supports the function of this domain as down-regulating cyclin A, since cyclin A expression initiates progression into S phase and subsequent DNA replication. A deletion mutant harboring the N-terminal 1-154 amino acids of MDM2 did not significantly alter the dynamics of BrdU incorporation from the effects seen by MDM2 del 491-110 in the three cell lines, suggesting that this extended domain of MDM2 may not possess DNA replication inhibitory ability. Extending the N-terminal portion of MDM2 to include ID1 by transfecting with MDM2 del 491-221 also does not have a significant effect on preventing BrdU incorporation in NIH3T3 and H1299 as it does in W138 cells. Although ID1 present in this deletion mutant arrests NIH3T3 cells at G0-G1/S, though inefficiently, but does not significantly prevent BrdU incorporation in these cells, this suggests that ID1 present in this deletion mutant may function downstream of initiation of DNA replication. Prior data from the laboratory suggested that deletion of the N-terminal 58 amino acids from MDM2 del 491-221 leads to more efficient growth arrest, suggesting that amino acids 1-58 may provide a signal that cooperates with the function of ID1. This signal is located within the defined tumorigenic domain of MDM2, and if ID1 functions after replication initiation it may play a role in this process. Finally, transfection with full-length MDM2 was found to efficiently inhibit BrdU incorporation in all three cell lines. Since further inclusion of ID2 prevents BrdU incorporation by the full-length protein in H1299 and NIH3T3 where ID1 and the cyclin A inhibitory domain do not, this suggests that ID2 prevents replication initiation. Thus, MDM2 possesses three inhibitory domains, each functioning potentially at a different step in the cell division cycle.
In determining the significance of these domains for the cell, a model can be proposed in which MDM2 is induced in response to oncogenic challenges in normal cells and provides protection against genetic damages by inhibiting cell cycle progression through three different mechanisms. Two scenarios can be envisioned. In one, the three inhibitory domains of MDM2 provide a way to arrest cells that have already committed to replicate and are at different stages of progression after passing the restriction point so that once genetic damage is sustained MDM2 has flexibility to inhibit cell growth independent of the cell cycle step in which the damage was conferred. In another scenario, MDM2 may possess the three inhibitory domains so that it provides multiple levels of protection for arresting growth of cells even when cellular defects exist that inactivate its function in the other growth inhibitory pathways. The hypothesis that multiple genetic damages increasingly desensitize cells to the growth arrest function of MDM2 is suggested by the data in Chapter 5 showing that tumor-derived cells which harbor more genetic damages require more MDM2 to achieve a growth arrest similar to that achieved with the less damaged NIH3T3 and, in turn, MDM2 can arrest normal WI38 cells with similar efficiency at even lower levels. The hypothesis that MDM2 may provide multiple levels of protection is reflected in the multiple mechanisms eukaryotic cells use to prevent rereplication of DNA during the normal cell division cycle. For example, eukaryotic cells employ cyclin-dependent kinases to phosphorylate the origin recognition complex, downregulate cdc6 activity and exclude the MCM2-7 complex from the nucleus to prevent re-initiation of DNA replication (74). Evidence presented in this dissertation suggests that defects present in the cellular pathways that MDM2 utilizes to induce growth arrest may inactivate its function. Ultimately, these damages may not
only eliminate MDM2's growth suppressive functions but enable MDM2's oncogenic function through the action of its tumorigenic domain.

To find novel protein interactions of MDM2 that may further elucidate MDM2's role in cellular processes, we performed proteomic analysis of MDM2 overexpression in H1299 cells. This endeavor identified interaction of MDM2 with elongation factor 1-alpha (ef1-α). The interaction was confirmed by immunoprecipitation and Western blotting. MDM2 was found to not significantly alter the expression of ef1-α in either normal WI38 or tumor-derived H1299 cells, suggesting that the function or dysfunction of the interaction is not to change level of protein expression. Confocal localization studies demonstrated that MDM2 and ef1-α colocalize in the cytoplasm, suggesting this is the place for the function of their association. Deletion mutant analysis suggested that two domains of MDM2 are required for interaction with ef1-α, one located between amino acids 1-58 and another between amino acids 221-325. The data presented in Chapter 5 identify and validate that MDM2 and ef1-α interact but leave the question open as to the functional significance of the interaction. Ef1-α is involved in catalyzing transfer of aminoacyl tRNAs to the A site during translation, but our preliminary data suggests that MDM2 overexpression in H1299 cells does not affect translation. This is also evidenced in the fact that our deletion mutant studies to determine the interaction domain, which relied on translabeling with ^{35}S methionine, led to visualization of labeled protein bands in the gel. MDM2 requires two domains to interact with ef1-α, one overlapping with the p53 interaction domain and another with a domain that harbors the acidic domain, ID2, and the p14ARF, sp1 and Rb binding sites. Since elimination of the N-terminal 58 amino acids of MDM2 prevents MDM2's interaction with p53 as well as
interaction with ef1-α, this may provide some clue to the function. It is also interesting that the other domain required for interaction involves p14ARF, and association of p14ARF with MDM2 has been shown to stabilize p53. Whether the MDM2-ef1-α interaction has any role in this process will have to be tested with further experiments.
N. Experimental Designs.

General Methods.

Plasmids and MDM2 deletion mutants. The MDM2 cDNA was a generous gift from Bert Vogelstein (23). Construction of plasmids expressing the full-length MDM2 and its deletion mutants has been described earlier in detail (9, 12). The plasmid pCMVp16 was constructed from a pbluescript p16 plasmid, which was a generous gift from Martine Roussel.

Cells. NIH3T3, WI38, H1299 cells were purchased from American Type Culture Collection and were maintained in media suggested by the supplier. NIH3T3 cells were maintained in DMEM with 10% calf serum. WI38 cells were maintained in Minimum Essential Media Eagle with 10% fetal bovine serum. H1299 cells were maintained in RPMI with 10% fetal bovine serum.

Immunofluorescent staining and confocal imaging. Immunofluorescent staining and confocal imaging were performed following a method reported by Lohrum et al (25). Briefly, normal diploid WI38 cells were seeded on sterile coverslips in a 6-well culture dish, and were transfected with 2 μg MDM2 expression plasmid per coverslip. The cells were fixed 48 hours after transfection using 4% paraformaldehyde for 10 minutes at room temperature with constant shaking. The cells were then washed three times in phosphate buffered saline (PBS), permeabilized in ice cold PBS containing 0.2% triton X-100 for 5 minutes and blocked in PBS containing 0.5% bovine serum albumin at room temperature for 30 minutes. For MDM2/cyclin A staining, the fixed cells were consecutively immunostained overnight at 4°C with a FITC-coupled anti-MDM2 antibody (N20, Santa Cruz) and a rhodamine-coupled anti-cyclin A antibody (SC751 TritC, Santa Cruz) in
blocking solution. The antibody used to detect Brg1 was purchased from Santa Cruz (SC-17796) followed by staining with a rhodamine goat anti-mouse secondary antibody from Jackson Immunoresearch (115-295-146). For detection of MDM2/ef1-α, cells were stained with a FITC-coupled anti-MDM2 antibody (N20, Santa Cruz) followed by staining with an anti-ef1-α primary antibody (Upstate, 05-235). The secondary antibody used against the primary ef1-α antibody was a rhodamine goat anti-mouse secondary antibody from Jackson Immunoresearch (115-295-146). The slides were then washed three times in PBS and once in water, air dried and mounted with Prolong Gold Antifade with DAPI (Molecular Probes). The coverslips were then analyzed under a confocal microscope (Zeiss) under 63X magnification.

**Western blot analysis.** Western blot analysis was carried out essentially as previously described (12, 17). Briefly, cells were washed three times in PBS and scraped from the plate in PBS and transferred to a microfuge tube. Cells were then spun at 900 rcf for 10 minutes. The supernatant was removed and cells were lysed in a volume of laemmli loading buffer equivalent to the size of the cell pellet and were boiled 5 minutes. Cells were separated in a 10% polyacrylamide gel and transferred to 0.45 μm nitrocellose. For detecting cyclin A in the blot, cyclin A antibody was purchased from Santa Cruz (SC-751). β-actin antibody was purchased from Sigma (A-5441). ef1-α was detected using an antibody from Upstate (05-235). The blots were developed using ECL purchased from Amersham.

**Chapter 3 Methods.**

**Transfections for flow cytometry.** Cells were seeded 18 to 24 hours before transfection at 0.4-1 X 10^5 cells per 10 cm dish and transfected by the calcium phosphate
method (9, 12, 17). The cells were transfected with plasmids (10 μg) that express wild type or deletion mutants of MDM2. Thirty to forty-eight hours after transfection the cells were collected and fixed. In the MDM2/cyclin A cotransfection experiment, WI38 cells were transfected with 10 μg of a plasmid expressing cyclin A along with 10 μg of a plasmid expressing MDM2 del 491-110.

**Antibodies.** The human cyclin D antibody was reactive for cyclin D1, D2 and D3 and was used to stain WI38 and H1299 cells. For cell cycle analysis in MDM2 Del 491-110 overexpressing cells a primary antibody to recognize the N-terminal epitope (N20, Santa Cruz) and a FITC conjugated secondary anti-rabbit IgG (Oncogene Science) were used. FITC conjugated N20 (to detect C-terminal deletion mutant MDM2 Del 491-110) and FITC conjugated SMP14 (to detect the N-terminal deletion mutants of MDM2) was purchased from Santa Cruz. For other flow cytometric experiments N20 was coupled with phycoerythrin (PE) using a phycoerythrin conjugation kit (Phycolink) from Prozyme. The PE-coupled antibody was separated from unconjugated PE and antibody using a Bio-Gel A-0.5m column. D-type cyclins were detected using FITC conjugated anti cyclin D antibody set from Pharmingen. The human cyclin D antibody was reactive for cyclin D1, D2 and D3. The FITC conjugated anti- cyclin E and A antibodies and anti Rb antibody for cyclin D- specific phosphorylation site (Ser 795) was purchased from Santa Cruz.

**Flow cytometry.** Cell cycle Analysis: Methods for cell cycle analysis have been described earlier (17, 18, 24). Cells were harvested 30 to 48 hours after transfection and fixed with 70% ethanol for overnight at 4°C. The fixed cells were incubated with appropriate antibody for 2 hours and an FITC-coupled secondary antibody for one hour.
in PBS, 0.5% BSA and 0.5% Tween 20. Cells were then washed three times in PBS and 0.5% BSA. To stain DNA with propidium iodide (PI), the cells were incubated with 0.1 mg per ml RNase A, 50 μg per ml propidium iodide, 0.1% NP40 in a trisodium citrate buffer for 30 minutes. The samples were analyzed in a fluorescence activated cell sorter (Elite, Coulter or FAC Star\textsuperscript{plus}, Becton Dickinson) in a flow cytometry core facility.

Relative levels of MDM2 expression per cell were determined by FITC-fluorescence intensity in the green channel (525 nm). Mock-transfected cells were used to determine the background fluorescence. Vector-transfected cells or cells transfected with a plasmid expressing an irrelevant protein did not induce growth arrest (17). Cells showing higher FITC-fluorescence intensity than mock-transfected cells were gated. The intensity of PI staining was recorded in the red channel.

**Comparison of cyclin expression.** To estimate and compare levels of cyclins or cyclin D-specific Rb phosphorylation, cells were incubated with FITC-coupled appropriate antibody or the respective matched IgG isotype after incubation with PE-coupled anti-MDM2 antibody. Intensities of the fluorescent dyes were plotted against cell number and compared as shown in the Figures. A minimum of 8,000 MDM2 overexpressing (transfected) cells was analyzed in each experiment. Experiments were repeated several times.

**Transfection with p16- or Brg1-siRNA.** For confocal experiments with siRNA, 5 μg of MDM2 expression plasmid was transfected with 80 pmol either p16- or Brg1-siRNA using a nucleofector from amaxa. WI38 cells were harvested and nucleofected using 3 X 10\(^6\) cells per transfection and nucleofector Program U-23. After nucleofection, cells were plated on sterile coverslips and fixed 48 hours later.
**Transient transfection promoter analysis.** Transient transfection promoter analysis was performed by a method published by Deb et al. (26). To determine cyclin A promoter activity, H1299 cells were transfected using Fugene 6 (Roche) with a luciferase reporter plasmid under the control of luciferase promoter (26) in the presence or absence of MDM2 or p16. For this experiment the amount of DNA used was 500 ng of vector, 100 ng of p16 expression plasmid, and 400 ng of MDM2 expression plasmid. Cell extracts containing equal amount of protein were assayed for luciferase activity. Luciferase assays were carried out using a commercial kit from Promega. Experiments were repeated several times.

**5’-deoxy-azacytidine treatment:** H1299 cells were treated for 72 hours with 5-aza-2’-deoxycytidine before transfection with MDM2 Del 491-110 expression plasmid, and the treatment was continued for another 48 hours after which the cells were harvested and fixed.

**Chapter 4 Methods.**

**Analysis of Bromodeoxyuridine incorporation:** Bromodeoxyuridine incorporation studies were performed by a method reported earlier (18, 24). Cells transfected using the calcium phosphate method with hMDM2 expression plasmids or mock-transfected cells were incubated with bromodeoxyuridine (BrdU, 10 μM) for 30 to 40 minutes 48 hours after transfection. Cells were then harvested by trypsinization, washed and fixed as described. Fixed cells were rehydrated and treated consecutively with HCl (2N) and sodium borate (0.1M) to partially denature DNA since the antibody can recognize BrdU in single-stranded DNA. A PE-coupled hMDM2 antibody and an FITC coupled anti-BrdU antibody were used to detect hMDM2 and BrdU respectively. The MDM2-PE
antibody was described previously in Chapter 3 methods. BrdU was detected using a FITC-coupled anti-BrdU monoclonal antibody set from Pharmingen. Cells were then incubated with 7AAD (20 μg per ml) to stain DNA and analyzed in a flow cytometry core facility.

**Flow cytometry.** Different concentrations of MDM2 expression plasmids were transfected by nucleofection into WI38, NIH3T3 and H1299 cells. Forty-eight hours after transfection the cells were harvested and fixed and stained for flow cytometric analysis as described previously using a primary antibody 2A10 (provided by Arnold Levine) and an anti-mouse IgG (goat) fluorescein conjugated secondary antibody (Calbiochem DC13L). DNA was stained using propidium iodide.

**Chapter 5 Methods.**

**Coimmunoprecipitation of MDM2-interacting proteins.** To identify MDM2-interacting proteins, cells were transfected with an MDM2 expression plasmid using the calcium phosphate method (9, 12, 17). Forty-eight hours after transfection, cells were washed in DPBS twice and the pellet was suspended in 990 μl Buffer A (10 mM Hapes, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, and 1X protease inhibitors Calbiochem Cocktail Set III (539134)). To this, 10 μl of 10% TritonX-100 was added and the suspension was incubated 5 minutes on ice. Cells were spun at 1700g at 4°C for 4 minutes. The supernatant was collected and spun at 20,000g at 4°C for 15 minutes for clarification, and the supernatant was taken as the cytoplasmic extract. The pellet generated from the Buffer A treatment (nuclei) was suspended in 190 μl Buffer A and 1mM CaCl₂. For every two plates harvested, 1 unit of micrococcal nuclease was added. The pellet was vortexed and incubated at 37° for 15 minutes. To
stop the reaction, 0.4µl of 250 mM EGTA was added for every two plates harvested. Cells were spun at 1700g at 4° for 4 minutes. To lyse the nuclei pellet, 1 ml of 10S buffer was added (50 mM Hepes, 0.1M NaCl, 0.3% NP40, 0.1M Triton-X-100, 10 mM NaPO₄ pH 7.0, 1 mM NaF, 0.14 mM PMSF, 0.5 mM DTT and 1X protease inhibitors Calbiochem Cocktail Set III (539134)). Lysate was passed through a 21 gauge needle, lysed on ice 5 minutes and spun at 1700g at 4° for 4 minutes. The supernatant was kept as the nuclear extract. Cytoplasmic and nuclear extracts were separately precleared in 1ml of their corresponding buffers using 12 µl normal mouse IgG agarose (Santa Cruz SC-2343) for 1.5 hours at 4° with tilting. The beads were pelleted at 800g for 2 minutes and MDM2 was immunoprecipitated with a monoclonal antibody coupled directly with agarose beads (Santa Cruz, SMP14 SC-965). The immunoprecipitate was washed two times to remove contaminating unbound proteins using 10S buffer, followed by two washes with 10 mM phosphate pH 6.8. For elution 40 µl of 100 mM glycine pH 2.5 was added to the beads and incubated 10 minutes at room temperature. The tubes were spun at 100 g for 2 minutes at room temperature and the supernatant was added to 4 µl of 0.5M phosphate pH 8.0. The elution step was then repeated one additional time. The fractions were then desalted using a Microcon filter device (42420) following the manufacturer’s protocol. Ammonium bicarbonate was then added to a final concentration of 0.1M and the associated proteins were then digested with trypsin and analyzed by the Peak Parking protocol described below. Proteins that bind nonspecifically to either the antibody or IgG coupled agarose act as false positives but can usually be identified as such by passing the sample through agarose beads coupled to irrelevant antibody. Only proteins that bind
nonspecifically to the antibody-coupled beads are detected in this experiment and can be subtracted from those found in the first experiment.

**Mass spectrometric analysis.** Mixtures were analyzed directly in Dr. Hunt's laboratory by employing "Peak Parking Technology" developed and patented at the University of Virginia (69-70). Immunoprecipitated and eluted samples were digested with trypsin and the resulting mixture of tryptic peptides (30 peptides/protein) were then analyzed by nanoflow-HPLC (5-200 nL/min) interfaced directly to electrospray ionization on a ThermoFinnigan, LC-Deca instrument. Peak Parking Technology under control of the mass spectrometer data system is employed to extend the sample peak width from 10 sec to 200 sec and thus to give the instrument time to record MS/MS spectra on more than a hundred different peptides that happen to elute in the same 10 sec chromatographic time window. Samples were fractionated initially at a flow rate of 200 nL/min on the microcapillary HPLC column and the mass spectrometer was scanned repetitively from m/z 300-2,000. When sample elutes from the column and the ion current detected by the instrument increases above background, the data system reduces flow rate to <10 nL/min and the instrument repetitively cycles through a program of six scans. (Software for this program was developed for Dr. Hunt's laboratory by ThermoFinnigan and is now commercially available.) The first spectrum (2 sec) records the masses of (M+H)+ and (M+2H)++ ions observed for the components eluting under the chromatographic peak. The next five scans (10 sec) record MS/MS spectra to generate sequence information on the five most abundant species observed in spectrum #1. These masses are then placed on an exclusion list along with the corresponding isotope peaks.
The next scan is used to re-measure ions characteristic of the component molecular weights and the next five scans record MS/MS spectra on the five most abundant species not already on the exclusion list. MS/MS spectra can be recorded on as many as 100 different co-eluting peptides using this protocol. Once the list of candidates to be fragmented is exhausted, the flow rate is changed back to 200 nL/min until the next group of peptides is detected. The above process is then repeated. At the end of the chromatographic run, proteins in the original mixture are identified by processing the complete set of MS/MS spectra against the protein and nucleic acid databases using the SEQUEST software program (71). Up to 6,000 sequences on tryptic peptides can be obtained in a single 4 hr chromatographic run with the above technology. Peptides present at the 5-10 fmol level in complex mixtures were readily identified with this approach.

**Translabeling and immunoprecipitation.** Cells were transfected with MDM2 expression plasmids using the nucleofection method. H1299 cells were nucleofected using program T-20 while program U-23 was used for WI38 cells. In both cases, 3 X10⁶ cells were used per transfection. Sixteen to 24 hours after transfection, cells were starved in methionine-free DMEM for one hour and incubated for two hours in 1.4 ml methionine-free DMEM with 10% dialyzed FBS and 16 µl³⁵S methionine. Plates were washed three times with PBS and lysed in 10S buffer on ice for 10 minutes. Cells were scraped from the plate and passed through a 21 guage needle and spun at 3220 g for 6 minutes at 4°C. The extracts were precleared for 1.5 hours using normal mouse IgG agarose (Santa Cruz SC-2343). Equal counts of each supernatant were immunoprecipitated with an agarose-conjugated anti-MDM2 antibody (Santa Cruz,
SMP14 SC-965) overnight at 4° with constant tilting. Beads were then washed three
times with 10S buffer, one time with 0.8M LiCl followed immediately by one wash with
TE buffer pH 8.0. After each wash cells were spun at 800 g. The beads were then boiled
in lammeli loading buffer and subjected to gel electrophoresis. The gel was incubated for
1 hour in Enhance (Perkin Elmer 6NE9701) followed by incubation in deionized water
for exactly 25 minutes and then a 10 minute incubation in 10% acetic acid/2%glycerol.
The gel was then dried for two hours and exposed to film.
O. Literature Cited.


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Rebecca Anne Frum was born on November 16, 1973 in Wheeling, WV and is a citizen of the United States. She graduated from Ashbrook High School, Gastonia, NC in 1992. She received her Bachelor of Science degree from North Carolina State University, Raleigh NC in 1998. After joining the laboratory of Swati Palit Deb at Wake Forest University in Winston-Salem NC in 1999, she moved with the lab to Virginia Commonwealth University in 2000 and subsequently entered the graduate program at VCU in 2002.