The Epithelial Cell Transforming Sequence 2, a Guanine Nucleotide Exchange Factor for Rho GTPases, Is Repressed by p53 via Protein Methyltransferases and Is Required for G1-S Transition

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Abstract

The epithelial cell transforming sequence 2 (ECT2), a member of the Dbl family of guanine nucleotide exchange factor for Rho GTPases, is required for cytokinesis. The tumor suppressor p53 plays a crucial role in coordinating cellular processes, such as cell cycle arrest and apoptosis, in response to stress signals. Here, we showed that ECT2 is negatively regulated by wild-type p53 but not tumor-derived mutant p53 or other p53 family members. In addition, ECT2 is down-regulated in multiple cell lines by DNA damage agents and Nutlin-3, an MDM2 antagonist, in a p53-dependent manner. Furthermore, we provided evidence that inhibition of protein methyltransferases, especially arginine methyltransferases, relieve the repression of ECT2 induced by DNA damage or Nutlin-3 treatment. Finally, we generated multiple cell lines in which ECT2 is inducibly knocked down and found that ECT2 knockdown triggers cell cycle arrest in G1. Taken together, we uncovered a novel function for ECT2 and provided a novel mechanism by which p53 represses gene expression via protein methyltransferases. (Cancer Res 2006; 66(12): 6271-9)

Introduction

Rho GTPases are essential for cellular processes ranging from membrane trafficking to transcriptional activation (1, 2). Rho proteins exist in an inactive (GDP-bound) state and an active (GTP-bound) state. Guanine nucleotide exchange factors (GEF) positively regulate Rho GTPase activity by enhancing the release of bound GDP (3). The epithelial cell transforming sequence 2 (ECT2), a member of the Dbl family of GEFs for Rho GTPases, is required for cytokinesis (4–6). Indeed, a dominant-negative C-terminally truncated ECT2 prevents contractile ring formation at cytokinesis and consequently generates multinucleated cells (7). Interestingly, a N-terminally truncated ECT2 is oncogenic in NIH-3T3 cells and nude mice (8, 9), underscoring the significance of ECT2 overexpression in cancer (10). ECT2 induction by growth factors is also cell cycle–dependent, occurring in S phase and reaching the maximum in G2-M phase (11). Furthermore, ECT2 activity is regulated by multiple kinases, including Cdk1 and Plk1 (12, 13).

In response to stress signals, such as DNA damage and hyperproliferation, the p53 tumor suppressor is stabilized and coordinates the induction of genes involved in cell cycle arrest (p21), apoptosis (IGFBP3 and PUMA), or its own regulation (MDM2; refs. 14, 15). Furthermore, p53 represses genes responsible for cell cycle progression (cyclin B1 and CDC25C), antiapoptotic activities (Bcl-2 and survivin), or other functions (RECA4 and α-fetoprotein; refs. 16–20). Interestingly, p53 down-regulates gene expression through diverse mechanisms, such as interference with transcriptional activators/coactivators and modification of the chromatin structure (21). For instance, p53 competes with Sp1 to access the cyclin B1 promoter (20), p53 down-regulates MAP4 and Myc by recruiting histone deacetylases (HDAC), which prevent access of the transcriptional machinery to the promoter (17, 22). Furthermore, p53 represses E2F-dependent genes (cyclin A and cdk1) via the induction of the CDK inhibitor p21, which results in the maintenance of inactive pRb/E2F complexes (19, 23).

In this study, we showed for the first time that ECT2 is negatively regulated by p53 and by DNA damage in a p53-dependent manner. We found that activated p53 binds the ECT2 gene in vivo in response to DNA damage and Nutlin-3 treatment. Furthermore, we provided evidence that inhibition of protein methyltransferases, especially arginine methyltransferases, relieve the repression of ECT2 induced by DNA damage or Nutlin-3 in a p53-dependent manner. Finally, we generated multiple cell lines in which ECT2 is inducibly knocked down and found that ECT2 knockdown triggers cell cycle arrest in G1. Taken together, we uncovered a novel function for ECT2 and provided a novel mechanism by which p53 represses gene expression via the mitotic spindles during metaphase, and in the cleavage furrow during telophase (10). ECT2 induction by growth factors is also cell cycle–dependent, occurring in S phase and reaching the maximum in G2-M phase (11). Furthermore, ECT2 activity is regulated by multiple kinases, including Cdk1 and Plk1 (12, 13).

Materials and Methods

Reagents. Nutlin-3 was purchased from Cayman Chemical Company (Ann Arbor, MI). Antibodies were from Santa Cruz (Santa Cruz, CA), except anti-phosphorylated ERK1/2 (Cell Signaling Technology, Danvers, MA), anti-ERK (BD Biosciences, San Diego, CA), anti-Myc, anti-HA, and anti-pRb (Covance, Berkeley, CA). Other reagents were from Sigma (St. Louis, MO).

Plasmids. pcDNA3 vectors expressing p21 and various p53 proteins have been previously described (24–26). The p21 promoter reporter was also previously described (27). For the ECT2 promoter reporter (−1511/+78), the ECT2 fragment was amplified by PCR using the forward primer 5′-ACCCGTTTATTTGTTAATCTAACTGCCACA-3′ and reverse primer 5′-GACGAGCTCGTTCCACCTCTAAGCGGACTCCTC-3′. ECT2 deletion constructs were generated by PCR using the above reverse primer and a forward primer for each individual construct. These are, 5′-GGTCTCTG-GCATCTGATTCCCCCTTCTTC-3′ (−989/+78 promoter), 5′-AAATCTTCA-CAGCTGACAGCAGCA-3′ (−438/+78 promoter), 5′-GTACGCAGAAG-CACCTGCTACTCCTT-3′ (−269/+78 promoter), 5′-CCCGAGTCGTTGGGCT-GCTTATTGGAACAC-3′ (−165/+78 promoter), 5′-CAGCCGCCCCTCGGA-GAAGCTCCTGCCT-3′ (−85/+78 promoter). To generate ECT2 short
hairpin RNA (shRNA), oligos 5′-GATCCGGAGACTGACTTGGCA-GACTCTTCAGAAGAGATCTGCAAGATGAGCTCTCTTTTGGAAA-3′ and 5′-AGCTTTTCAAAAGAGGATCTGCTGGCAAGACTCTTCTTTTGAAGGAA-AGAGTCGCAAGACGTGACGG-3′ were designed to target the ECT2 first exon (in boldface). The oligos were annealed and cloned into pTER, a PoIII promoter-driven shRNA expression vector (28). The resulting vector was named pTER-ECT2.

Cell lines. The dual inducible H1299 cell lines for wild-type and/or mutant p53 expression, the inducible H1299 and MCF7 cell lines for p63 or p73 expression, H1299, MCF7, MCF7-p53-KD-7, HCT116 p53+/−, and p53−/− cell lines, were maintained as described previously (24, 29, 30). The MCF7 cell line containing the pCDNA6-TR vector (Invitrogen, Grand Island, NY), named MCF7-pTER-7, was generated in our laboratory. MCF7-pTER-7 cell lines that inducibly express ECT2 shRNA were generated by transfection with pTER-ECT2 and selected with medium containing 200 µg/mL of zeocin. Individual clones were screened for inducible ECT2 knockdown by Western blot analysis and two representative clones MCF7-ECT2-KD-25/−58 were chosen for subsequent studies.

Northern blot analysis. Total RNAs were isolated using TRIzol (Invitrogen) and used for Northern blot analysis as described previously (31). The p21 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes were described previously (31). The ECT2 probe was generated by PCR with the primers 5′-GAGGCTACGGTGGCAAGCAGC−3′ and 5′-TAAATCGCACCCATGTTGAT-3′.

Western blot analysis. Cells were washed twice with PBS, resuspended with 2× SDS sample buffer, incubated at 95°C for 5 minutes, and used for Western blot analysis as described previously (31).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (CHIP) was done as previously described (32). Protein-DNA complexes were immunoprecipitated with anti-HA antibody or a mixture of anti-p53 antibodies (DO-1, PAh21, PAh1801, and PAh240). PCR primers to amplify a p53-responsive region in the ECT2 gene (+29/+345) were 5′-CGAG-GAAATGCGGTATTTGTGAGA−3′ and 5′-AGAGGCAACAGTGGGAGC-GAAG-3′. PCR primers to amplify the p53-RE1 within the p21 promoter are 5′-CAGGCTGGTCTGATGGG-3′ and 5′-TCCAGAATAGGCTTTAGG-3′.

Growth rate and DNA histogram analysis. To determine cell growth rate, 1 × 10⁵ cells were seeded per six-well plate. After 24 hours, cells were untreated or treated with tetracycline. At the times indicated, cells were collected and counted using a Coulter cell counter (Beckman Coulter Corporation, Miami, FL). For DNA histogram analysis, 2 × 10⁵ cells were collected per 90-mm-diameter plate and were untreated or treated with tetracycline for 2 days. Cells were collected and stained with propidium iodide as previously described and examined by fluorescence-activated cell sorter (BD FACS Calibur, San Jose, CA; ref. 25).

Luciferase assay. Dual-luciferase reporter assay was done according to the manufacturer’s instructions (Promega, Madison, WI). Cells (3 × 10⁴) were plated on a 24-well plate, cultured for 4 hours, and then transfected with pGL2-basic reporter (250 ng), an activator in pCDNA3 vector (250 ng), and Renilla luciferase vector (2.1 ng). Luciferase assay was done on triplicate wells after 2 days.

Results

ECT2 is down-regulated by p53 but not mutant p53 or other p53 family members. To identify novel target genes differentially regulated by p53 and tumor-derived mutant p53, an Affymetrix GeneChip assay was done on RNA samples from two dual inducible H1299 cell lines (#317 and #268; ref. 33), which were induced to express wild-type p53 and/or mutant p53 for 24 hours. As expected, many known genes up-regulated by wild-type p53 were detected in this assay, including p21 and MDM2. In contrast, ECT2 was found to be down-regulated by wild-type p53.

To confirm this, Northern blot analysis was done using the same RNA samples as for the GeneChip assay. We found that ECT2 expression was significantly decreased in both #317 and #268 cells upon wild-type p53 induction (Fig. 1A, compare lanes 1 and 5 with lanes 2 and 6, respectively). In contrast, ECT2 expression was slightly increased in #317, but not in #268, following mutant p53 induction (Fig. 1A, compare lanes 1 and 5 with lanes 3 and 7, respectively). However, when wild-type and mutant p53 were simultaneously expressed, ECT2 repression by wild-type p53 was attenuated in both #317 and #268 cells (Fig. 1A, compare lanes 2 and 6 with lanes 4 and 8, respectively). To ascertain that wild-type p53 is transcriptionally active, p21 expression was assessed and found to be up-regulated upon wild-type but not mutant p53 induction (Fig. 1A, compare lanes 2 and 6 with lanes 3 and 7, respectively). The GAPDH level was determined as a loading control (Fig. 1A).

Next, we did Western blot analysis to examine whether the alteration in ECT2 transcript level is accompanied by a change in ECT2 protein level. We found that upon wild-type p53 induction, ECT2 was significantly reduced in both #317 and #268 cells (Fig. 1B, compare lanes 1 and 5 with lanes 2 and 6, respectively). In contrast, mutant p53 had no effect (Fig. 1B). Interestingly, when both wild-type and mutant p53 were induced, ECT2 was less efficiently repressed by wild-type p53 (Fig. 1B, compare lanes 2 and 6 with lanes 4 and 8, respectively). This is consistent with previous studies that mutant p53 inhibits the transcriptional activity of wild-type p53 through the formation of heterotetramers (33). Indeed, we observed a similar dominant-negative effect of mutant p53 on p21 induction (Fig. 1B, p21). The level of actin was used as a loading control.

p63 and p73 share a high similarity to p53, especially in their DNA-binding domains, and transactivate many p53 targets, such as p21 and MDM2 (29, 34). Thus, we examined whether p63 and p73 regulate ECT2 expression. As shown in Fig. 2A, p63, p63y, p73a, p73β, and ΔNp73β were unable to significantly regulate ECT2 expression, although ECT2 was slightly reduced by p63β. In contrast, p63 and p73 isoforms significantly induced p21 (Fig. 1C). Taken together, these results suggest that ECT2 is specifically regulated by p53.

ECT2 is repressed by DNA damage agents and Nutlin-3 in a p53-dependent manner. To investigate whether endogenous p53 represses ECT2, MCF7 cells were treated with DNA damage agents, i.e., camptothecin, doxorubicin, and etoposide. We found that following treatment for 24 hours, each DNA damage agent stabilized endogenous p53, which resulted in a decrease in ECT2 following treatment for 24 hours, each DNA damage agent regulated by p53.

In contrast, ECT2 repression by wild-type p53 through the formation of heterotetramers (33). Indeed, we observed a similar dominant-negative effect of mutant p53 on p21 induction (Fig. 1B, p21). The level of actin was used as a loading control.
**ECT2 Repression by p53 through Protein Methylation**

**Figure 1.** ECT2 was down-regulated by wild-type but not mutant p53, p63, or p73. A, the level of ECT2 transcripts was decreased by p53. Northern blots were prepared using total RNAs isolated from H1299 cells uninduced (−) or induced (+) to express wild-type p53, mutant p53, or both for 24 hours. The blots were probed with cDNAs derived from ECT2, p21, and GAPDH genes, respectively. B, the level of ECT2 protein was decreased by p53. Western blots were prepared using extracts from H1299 cells uninduced (−) or induced (+) to express wild-type p53, mutant p53, or both for 24 hours. ECT2, p53, p21, and actin were detected using anti-ECT2, anti-p53, anti-p21 and anti-actin, respectively. C, ECT2 was not regulated by p63 or p73. Western blots were prepared using extracts from H1299 and MCF7 cells uninduced (−) or induced (+) to express various p63 and p73 isoforms for 24 hours. The levels of p63 and p73 were detected using anti-Myc and anti-HA, respectively.

Effect on ECT2 or p21 expression in MCF7-p53-KD-7 cells (Fig. 2C). Together, this suggests that endogenous p53 is necessary and sufficient to down-regulate ECT2 expression.

The ECT2 gene is bound and repressed by p53 but is slightly repressed by p21. To determine whether p53 directly inhibits ECT2 transcription, luciferase assays were done on the ECT2 promoter reporter (−1511/+78). As shown in Fig. 3A (left), the ECT2 promoter activity was reduced by 80% in the presence of wild-type but not DNA-binding mutant p53. As a control, the p21 promoter reporter was highly activated by wild-type but not mutant p53 (Fig. 3A, middle). Western blot analysis was done and showed that wild-type and mutant p53 were expressed at equivalent levels (Fig. 3A, right).

It is well-known that p53 regulates gene expression through a p53-responsive element (p53-RE) composed of two copies of the 10-bp half-site RNN[AT][AT][AT][AT]GYY (where R, purine; and Y, pyrimidine), separated by up to 13 bp (36). We searched for, but failed to find, such an element in the ECT2 promoter. Nevertheless, we further characterized the ECT2 promoter through a series of deletions. As shown in Fig. 3B, the activity of an ECT2 promoter (−853+/78) was still significantly reduced by p53. Shorter deletion constructs were generated, which could not be examined due to low basal promoter activity.

Recent studies showed that p53 represses certain genes indirectly through p21 (19, 23). To examine whether p21 alone is sufficient to inhibit ECT2, luciferase assay was done using the ECT2 promoter (−1511/+78) reporter. As shown in Fig. 3C (left), the ECT2 promoter activity was reduced by p21 (≤45%), which is considerably less than that (80%) by p53. As a control, the p21 promoter was found to be activated by p53 but not p21 (Fig. 3C, right). Therefore, p21 could specifically inhibit the ECT2 promoter, but this effect alone does not account for the strong repression of ECT2 by p53.

p53 is a modular transcription factor composed of two transcriptional activation domains (AD1 and AD2), a DNA-binding domain, a tetramerization domain, and a regulatory basic domain (37). Recent studies showed that the activation and the basic domains play a crucial role in the differential transactivation of p53 target genes (25, 31). Above, we showed that an intact DNA-binding domain is required for p53-mediated ECT2 repression (Fig. 3A). To determine what other domains are necessary for ECT2 repression, we examined the effect of p53ΔBD, which lacks the basic domain; p53ΔAD1ΔBD, which lacks the AD1 and basic domain; p53ΔAD2ΔBD, which lacks AD2 and the basic domain; and p53ΔAD1ΔAD2ΔBD, which lacks AD1 and basic domain along with mutations in the AD2. In Fig. 3D (left), we showed that the ECT2 promoter was markedly repressed by p53, p53ΔBD, and p53ΔAD1ΔBD, but less efficiently by p53ΔAD2ΔBD and p53ΔAD1ΔAD2ΔBD. These results suggest that AD2 is essential for the efficient repression of ECT2 by p53. In contrast, the p21 promoter was activated efficiently by p53, p53ΔBD, and p53ΔAD1ΔBD, but less efficiently by p53ΔAD2ΔBD and p53ΔAD1ΔAD2ΔBD (Fig. 3D, right), consistent with our earlier studies that both p53ΔAD1 and p53ΔAD2 play a role in p21 induction (24).

To address whether endogenous p53 binds the ECT2 gene in vivo, we did ChIP assays. MCF7 cells were treated with Nutlin-3 or the DNA damage agent cisplatin for 8.5 hours and the p53-DNA complexes were immunoprecipitated with anti-p53 antibodies or a control anti-HA antibody. PCR was done to scan the promoter, first exon, and first intron of ECT2. As a control, PCR was done on a
sequence containing the upstream p53-RE in the p21 promoter (Fig. 3E, left). We found that an ECT2 sequence (+29/+345) was significantly enriched upon p53 induction (Fig. 3E, right). As expected, the p21 promoter fragment was highly enriched upon p53 induction (Fig. 3E, right). No DNA fragment was enriched by the control anti-HA antibody (Fig. 3E, right). These results suggest that endogenous p53 directly binds the ECT2 gene in vivo.

Protein methyltransferases, especially arginine methyltransferases, mediate p53 repression of ECT2. p53 is known to repress certain genes through interaction with HDACs. To assess whether HDACs are involved in ECT2 repression, we did Western blot analysis on MCF7 cells treated with an HDACs inhibitor, trichostatin A or sodium butyrate, along with camptothecin. We found that HDAC inhibitors had no effect on ECT2 expression.

Next, we investigated whether protein methylation is involved in ECT2 repression. Cells were pretreated with methylthioadenosine, an inhibitor of protein methyltransferases, for 6 hours, followed by treatment with camptothecin for 24 hours (38). We showed that in MCF7 cells treated with camptothecin, endogenous p53 was stabilized, leading to ECT2 repression and p21 induction (Fig. 4A, compare lanes 1 and 2). In contrast, methylthioadenosine alone had no effect on ECT2 or p21 (Fig. 4A, compare lane 1 with lanes 3, 5, and 7). Interestingly, whereas lower levels of methylthioadenosine had no effect on p53 stabilization, a higher level was able to stabilize p53, which was still not fully active to induce p21 (Fig. 4A, compare lanes 1, 3, 5, and 7). Furthermore, methylthioadenosine had no significant effect on the level of p53 activated by camptothecin (Fig. 4A, p53). Surprisingly, we found that methylthioadenosine attenuated the repression of ECT2 by camptothecin in a dose-dependent manner (Fig. 4A, compare lane 2 with lanes 4, 6, and 8). We also found that methylthioadenosine inhibited, to some extent, the induction of p21 by camptothecin (Fig. 4A, p21).

However, in MCF7-p53-KD-7 cells, there was no change in ECT2 and p21 expression upon treatment with camptothecin, methylthioadenosine, or both agents (Fig. 4A, lanes 9-16). This suggests that protein methylation is involved in the repression of ECT2 by DNA damage in a p53-dependent manner.

To further determine how methylthioadenosine relieves ECT2 repression upon DNA damage, we examined the kinetics of methylthioadenosine function in MCF7 cells pretreated with 250 μmol/L of methylthioadenosine for various times, followed by camptothecin treatment for 24 hours. We found that pretreatment with methylthioadenosine for 1 hour was most efficient in relieving ECT2 repression, followed by 3, 6, and 0 hours of pretreatment (Fig. 4B, compare lane 2 with lanes 6, 8, 10, and 12). Interestingly, pretreatment with methylthioadenosine for 9 hours had little effect (Fig. 4B, compare lanes 2 and 4). Therefore, subsequent experiments were done with 1 hour of pretreatment.

Next, we examined whether protein methylation plays a role in Nutlin-3-mediated repression of ECT2 using isogenic HCT116 p53+/+ and p53−/− cells pretreated with 250 μmol/L of methylthioadenosine for 1 hour, followed by treatment with Nutlin-3 for 24 hours. In HCT116 p53+/+ cells, methylthioadenosine alone had no effect on ECT2 and p21 expression, although the level of p53 was slightly increased (Fig. 4C, compare lanes 1 and 2), consistent with the results obtained in MCF7 cells (Fig. 4A, compare lanes 1 and 7). However, p53 was stabilized by Nutlin-3 treatment, leading to a significant repression of ECT2 and induction of p21 (Fig. 4C, compare lane 1 with lanes 3 and 5). In addition, methylthioadenosine slightly enhanced the p53 stabilization by Nutlin-3 (Fig. 4C, p53, compare lanes 3 and 5 with lanes 4 and 6, respectively). Most interestingly, we found that methylthioadenosine inhibited ECT2 repression as well as p21 induction by Nutlin-3 (Fig. 4C).

In contrast, in HCT116 p53−/− cells, there was no detectable level of endogenous p53, and therefore, no repression of ECT2 or induction of p21 by Nutlin-3 (Fig. 4C, compare lanes 7 and 8). In addition, methylthioadenosine had no effect on ECT2 expression in the presence or absence of Nutlin-3 (Fig. 4C, lanes 7-12).

To address whether protein methylation plays a role in the regulation of other p53 target genes, we examined cyclin B1 (p53-repressed gene) and MDM2 (p53-induced gene). In HCT116 p53+/+ cells, methylthioadenosine alone had no effect on cyclin B1 and MDM2 expression (Fig. 4C, compare lanes 1 and 2). As expected, endogenous p53 activated by Nutlin-3 decreased cyclin B1 and increased MDM2 expression (Fig. 4C, compare lanes 1 with lanes 3 and 5). Interestingly, we found that methylthioadenosine markedly prevented cyclin B1 repression by Nutlin-3 (Fig. 4C, compare lanes 3 and 5 with lanes 4 and 6, respectively).
Methylthioadenosine also reduced MDM2 induction by Nutlin-3 but to a lesser extent than p21 induction (Fig. 4C, p21 and MDM2). Likewise, the effect of methylthioadenosine on the regulation of cyclin B1 and MDM2 by Nutlin-3 was found to be p53-dependent (Fig. 4C, lanes 7-12).

Next, we wanted to examine whether methylthioadenosine is capable of attenuating the repression of the ECT2 promoter by p53. To test this, the luciferase activity for the ECT2 promoter (−1511/+78) was measured in cells pretreated with methylthioadenosine for 0 to 5 hours, followed by p53 expression. We found that p53 repression was attenuated by methylthioadenosine pretreatment (Fig. 4D).

Protein methylation is catalyzed by two groups of enzymes, lysine and arginine methyltransferases (39). Methylthioadenosine, an analogue of S-adenosyl-L-methionine, is a broad inhibitor of methyltransferases (38). To explore whether arginine methylation is involved, MCF7 cells were pretreated for 1 hour with arginine methyltransferase inhibitor 1 (AMI-1), a specific inhibitor of arginine methyltransferases, followed by treatment with camptothecin for 24 hours (40). Pretreatment with methylthioadenosine
was used as a control. We found that AMI-1 alone had no effect on ECT2 expression (Fig. 4E, compare lane 1 with lane 5 and 7). In addition, endogenous p53 was stabilized efficiently in the presence or absence of AMI-1 (Fig. 4E, compare lane 2 with lanes 6 and 8). However, we found that p53 repression of ECT2 was attenuated by AMI-1 in a manner similar to methylthioadenosine (Fig. 4E, compare lane 2 with lane 4, 6, and 8). In addition, AMI-1 inhibited, to some extent, p21 induction (Fig. 4E, compare lane 2 with lanes 6 and 8). These results revealed that p53 regulates the expression of some target genes through a mechanism that involves arginine methyltransferases.

In response to cellular insults, p53 transcriptional activity is posttranslationally modified, including phosphorylation and acetylation (37). To determine whether p53 is arginine-methylated, we did immunoprecipitation on MCF7 cells treated with Nutlin-3 by Western blot analysis with either anti-p53 or anti-methylated-p53 was immunoprecipitated with anti-p53 antibodies followed by Western blot analysis with either anti-p53 or anti-methylated-arginine antibodies. We found that upon treatment with Nutlin-3, camptothecin, or doxorubicin for 8 hours, endogenous p53 was immunoprecipitated with anti-p53 antibodies followed by Western blot analysis with either anti-p53 or anti-methylated-arginine antibodies. We found that upon treatment with Nutlin-3 or DNA damage, there was no detectable level of arginine-methylated p53, suggesting that p53 is unlikely to be arginine-methylated.

Knockdown of ECT2 induces cell cycle arrest in G1. To determine the role of ECT2 in the p53 pathway, we generated MCF7 cell lines, MCF7-ECT2-KD-58/-25, in which ECT2 is inducibly knocked down by the tetracycline-inducible shRNA expression system. We showed that the induction of ECT2 shRNA for 24 hours elicited a significant decrease in ECT2 levels but little if any change in p53 or p21 levels (Fig. 5A, compare lanes 1 and 3 with lanes 2 and 4, respectively). To rule out the possibility that shRNA elicits an IFN response, we did reverse transcription-PCR to measure the induction of 2′,5′-oligoadenylate synthetase, a classic IFN target gene (41), and found that 2′,5′-oligoadenylate synthetase was not induced upon shRNA expression. To determine whether ECT2 knockdown has an effect on cell proliferation, colony formation assay was done. We found that cell proliferation was markedly inhibited by ECT2 knockdown (Fig. 5B). As a control, we examined the growth rate of parental MCF7-pTER-7 cells and found that tetracycline had no effect (Fig. 5B). To further characterize this effect, a 5-day growth curve was done (Fig. 5C). As expected, tetracycline had no effect on the growth rate of parental MCF7-pTER-7 cells (Fig. 5C). Likewise, the growth rate of MCF7-ECT2-KD-58/-25 cells was markedly inhibited by ECT2 knockdown (Fig. 5C). Next, we did a DNA

**Figure 4.** Protein methylation is required for p53 repression of ECT2. A, methylthioadenosine (MTA) attenuates ECT2 repression by DNA damage in a p53-dependent manner. Western blots were prepared using extracts from MCF7 or MCF7-p53-KD-7 cells that were pretreated with various doses of methylthioadenosine (5-250 μmol/L) for 6 hours, followed by treatment with 0.2 μmol/L of camptothecin (CPT) for 24 hours. B, time-dependent release of ECT2 repression by methylthioadenosine. Western blots were prepared using extracts from MCF7 cells that were pretreated with 250 μmol/L of methylthioadenosine for various times, followed by treatment with 0.2 μmol/L of camptothecin for 24 hours. C, methylthioadenosine attenuates ECT2 repression by Nutlin-3 in a p53-dependent manner. Western blots were prepared using extracts from HCT116 p53+/− and p53−/− cells that were pretreated with 250 μmol/L of methylthioadenosine for 1 hour, followed by treatment with 5 to 10 μmol/L of Nutlin-3 for 24 hours. D, p53 repression of the ECT2 promoter was attenuated by methylthioadenosine. H1299 cells were cotransfected with the ECT2 promoter (−1511/+78) reporter along with pcDNA3 or pcDNA3 expressing p53. Cells were preincubated with 250 μmol/L of methylthioadenosine for various times (0-5 hours) before transfection. E, AMI-1 attenuates ECT2 repression by DNA damage. Western blots were prepared using extracts from MCF7 cells that were pretreated with 250 μmol/L of methylthioadenosine, or 50 to 100 μmol/L of AMI-1 for 1 hour, followed by treatment with 0.2 μmol/L of camptothecin for 24 hours.
histogram assay to determine the cell cycle profile of cells induced to express ECT2 shRNA for 48 hours. We found that upon ECT2 knockdown, the number of cells in G1 phase was markedly increased, concomitantly with a decrease in the number of cells in S phase (Fig. 5D). There was no increase in the number of apoptotic cells (sub-G1).

The above data clearly indicate that ECT2 knockdown induces G1 arrest. Thus, we analyzed the effect of ECT2 knockdown on several regulators necessary for G1-S phase transition, including pRb, p130, and E2F1. The level of these proteins was measured in MCF7-ECT2-KD-58 cells induced to express ECT2 shRNA for 1 to 4 days in the presence or absence of camptothecin. As expected, endogenous p53 was stabilized by camptothecin after 1 day, which resulted in ECT2 repression and p21 induction (Fig. 6A, compare lanes 1 and 2). Furthermore, we detected an increase in active, hypophosphorylated pRb and p130 and a decrease in E2F1. These responses to camptothecin were maintained during the 4-day testing period (Fig. 6A, pRb, p130, and E2F1). Furthermore, upon simultaneous treatment with camptothecin and ECT2 knockdown, endogenous p53 was efficiently activated, concomitantly with a stronger effect on these proteins than each individually (Fig. 6A). These results suggest that ECT2 is a novel mediator of G1 arrest by p53. Therefore, overexpression of ECT2 may exert an inhibitory effect on p53-mediated G1 arrest. To test this, we generated MCF7 cell lines in which ECT2 is inducibly expressed by the tetracycline-inducible system. We found that ECT2 overexpression alone had no effect on cell proliferation and was not sufficient to inhibit the strong G1 arrest mediated by p53 in response to DNA damage. To further test this, we generated dual-inducible H1299 cell lines, which inducibly express p53 and/or ECT2. We found that ECT2 overexpression alone was unable to attenuate the G1 arrest mediated by p53 overexpression. These results suggest that overcoming p53 repression of ECT2 is not sufficient to abrogate p53-dependent G1 arrest mediated by other p53 targets, such as p21.

ECT2 is a GEF for Rho GTPases, which are necessary for the activation of extracellular signal-regulated kinase (ERK). To determine whether ECT2 is necessary for ERK activation, we examined the level of activated ERK1/2 on ECT2 knockdown for 2 to 4 days. We found that ECT2 knockdown led to a significant decrease in

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**Figure 5.** ECT2 is required for G1-S transition. 
A, generation of ECT2 knockdown MCF7 cell lines. Western blots were prepared using extracts from MCF7-ECT2-KD-58/-25 cells uninduced (−) or induced (+) to knock down ECT2 for 24 hours. 
B, ECT2 is required for colony formation. MCF7-pTER-7 and MCF7-ECT2-KD-58/-25 cells were untreated or treated with tetracycline and cultured for 14 days. 
C, ECT2 is required for cell proliferation. Growth rate of MCF7-pTER-7 and MCF7-ECT2-KD-58/-25 cells were uninduced or induced to knock down ECT2 for 48 hours. The percentage of cells in each phase of the cell cycle from three independent experiments was determined by DNA histogram analysis.
active, phosphorylated ERK1/2 (Fig. 6B, compare lanes 1, 3, and 5 with lanes 2, 4, and 6, respectively). Importantly, there was no change in the level of total ERK1/2 protein. This suggests that ECT2 knockdown ultimately impinges on the activation of the mitogen-activated protein kinase signaling pathway, which is necessary for cell cycle progression in G1 phase (42).

Discussion

In response to receptor-mediated stimulation (growth factors and extracellular matrix proteins), Rho GTPases are activated via GEFs, which promote cell proliferation, migration, and morphogenesis. However, little is known about how GEFs are themselves regulated. For the first time, to our knowledge, a GEF, i.e., ECT2, was found to be regulated by p53. Specifically, we found that ECT2 is down-regulated by endogenous p53 activated by DNA damage or Nutlin-3. We also found that ECT2 knockdown induces G1 arrest. Thus, in addition to its function in cytokinesis, ECT2 possesses a novel function in the cell cycle transition from G1 to S phase. Furthermore, we found that ECT2 is necessary for ERK1/2 activation and ECT2 knockdown leads to hypophosphorylation of the pRb family proteins and decreased E2F1 expression. Taken together, we postulate that in response to stress signals, p53 is activated and represses ECT2, which then inhibits the activation of Rho GTPases, especially RhoA, leading to a diminished level of active ERK1/2, and subsequently, an increased level of active pRb family proteins and a block of G1-S transition (Fig. 6C). Therefore, p53 repression of ECT2 provides a novel mechanism by which p53 regulates the activity of Rho proteins and ultimately functions as a tumor suppressor.

In this study, we found a p53-responsive region (+29/+345) in the ECT2 gene, which is selectively bound by endogenous p53 in response to DNA damage or Nutlin-3. This region does not contain a consensus p53-RE, although similar sequences containing mismatches in noncritical positions were identified. Nonetheless, we may speculate that p53 inhibits ECT2 transcription by directly binding to one of these nonconsensus elements and recruiting transcriptional repressors. Alternatively, p53 may interact with promoter-bound transcriptional activators/coactivators as shown for other p53-repressed genes, such as cyclin B1 and cdk1 (20, 43). Here, we also found that p21 down-regulates, to some extent, ECT2 promoter activity. However, p21 alone does not account for the strong repression induced by p53. Furthermore, we showed that ECT2 is repressed through a mechanism by which p53 transcriptional activity is modulated by protein methylation. Interestingly, a recent study showed that lysine methylation at K372 via Set9 stabilizes p53 and stimulates its ability to transactivate p21 and Bax (44). Therefore, it is reasonable to speculate that methylation of p53 by Set9 is necessary for the repression of ECT2 and induction of p21. However, we also found that an arginine methyltransferase inhibitor attenuated p53-mediated ECT2 repression and, to a limited degree, p21 induction. In addition, we were unable to detect endogenous arginine-methylated p53. Thus, we speculate that arginine methylation of p53 cofactors or histones modulate p53 transcriptional activity.

We showed that a second activation domain in p53 is required for efficient repression of the ECT2 promoter. Interestingly, the second activation domain is also required for p53-mediated activation of proapoptotic target gene IGFBP3 (32). Previously, we have shown that deletion of the second activation domain abolishes...
Ect2 Repression by p53 through Protein Methylation


The Epithelial Cell Transforming Sequence 2, a Guanine Nucleotide Exchange Factor for Rho GTPases, Is Repressed by p53 via Protein Methyltransferases and Is Required for G₁-S Transition

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