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Advances in Brief

Cul4A Physically Associates with MDM2 and Participates in the Proteolysis of p53

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Abstract

The *cullin 4A* (*Cul4A*) gene is amplified and overexpressed in breast and hepatocellular carcinomas. Cul4A functions as an E3 ligase and participates in the proteolysis of several regulatory proteins through the ubiquitin-proteasome pathway. Here, we show that Cul4A associates with MDM2 and p53. Depletion of Cul4A leads to an accumulation of p53. Moreover, expression of Cul4A increases the decay-rate of p53 and delays the accumulation of p53 in response to DNA damage. Cul4A fails to increase the decay of p53 in mouse embryonic fibroblasts lacking MDM2. In addition, the Cul4A-mediated rapid decay of p53 is blocked by p19ARF. The results provide evidence for a role of Cul4A in the MDM2-mediated proteolysis of p53.

Introduction

The tumor suppressor p53 is mainly regulated at the level of protein stability (reviewed in ref. 1). The p53 protein is constantly ubiquitinated and is degraded by the ubiquitin-proteasome pathway (2). Several pathways for the ubiquitination of p53 have been identified (1–4). The most widely studied mechanism for ubiquitination of p53 is that of MDM2. MDM2 physically associates with the NH_2-terminal activation domain of p53 to induce ubiquitination by acting as an E3 ligase (5–8). The mechanisms that activate the tumor suppression functions of p53 do so by blocking proteolysis of p53. The stabilization of p53 has been studied extensively in the context of damaged-DNA response and oncogenic stress. Cells activate the checkpoint pathways after exposure to damaged DNA. The checkpoint kinases phosphorylate p53 at multiple sites that disrupts the interaction with MDM2, leading to stabilization and activation of p53 (reviewed in ref. 9). In addition, oncogenic stress activates expression of the tumor suppressor ARF, which is a specific inhibitor of the E3 ligase function of MDM2 (reviewed in ref. 10). ARF directly binds to MDM2 and inhibits its ability to ubiquitinate p53, leading to an increased stabilization of p53.

The cullin-family member *Cul4A* is amplified in cancers (11, 12) and has been implicated in the ubiquitination and proteolysis of DDB2 (13), CDT1 (14), c-jun (15), and the STAT proteins (16). Here, we provide evidence that Cul4A physically associates with MDM2 and participates in the proteolysis of p53.

Materials and Methods

Antibodies, Immunoprecipitation, and Western. Anti-cullin 4A and DDB1 antibodies have been described previously (13). Anti-FLAG antibodies and M2-agarose beads were purchased from Sigma (St. Louis, MO). Anti-p53 monoclonal antibody (Ab6) was from Oncogene (Boston, MA), anti-mdm2 (SMP14) was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-green fluorescent protein (GFP) and antitubulin antibody was obtained from Labvision (Freemont, CA).

Construction of Recombinant Adenoviruses. Recombinant adenovirus expressing ARF has been described previously (17). The Cul4A virus was generated by with the Adenovator system from Q-Biogene according to the manufacturer's instructions.

Generation of Stable Cell Lines. Plasmid DNA expressing Cul-4A–V5-Flag was transfected in 293a and HeLa cells with LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA). Transfected cells were selected in medium containing 100 μg/mL zeocin for at least 20 days followed by expression analysis of the recombinant protein.

Analysis of the Decay Rate. HeLa cells were equally split and grown for 16 to 18 hours until they were ~70% confluent. Cells were infected (30 plaque-forming units per cell) with the respective adenoviruses. Sixteen hours after the adenovirus infection, cells were treated with 50 μg/mL cycloheximide and harvested at the indicated time points. Cell extracts were subjected to Western blot analysis as described previously (13). For mouse embryonic fibroblasts, cells were transfected with GFP-p53 expression plasmid. Sixteen hours after transfection, cells were trypsinized, pooled, and replated equally. Eight hours after replating, cells were infected either with Cul 4A or control virus.

The Small-interfering RNA (siRNA)-Mediated Knockdown of Cullin 4A. Cells were transfected with a total of 1 nmol/L siRNA oligos (per 10-cm dish) with the Oligofectamine Reagent (Invitrogen) according to manufacturer's instructions. The following sequences were used: human Cul4A, 5′AAGAAGAUUAACACGUGCUGG3′; human Cul4B, 5′AAGCCUAAAUUACCAGAAAUU3′; and control scramble siRNA sequence, 5′ AACAGUCGCGUUUGCGACUGGdTdT 3′.

Results and Discussion

To identify the protein partners and targets of Cul4A, we constructed cell lines that express Flag-epitope tagged Cul4A. Extracts of the cells (HeLa and 293) expressing Flag-Cul4A or of the parental cell lines were subjected to immunoprecipitations with a monoclonal antibody (M2) against the Flag-epitope. The immunoprecipitates from the Flag-Cul4A–expressing cells contained the known Cul4A binding proteins, including the subunits of COP9/Signalosome and DDB (data not shown). Interestingly, we observed that the immunoprecipitates from the Flag-Cul4A–expressing cells also contained MDM2 and p53 (Fig. 1*A*). The evidence for associations of MDM2 and p53 with Flag-Cul4A or Cul4A was observed also in reciprocal immunoprecipitation experiments in which extracts from Flag-Cul4A–expressing HeLa cells or parental 293 cells were immunoprecipitated with p53- or MDM2-antibodies and probed for Flag-Cul4A or Cul4A in Western blot assays (Fig. 1*B*). The observed interaction between the three proteins prompted us to investigate a possible role of Cul4A in maintaining the steady-state level of p53. We used siRNA to knock down the level of Cul4A in HeLa cells. Because our antibody against Cul4A did not distinguish between the highly homologous Cul4A and Cul4B, we used siRNA to knock down both proteins. As seen in Fig. 1*C*, the depletion of Cul4 (Fig. 1, *A* and *B*) resulted in an increase in the steady-state level of p53. Similar results were obtained also in U2OS cells (data not shown).

To additionally investigate the mechanism by which Cul4A regu-

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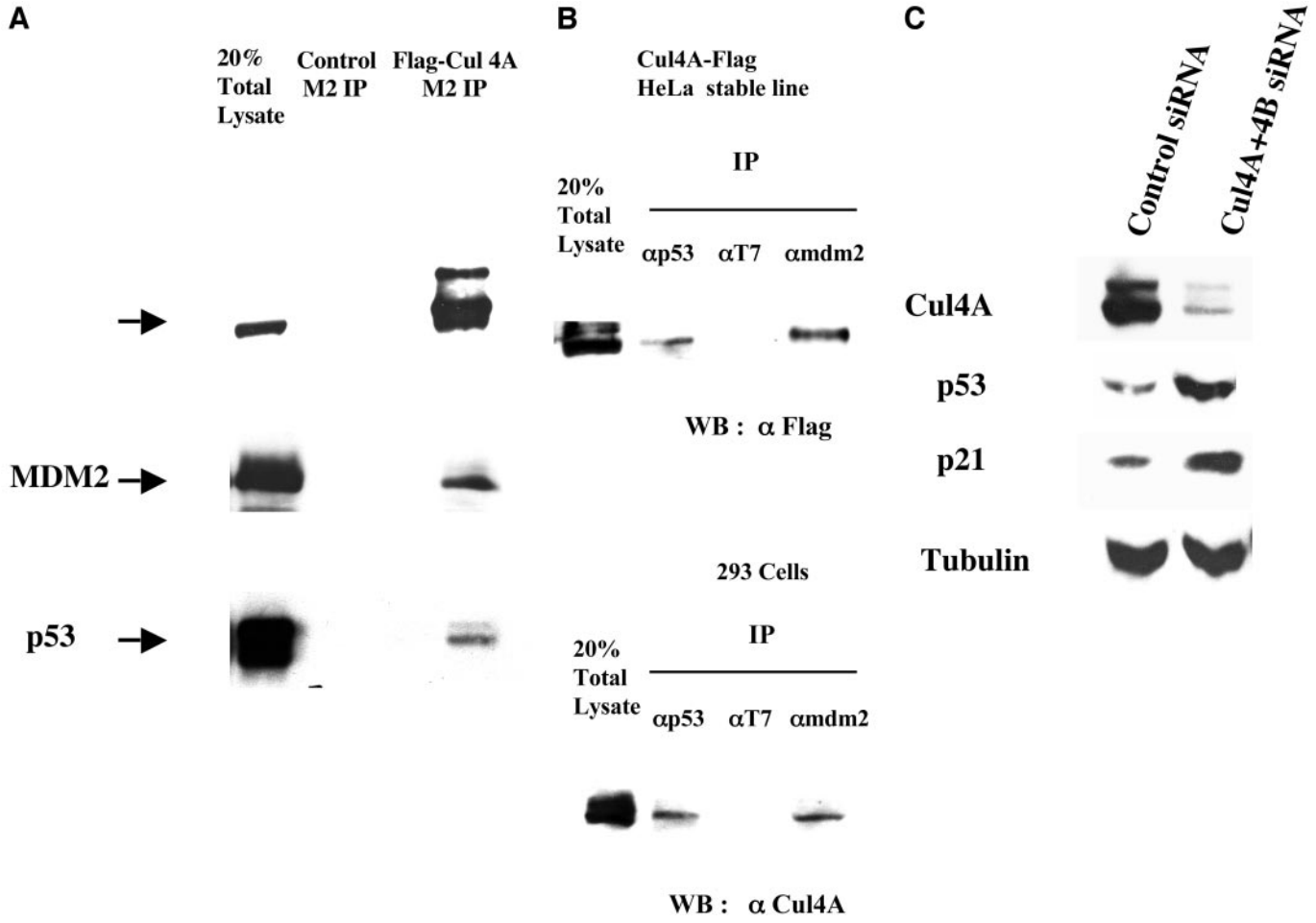

Fig. 1. Cul4A functionally interacts with p53 and MDM2. *A.* 293 cells stably expressing Cul4A-Flag were immunoprecipitated with Flag antibody covalently linked to agarose beads (M2 beads) as described in Materials and Methods. Immunoprecipitates were eluted with FLAG peptide and subjected to Western blot assays with Flag, MDM2, and p53 antibodies. *B*. Total extracts from HeLa cells stably expressing Cul4A-Flag or extracts from 293a cells were precleared with protein G-Sepharose followed by immunoprecipitation with anti-p53, mdm2, and a control antibody. The immunoprecipitates were eluted with gel loading buffer and immunoblotted with either Flag or Cul4A antibodies. *C*. HeLa cells were transfected with siRNAs specific for cullin 4A and 4B or with control siRNA. Forty-eight hours after transfection, cells were harvested, and the levels of p53, p21, and tubulin were analyzed by Western blots.

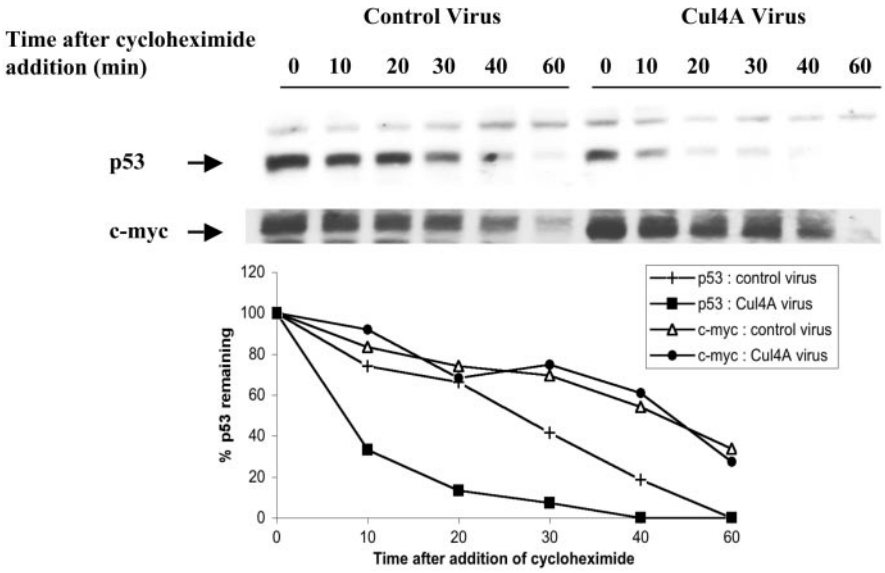

Fig. 2. Cul 4A enhances the decay rate of endogenous p53. HeLa cells were infected with either Cul4A or a control adenovirus. Sixteen hours after infection, cycloheximide was added to the medium to a final concentration of 50 μg/mL, and the cells were harvested at the indicated time points. Total cell extracts were subjected to Western blot assay with anti-p53 and anti-myc antibodies as described in Materials and Methods.

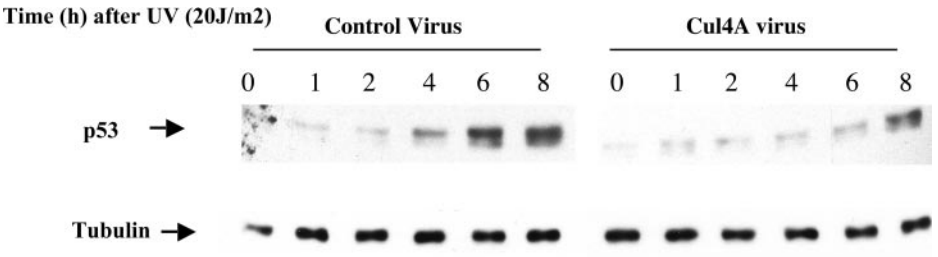

Fig. 3. Cul4A overexpression delays the up-regulation of p53 after UV treatment. HeLa cells were infected with either Cul4A or a control virus. Sixteen hours after infection, cells were treated with UV (20J/m2). The irradiated cells were harvested at indicated time intervals. Total cell lysates were analyzed for the level p53 and tubulin.

lates the level of p53, we sought to determine the effect of Cul4A on the decay rate of p53. We expressed Cul4A in HeLa cells with recombinant adenovirus. HeLa cells were infected with adenovirus expressing Cul4A or the Tet-activator protein (TA-virus). Sixteen hours after infection, cells were treated with cycloheximide to inhibit new protein synthesis. At different times after addition of cycloheximide, the cells were harvested, and the extracts were assayed for the levels of p53 and c-Myc. Expression of Cul4A significantly increased the decay rate of p53 (Fig. 2). The first order decay constants of p53 calculated from the apparent half-life are 0.09 ± 0.018/minutes in the presence of Cul4A and 0.03 ± 0.003/minute in the absence of Cul4A expression. When the same blot was probed for c-Myc, no significant change in the decay rate of c-Myc was observed. Thus, Cul4A specifically increased the decay rate of p53. Because DNA damage is known to increase the stability of p53, we investigated whether Cul4A expression had any effect on the accumulation of p53. HeLa cells were infected with control virus (TA) or Cul4A-expressing virus. Twelve hours after infection, the cells were treated with UV irradiation (20 J/m2) and then maintained in culture medium. At different times after irradiation, the cells were harvested, and the extracts were assayed for the accumulation of p53. In the control virus infected cells, a significant accumulation of p53 was observed within 4 to 6 hours, whereas a clear delay in the accumulation of p53 was observed in the Cul4A virus infected cells (Fig. 3).

The delay in accumulation of p53 after UV irradiation is consistent with a role of Cul4A in the MDM2 pathway of p53 proteolysis. Therefore, we investigated whether the Cul4A-induced proteolysis of p53 involves MDM2. To investigate the involvement of MDM2 in the Cul4A-induced proteolysis of p53, we compared the decay of p53 in the wild-type and in the p53-/MDM2-mouse embryonic fibroblasts. The mouse embryonic fibroblasts were transfected with a plasmid expressing GFP-tagged p53. Twelve hours after transfection, the cells from each group were pooled and equally divided into four plates to equalize for the transfection variations. The replated cells were then

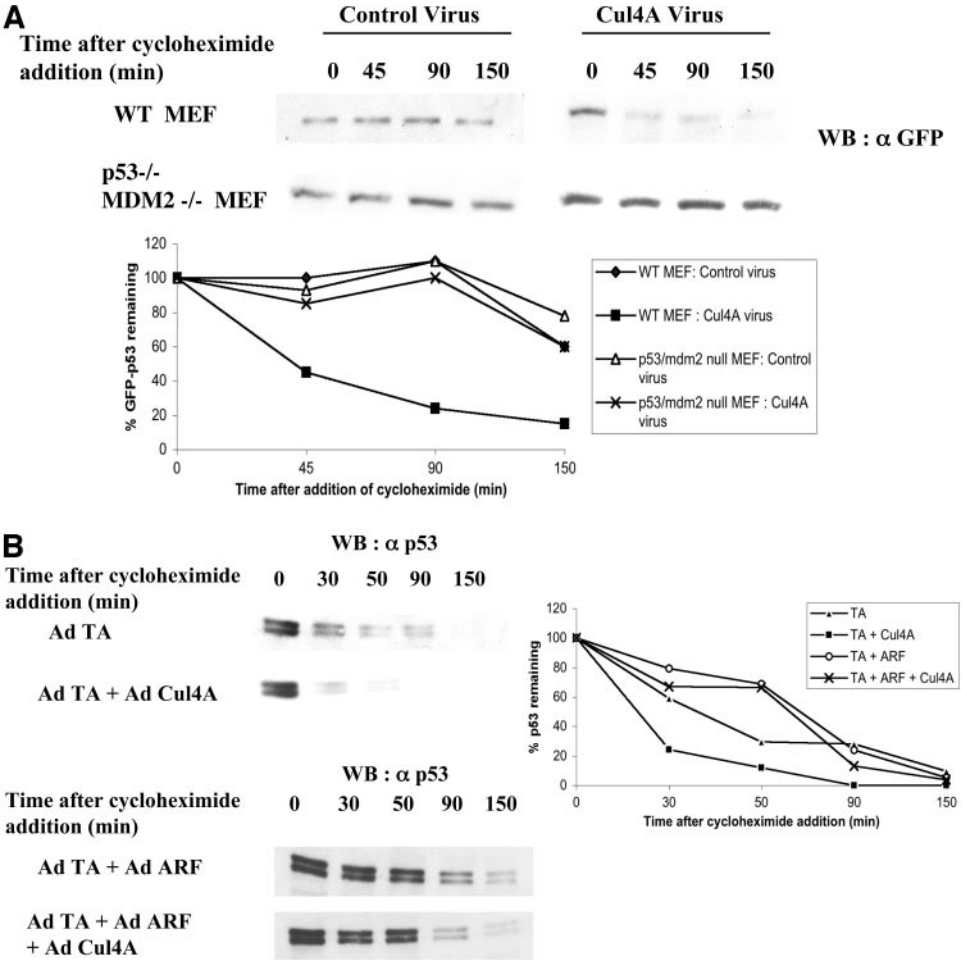

Fig. 4. Cul4A requires MDM2 for the degradation of p53. *A*. Mouse embryonic fibroblasts (WT and p53/MDM2 double null) were transfected with GFP-p53. Decay rate of GFP-p53 was studied according to the procedure described in Materials and Methods. *B*. HeLa cells were infected with the indicated combination of the adenoviruses, and the decay rates of p53 were analyzed as mentioned in Materials and Methods. The total virus was maintained at 100 plaque-forming units per cell with the TA virus. (*MEF*, mouse embryonic fibroblast; *WT*, wild-type; *WB*, Western blot.)

infected with virus-expressing Cul4A. Sixteen hours after infection, the mouse embryonic fibroblasts were treated with cycloheximide, and at different times, cells were harvested. The cell-extracts were assayed in a Western blot assay with GFP-antibody. A rapid decay (first-order decay constant 0.017 ± 0.001/minute) of the GFP-p53 was evident in the wild-type mouse embryonic fibroblasts that were infected with Cul4A virus compared with that in the mouse embryonic fibroblasts infected with a control virus (decay constant 0.004 ± 0.001/minute). However, very little Cul4A-induced decay of the GFP-p53 protein was observed in the p53-/MDM2-mouse embryonic fibroblasts within the time frame of the experiment (Fig. 4*A*). To additionally confirm the requirement for MDM2 in the Cul4A-mediated proteolysis of p53, we used recombinant adenovirus-expressing p19ARF to inhibit the E3 ligase function of MDM2. HeLa cells were infected with Cul4A virus in the presence or absence of ARF virus. Sixteen hours after infection, the cells were treated with cycloheximide to inhibit new protein synthesis, and the decay rates of p53 were analyzed by a Western blot assay. ARF expression had no effect on the expression of Cul4A from the recombinant virus (data not shown). Infection with the ARF virus efficiently blocked the rapid decay of p53 induced by the Cul4A virus (Fig. 4*B*). In the presence of ARF, the calculated first-order decay constant of p53 (0.01 ± 0.001/minute) was not significantly altered by Cul4A expression. Taken together, these observations suggest that Cul4A is involved in the MDM2-mediated proteolysis of p53.

The observations on Cul4A regulating the level of p53 are significant because the *Cul4A* gene is amplified or overexpressed in cancers (11, 12). It is likely that the overexpressed Cul4A reduces the steady-state level of p53 contributing to genomic instability and to eventual cancer development. The physical association of Cul4A with MDM2 and the requirement of MDM2 in the Cul4A-induced proteolysis of p53 indicate a potential collaboration between Cul4A and MDM2. MDM2 preferably generates monoubiquitinated p53 (18). It was shown that p300, acting as an E4 ligase, participates in the MDM2-mediated proteolysis of p53 (19). It is possible that Cul4A, like p300, cooperates with MDM2 to induce polyubiquitination of p53, and that the Cul4A-MDM2 interaction offers an alternative pathway of p53-proteolysis. Given the importance of the level of p53 for survival and normal behavior of cells, it is not surprising that mammalian cells will regulate p53 through multiple pathways. It is, however, possible that p300 and Cul4A collaborate with MDM2 in different contexts. For example, p300 might be important in targeting p53 in the context of transcription because it is believed to be a transcriptional coactivator of p53 (20). Because Cul4A is not known to be a transcriptional coactivator, the Cul4A-MDM2 interaction might be responsible for targeting the p53 molecules not engaged in transcription. However, a potential collaboration between MDM2, p300, and Cul4A cannot be ruled out.

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