Enigma negatively regulates p53 through MDM2 and promotes tumor cell survival in mice

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The human E3 ubiquitin ligase murine double minute 2 (MDM2) targets the tumor suppressor p53 for ubiquitination and degradation but also promotes its own ubiquitination and subsequent degradation. As the balance between MDM2 and p53 levels plays a crucial role in regulating cell proliferation and apoptosis, we sought to identify factors selectively inhibiting MDM2 self-ubiquitination. Here we have shown that the LIM domain protein Enigma directly interacts with MDM2 to form a ternary complex with p53 in vitro and in human hepatoma and colon carcinoma cell lines and mouse embryonic fibroblasts. We found that Enigma elicits p53 degradation by inhibiting MDM2 self-ubiquitination and increasing its ubiquitin ligase activity toward p53 in cells. Moreover, mitogenic stimuli such as serum, FGF, and HGF increased Enigma transcription via induction of serum response factor (SRF), leading to MDM2 stabilization and subsequent p53 degradation. We observed similar results in the livers of mice treated with HGF. In humans, we found SRF and Enigma coexpressed with MDM2 but not p53 in several liver and stomach tumors. Finally, we showed that Enigma promoted cell survival and chemoresistance by suppressing p53-mediated apoptosis in both cell lines and a mouse xenograft model. Our findings suggest a role for Enigma in tumorigenesis and uncover a mechanism whereby mitogens attenuate p53 antiproliferative activity through an SRF/Enigma/MDM2 pathway.

Introduction

The p53 tumor suppressor is a transcription factor that activates many genes involved in cell growth arrest and apoptosis and thereby prevents incidence of abnormal cells and tumorigenesis (1). Tight regulation of the antiproliferative activities of p53 is essential for cell viability. This is mostly achieved by the mouse/human double minute 2 (Mdm2/Hdm2) E3 ubiquitin ligase, which targets p53 for degradation (2–4). MDM2 also targets itself for ubiquitination and degradation (5, 6). Since MDM2 is a transcriptional factor for p53, these two molecules form an autoregulatory loop (7, 8). Recently, the p300/CBP-associated factor (PCAF) and/or unidentified E3 ligases have been shown to promote ubiquitin-mediated proteolysis of MDM2 (9, 10).

Factors that differentially regulate ubiquitination of MDM2 and p53 may affect cell fate profoundly, as they can change the ratio between MDM2 and p53 (11). Many proteins have been discovered that regulate MDM2 and p53 levels by multiple mechanisms (12–14). However, cellular factors that selectively inhibit self-ubiquitination of MDM2 and thereby promote ubiquitination and degradation of p53 have been elusive. These factors likely play a positive role in cell growth and tumorigenesis. Indeed, MDM2 is overexpressed in various human tumors and correlates with increased cell proliferation and tumor growth (15).

The LIM domain protein Enigma, PDLIM7, structurally contains one amino-terminal PDZ domain and three carboxyterminal LIM domains (16). The PDZ domain binds actin-binding proteins such as β-tropomysin (17). The Enigma LIM domains interact with proteins involved in mitogenic or insulin signaling such as protein kinases (18–21). Enigma enhances bone morphogenetic responsiveness by interacting with Smad ubiquitin-regulatory factor 1 (22). Recently, pdlim7 in zebrafish has been shown to be involved in heart and skeletal muscle development (23). However, the exact cellular functions of Enigma remain enigmatic.

The Enigma gene is one of many candidates that have been identified from human liver and stomach tumors through the Functional Genome Project in Korea. We found that overexpression of Flag-tagged Enigma (F-Enigma) significantly reduced the expression of luciferase (luc) reporter gene under the control of an artificial promoter containing multiple p53 response elements (REs). We hypothesized, therefore, that Enigma might regulate the MDM2/p53 pathway.

Results

Enigma regulates p53 and p21 protein levels through MDM2. To identify association of Enigma with the MDM2/p53 pathway, we examined MDM2 and p53 protein levels in HLK3 hepatoma cells with or without expression of F-Enigma in the presence or absence of MG132, a proteasome inhibitor (Figure 1A). We also examined protein levels of p21, a transcriptional target of p53, which induces cell growth arrest (24). The MDM2 level was increased without F-Enigma expression in the presence of MG132, confirming previous reports that MDM2 is susceptible to proteasomal degradation (5, 6). In the absence of MG132, F-Enigma expression resulted in a dose-dependent increase in MDM2 levels, with concomitant decrease in p53 and p21 levels (Figure 1A). To test whether altera-
Enigma regulates p53 and p21 protein levels through MDM2. (A) Enigma stabilizes MDM2. HLK3 cells were transfected with F-Enigma vector (0, 2, 5 μg [designated −, +, ++]), incubated for 12 hours in the presence or absence of 10 μM MG132, and immunoblotted as indicated. (B) p53 levels are reflected in p533 activity. We transfected cells with the indicated reporter constructs (0.5 μg each) and with or without F-Enigma vector, and incubated them with or without MG132 for 12 hours. A luciferase assay was performed. Rel. luc. unit, relative luciferase units. (C) The effects of Enigma on mRNA levels of MDM2, p53, and p21. We transfected HLK3 cells with or without F-Enigma vector, prepared cell lysates or extracted total RNAs after 48 hours, and performed Northern blotting (NB) or IB as indicated. We stained 28S RNA with ethidium bromide as a control to indicate equivalent loading. (D) Enigma regulates p53 levels Mdm2-dependently. We transduced MEFs with Ad-p53 at an MOI of 50, or Ad-siEnigma or Ad-siControl at an MOI of 100, and incubated them for 32 hours before IB as indicated. (E) Enigma regulates p53 and p21 levels MDM2-dependently. We transfected cells with F-Enigma (5 μg), F-EniLIM3 (5 μg), siEnigma (10 μg), or siControl (10 μg) vectors, and incubated them for 48 hours before IB as indicated.

These effects of Enigma on p53 and p21 levels were not detected in Mdm2−/− MEFs (Figure 1D). p53 induces MDM2 expression at the mRNA level (7, 8). p21 expression was shown to be regulated by a p53-independent mechanism (27). We therefore tested whether Enigma-mediated changes in MDM2 and p21 levels occurred p53-dependently (Figure 1E). We used an F-Enigma mutant without the LIM3 domain (F-EniLIM3) as a control, as the LIM3 domain was essential for binding of Enigma to MDM2 in vitro and in vivo (Figure 2, C, E, and F). The MDM2 level was increased by F-Enigma overexpression and was decreased by Enigma depletion in both p53−/− and p53−/− cells (Figure 1E). The p21 level was decreased by F-Enigma overexpression and was increased by depletion of Enigma in p53+/− but not p53+/− cells (Figure 1E).

Overexpression of Enigma increased the half-life of MDM2 and decreased that of p53, whereas depletion of Enigma induced the opposite effect (Supplemental Figure 2). Collectively, these results suggest that Enigma stabilizes MDM2 p53-independently and regulates the p53/p21 pathway negatively through MDM2 at a posttranscriptional level. Enigma interacts with p53 through MDM2 in vitro and in vivo. To identify how Enigma regulates the p53 pathway MDM2-dependently, we first tested whether Enigma interacted with MDM2 and/or p53. Endogenous Enigma was coimmunoprecipitated with endogenous MDM2 in the presence or absence of endogenous p53 and formed a ternary complex with endogenous MDM2 and p53 proteins (Figure 2A). To test whether Enigma directly interacts with
Figure 2
Enigma interacts with p53 through MDM2 in vitro and in vivo. (A) We immunoprecipitated and/or immunoblotted HCT116 p53+/+ or p53−/− cells. (B) We transduced Mdm2−/−p53−/− (Mdm2−/−) and Mdm2+/−p53−/− (Mdm2+/−) MEFs with Ad-p53 at an MOI of 100 and incubated them for 24 hours before IP and/or IB. (C) We mixed His-MDM2 (1 μg), Enigma (0.5 μg), EniLIM3 (0.5 μg), GST-p53 (1 μg), and/or GST (1 μg) proteins for IP or GST pull-down and IB. (D and E) Various GST-MDM2 fusion (D) or F-Enigma (E) vectors are illustrated in the top panel. (D) We transfected 293−F-Enigma cells with the indicated vectors (5 μg each), and performed GST pull-down/IB and IB at 24 hours after transfection. MDM2 domains necessary for the interaction with Enigma and for binding to p53 are indicated at the top. The RING domain of MDM2 is shown in gray. (E) We transfected 293T cells with or without various F-Enigma (5 μg each) and with GST-MDM2 (5 μg each) vectors and incubated them for 24 hours before IB and IP/IB. The PDZ and 3 LIM domains of Enigma and the Enigma domain required for binding to MDM2 are shown at the top. The results from the binding assay are summarized on the right of the top panels in (D) and (E). (F) We transfected HLK3 cells with the indicated vectors (5 μg each), which are illustrated in (E), and incubated them for 48 hours before GST pull-down/IB and IB. (G) Schematic illustration of the Enigma-MDM2-p53 ternary complex.
p53, we expressed p53 in p53−/− MEFs with or without the Mdm2 gene and analyzed the interactions between them (Figure 2B). Enigma interacted with Mdm2 and formed a complex with p53 in Mdm2−/− but not Mdm2+/− MEFs, suggesting that the interaction between Enigma and p53 is Mdm2-dependent. To substantiate these results in vitro, we analyzed the interactions among Enigma, MDM2, and/or p53 proteins purified from bacteria (Figure 2C). MDM2 interacted with p53, indicative of functionality of MDM2 and p53 proteins from bacteria. Enigma interacted with MDM2, but not with p53, and bound to p53 when MDM2 was present (Figure 2C). These results suggest that Enigma interacts with p53 through MDM2 in vitro and in vivo.

To identify the molecular basis for MDM2-mediated interaction of Enigma with p53, we delineated domains for binding between MDM2 and Enigma. The C terminus of MDM2 (amino acids 401–491) including the RING finger domain was sufficient for its binding to Enigma (Figure 2D). The LIM3 domain of Enigma, but not the PDZ, LIM1, or LIM2 domain, was found to bind to MDM2 (Figure 2C, E, and F). The interaction between MDM2 and p53 was shown to occur through their N termini (28). Thus, these findings suggest that Enigma, MDM2, and p53 proteins form a ternary complex through the interaction between the C termini of Enigma and MDM2 and through the contact between the N termini of MDM2 and p53 (Figure 2G).

Enigma homolog (ENH) has a single PDZ domain and 3 LIM domains and is closely related to Enigma (16). Moreover, the LIM domains of these two proteins were shown to interact with identical protein kinases (20, 21). Therefore, we tested whether ENH could act like Enigma. ENH neither interacted with MDM2 nor stabilized it (Supplemental Figure 3), suggesting that Enigma specifically interacts with MDM2.

Enigma inhibits MDM2 self-ubiquitination and enhances MDM2-mediated ubiquitination of p53. Enigma-mediated regulation of MDM2 and p53 levels and formation of the Enigma-DMM2-p53 ternary complex led us to test whether Enigma affected self- and p53 ubiquitination of MDM2. Overexpression of F-Enigma but not F-EniLIM3 reduced MDM2-Ub and increased p53-Ub conjugates in a dose-dependent manner in cells, while depletion of Enigma increased MDM2-Ub and decreased p53-Ub conjugates (Figure 3A). Since MDM2 levels were not greatly affected in the presence of MG132 (Figure 1A), the finding that Enigma dose-dependently increased p53-Ub conjugates suggests that Enigma may activate MDM2-dependent ubiquitination of p53 in cells.

Next, we examined the effects of Enigma on self-ubiquitination of MDM2 and on PCAF-mediated ubiquitination of MDM2 in vitro using protein components and in cells. Virtually all the MDM2 molecules appeared to bind to Enigma in MDM2 self-ubiquitination assay conditions (Supplemental Figure 4). In line with this, Enigma, but not EniLIM3, efficiently inhibited self-ubiquitination of MDM2 in vitro (Figure 3B and Supplemental Figure 5). Enigma bound to MDM2(C464A), a catalytically inactive mutant of Hdm2, but had no effect on it (Figure 3B). PCAF ubiquitinated MDM2(C464A) in vitro (Figure 3B), and PCAF expression decreased the protein level of MDM2(C464A) in Mdm2−/− MEFs (Figure 3C), as previously reported (10). However, Enigma did not greatly affect the PCAF-mediated ubiquitination and degradation of MDM2(C464A) in vitro and in cells.

We next tested the effect of Enigma on MDM2-mediated ubiquitination of p53 in vitro (Figure 3D). Enigma bound to MDM2 and p53, stimulated MDM2-mediated ubiquitination of p53 in a dose-dependent manner, and inhibited self-ubiquitination of MDM2. This effect of Enigma on p53 ubiquitination was not detected with MDM2(C464A) (Figure 3D). Collectively, these results suggest that Enigma specifically inhibits self-ubiquitination of MDM2 and increases the stability and activity of MDM2 E3 ligase, thereby enhancing MDM2-dependent ubiquitination and degradation of p53 in cells.

Proliferation signal attenuates p53 via the SRF/Enigma/MDM2 pathway. Activation of cell proliferation or survival pathways has been shown to increase MDM2 levels by various mechanisms (29). We identified serum response element (SRE) in the upstream regulatory region of the Enigma gene (Supplemental Figure 6). Many immediate early genes responsive to growth factors contain SRE in their promoters to which serum response factor (SRF) binds (30). Therefore, we hypothesized that increased MDM2 levels in response to cell proliferation signals might be attributed to SRF-mediated induction of Enigma expression. Indeed, we found that serum, FGF, or HGF activates the Enigma promoter through the SRE to which SRF binds (Supplemental Figure 6). This SRF requirement for Enigma expression is consistent with the previous finding that Enigma is downregulated in the embryonic heart of Srf-knockout mice (31).

To test whether cell proliferation signals negatively regulate p53 through the SRF/Enigma/MDM2 pathway, we stimulated serum-starved cells with serum and examined time-dependent alterations in mRNA and protein levels of those molecules. Serum induced Enigma expression at the mRNA and protein levels following early induction of SRF expression in the presence or absence of p53 (Figure 4, A and B). Serum did not greatly affect the mRNA level of P1-MDM2, but decreased that of P2-MDM2 (Figure 4B), presumably due to the decreased protein level of p53 after serum stimulation (Figure 4A). The protein level of p53 was gradually and slightly increased after an initial decrease at 2 hours after serum stimulation, while the MDM2 level was steadily increased by 6 hours (Figure 4A). This may have been the result of molecular events caused by proliferation signaling, which negatively regulates MDM2-mediated degradation of p53 (32). However, serum stimulation increased protein levels of MDM2, which led to an overall decrease in p53 levels (Figure 4A). Serum withdrawal from proliferating cells resulted in decreasing mRNA and protein levels of SRF and Enigma in a p53-independent manner, and concomitantly resulted in a decreasing MDM2 protein level and increasing p53 protein level (Supplemental Figure 7). These alterations in MDM2 and p53 protein levels in serum-starved cells occurred at the posttranscriptional level, as serum removal increased the P2-MDM2 mRNA level in p53−/− cells and did not significantly affect mRNA levels of P1-MDM2 and p53 (Supplemental Figure 7B).

Treatment of serum-starved cells with HGF also increased protein levels of SRF, Enigma, and MDM2 in a dose-dependent manner, resulting in a decrease in p53 levels (Figure 4C). When SRF or Enigma was depleted, the effects of HGF on MDM2 and p53 levels were attenuated (Figure 4C). Forced expression of SRF increased protein levels of Enigma and MDM2 in a dose-dependent manner and decreased p53 protein level (Figure 4D), suggesting that SRF is directly involved in transcriptional activation of the Enigma gene. Growth factor-mediated activation of the MAP kinase cascade induces or activates expression of SRF (30, 33). We therefore used a specific MEK inhibitor, PD98059, to examine whether the MAP kinase pathway might regulate p53 through the SRF/Enigma pathway. When Mdm2+/+ p53−/− MEFs expressing p53 were treated...
with PD98059, the effects of HGF on SRF, Enigma, Mdm2, and p53 levels were abrogated (Figure 4E). Collectively, these results suggest that cell proliferation signals induce SRF expression through activation of the MAP kinase cascade and subsequently increase Enigma expression, resulting in stabilization of MDM2 that enhances ubiquitin-mediated degradation of p53.

HGF infusion has been shown to induce liver growth in normal mice (34). We identified that the SRE is conserved in the Enigma
promoters of human and mouse and then examined whether HGF might regulate p53 levels through the SRF/Enigma/Mdm2 pathway in vivo. To detect HGF-mediated alterations in Mdm2 and p53 levels more clearly, we initially injected mice with or without Ad-p53, treated them with HGF, and examined time-dependent alterations in SRF, Enigma, Mdm2, and p53 levels in mouse liver. HGF increased mRNA and protein levels of Enigma following induction of SRF expression with or without Ad-p53 injection (Figure 5, A and B). The protein level of Enigma was positively correlated with that of Mdm2 between 3 and 16 hours, at which time p53 protein levels were decreased (Figure 5A). HGF treatment decreased exogenous or endogenous p53 protein levels at 2 hours, at which time Enigma but not Mdm2 protein was detected (Figure 5A). This may result from the fact that the Mdm2 level at 2 hours might have been too low to be detected. Alternatively, there are unidentified mechanisms by which HGF may decrease p53 levels together with the Enigma/Mdm2 axis in mouse liver (35). To further verify that Enigma is directly involved in the HGF-mediated regulation of the Mdm2/p53 pathway in vivo, we pretreated mice with or without Ad-p53 and then injected one group of mice with Ad-F-Enigma and another group with Ad-siEnigma. Mice with Ad-F-Enigma were left untreated. Mice with Ad-siEnigma were treated with HGF. We then examined protein levels of Enigma, Mdm2, and p53 in mouse liver (Figure 5C). HGF increased endogenous Enigma and Mdm2 protein levels and decreased exogenous and endogenous p53 levels (Figure 5C). These effects of Enigma on Mdm2 and p53 levels were abolished when endogenous Enigma was depleted by Ad-siEnigma. Overexpression of F-Enigma increased endogenous Mdm2 protein levels and decreased exogenous and endogenous p53 protein levels (Figure 5C). Thus, these results suggest that Enigma is directly involved in the HGF-mediated decrease in p53 levels and support the in vivo presence of an SRF/Enigma/Mdm2 pathway by which mitogenic signals negatively regulate p53.

**SRF and Enigma are coexpressed with MDM2 in human tumors.** SRF is highly expressed in advanced human liver cancer and correlates with tumor progression (36) and increases expression of E2-EPF UCP, which targets the VHL tumor suppressor for degradation and promotes tumor growth (33, 37). It was shown that MDM2 is highly expressed in tumors with wild-type p53 (38). Thus, activation of the SRF/Enigma axis in proliferating cells (Figures 4 and 5) suggests that SRF and Enigma are likely coexpressed with MDM2 but not p53 in certain human tumors, presumably because MDM2 targets wild-type p53 for degradation. It was also shown that MDM2 levels are high in some human tumors where p53 levels are elevated (39–42). Moreover, the p53 gene is mutated in more than 50% of human tumors (38), and the mutated p53 proteins are unable to induce target genes through the p53RE and are often accumulated in tumor cells (43, 44). These previous findings suggest that the SRF/Enigma axis may be partly responsible for coexpression of MDM2 and p53 proteins in some tumors, where p53 proteins lose their ability to
induce target genes or MDM2 interferes with transcriptional activation of p53 (45). We thus preselected SRF- or Enigma-positive human tumors from 27 liver and 18 stomach tumors of advanced stage by primary screening, and we examined SRF/Enigma/MDM2/p53 levels in those tumors and their normal counterparts (Figure 6A). Indeed, SRF and Enigma were coexpressed with MDM2 in 10 cases of human tumors, where p53 was undetected, and were also coexpressed with MDM2 and p53 proteins in 7 cases of human tumors. Thus, these results suggest that the SRF/Enigma axis may stabilize MDM2 in human tumors, thereby ablating wild-type p53.

Tissue array experiments revealed that Enigma was colocalized with MDM2 at apical cytoplasm of human stomach and colorectal tumor cells (Figure 6B). Immunostaining experiments also revealed that Enigma and MDM2 were colocalized mainly in the cytosol of the cells treated with 10% serum (Figure 6C). Coexistence of Enigma and MDM2 was also detected in the cytosol of the serum-starved cells (0% serum). Enigma appeared to exist in the nucleus in proliferating cells at low levels (10% serum). Since Enigma and MDM2 are colocalized in the cytosol in human tumor cells and a tumor cell line, we examined whether Enigma, MDM2, and p53 proteins were present as a ternary complex in the cytosol. To that end, we prepared cytosolic and nuclear fractions from cells incubated with or without 10% serum. Protein levels of Enigma, MDM2, and p53 and the interaction between them were examined in those fractions (Supplemental Figure 8). After serum stimulation, protein levels of Enigma, MDM2, and p53 were increased in the cytosol compared with those in the nucleus. Enigma was detected in the nuclear fraction from cells with 10% serum, albeit at a low level, which is consistent with the immunostaining data (Figure 6C). The Enigma-MDM2-p53 ternary complex was detected in both the cytosolic and nuclear fractions from serum-starved and serum-stimulated cells (Supplemental Figure 8). Thus, these results suggest that the Enigma/MDM2 axis may function to attenuate p53 activity mainly in the cytosol in proliferating cells and may act also in the nucleus to suppress p53 functionality.

Enigma promotes tumor cell viability in vitro and in vivo. The presence of the Enigma/MDM2 axis in proliferating cells and human tumors led us to test whether Enigma might indeed affect tumor cell viability. To that end, we treated HLK3 tumor cells with or without adriamycin (ADR). In the absence of ADR, overexpression of F-Enigma appeared not to significantly promote cell growth, but depletion of Enigma resulted in decreased cell viability (Figure 7A). The results suggest that MDM2 may act as a survival factor in proliferating cells, as previously reported (46). The ADR-mediated decrease in cell viability was rescued by F-Enigma overexpression and became severe with Enigma depletion. F-Enigma overexpression increased the MDM2 level, decreased the p53 level, and promoted cell cycle progression in the presence or absence of ADR, while Enigma depletion decreased the MDM2 level and increased the p53 level and population of apoptotic cells (Figure 7, B and C). To substantiate this effect of Enigma on cell viability in vivo, we generated subcutaneous tumors in nude mice and examined the effect of Enigma on tumor growth after ADR injection (Figure 7, D and E). ADR significantly inhibited tumor growth in mice (Figure 7D, lines 1 and 2). This ADR-mediated inhibition of tumor growth was weakened when F-Enigma was overexpressed (line 3) and became stronger with Enigma depletion (line 5). Protein levels of MDM2 and p53 in tumors excised from mice (Figure 7F) sug-
gest that the in vivo tumor growth rate may be positively correlated with MDM2 level and be inversely linked with p53 level.

Next, we tested whether Enigma regulated tumor cell viability p53-dependently. To this end, we used HCT116 isogenic cell lines from human colon carcinoma, in which only p53 status is different. ADR decreased MDM2 level in both p53+/− and p53−/− cells (Figure 8A), suggesting that damage-activated kinases may accelerate MDM2 autodegradation, as previously reported (47). This ADR-
mediated decrease in MDM2 levels was rescued by F-Enigma overexpression and was further intensified by Enigma depletion in p53\(^{+/+}\) and p53\(^{-/-}\) cells (Figure 8A). Viability of p53\(^{+/+}\) cells was increased by F-Enigma overexpression and was decreased by Enigma depletion (Figure 8B). This effect of Enigma on cell viability was not observed in p53\(^{-/-}\) cells (Figure 8B). Depletion of Enigma increased the p53 level and population of apoptotic cells in p53\(^{+/+}\) but not p53\(^{-/-}\) cells (Figure 8, A and C), suggesting that Enigma enhances tumor cell viability by suppressing p53-mediated apoptosis.

**Discussion**

Here we demonstrated that Enigma specifically inhibits self-ubiquitination of MDM2 and increases the stability and activity of MDM2 E3 ligase, thereby enhancing MDM2-mediated ubiqui-
tination and degradation of p53 in cells. Moreover, Enigma promotes cell survival and chemoresistance through its effects on the MDM2/p53 pathway. In addition, our findings may provide a molecular basis for MDM2 overexpression in human tumors through the SRF/Enigma axis.

A number of factors have been shown to regulate MDM2 positively and p53 negatively (12–14). However, Enigma is unique and distinct among those factors in its abilities to form a complex with p53 through MDM2 and enhance the stability and activity of MDM2 E3 ligase. Mdm2-null mice are embryonic lethal but are rescued by p53 elimination (48, 49), indicating that Mdm2 is the central node in the p53 pathway. In this context, Enigma may act as an integral part of the MDM2/p53 pathway in proliferating cells through its ability to switch the E3 ligase specificity of MDM2 from itself to p53.

How does Enigma differentially regulate self- and p53 ubiquitination of MDM2? The MDM2 RING domain has been shown to be essential for oligomerization of MDM2 (50–52). MDM2 E3

Figure 8
Enigma suppresses p53-mediated apoptosis. (A) We transfected cells with pIRES2-eGFP (2 μg) and with or without F-Enigma (5 μg), F-EniΔLIM3 (5 μg), siEnigma (10 μg), or siControl (10 μg) vector. After 16 hours, culture media were changed, and cells were further grown for 20 hours and then treated with or without 2 μg/ml ADR for 12 hours. Cell lysates were immunoblotted as indicated. (B and C) We transfected cells with indicated vectors as described in Methods (B). (C) At 16 hours after transfection, culture media were changed, and cells were further grown for 8 hours and then treated with or without 20 μg/ml ADR. After 24 hours, we performed TUNEL assay (original magnification, ×100) or analyzed cells by flow cytometry.
ligase likely recognizes itself as a substrate via homo(di)merization to self-ubiquitinate (53). The binding of MDM2 to the N terminus of p53 has been proposed to cause a conformational change in MDM2 that enables binding of the central acidic region of MDM2 to a “ubiquitination signal” in the core DNA-binding domain of p53 (54). Thus, the interaction of Enigma with the RING domain of MDM2 may interfere with homo(di)merization of MDM2 and induce conformational changes in MDM2 that enhance p53 ubiquitination. This notion is supported by MDM2-dependent interaction of Enigma with p53 and by the previous findings that the self- and p53 ubiquitination activities of MDM2 reside in its RING domain and the self-ubiquitination activity of MDM2 is separate from its ability to drive the degradation of p53 (5, 6).

Data from knock-in mice of the RING mutant of Mdm2(C462A) suggest that Mdm2 is regulated by PCAF or another E3 ligases but does not regulate its own stability by self-ubiquitination (9). However, MDM2 exhibits many p53-independent functions (55) that may also be in part essential for survival of the knock-in mice with p53 elimination. Only the knock-in mice that can properly regulate Mdm2(C462A) levels are likely to survive, as uncontrolled accumulation of Mdm2(C462A) protein would be detrimental. Thus, MDM2 level is likely regulated by MDM2 itself, PCAF, and/or or unidentified E3 ubiquitin ligases. It would depend on cellular status which E3 ubiquitin ligase acts dominantly for MDM2 and p53 levels to be properly balanced for cell viability. The balance between MDM2 and p53 levels in proliferating cells such as tumor cells is likely to be regulated by Enigma that inhibits self-ubiquitination of MDM2, as evidenced by this study.

Growth factor-mediated activation of the phosphatidylinositol 3-kinase/Akt or MAPK pathway has been shown to phosphorylate MDM2 and regulate nuclear import or export of MDM2, thereby negatively regulating p53 (56–59). Akt-mediated phosphorylation of MDM2 leads to inhibition of self-ubiquitination and stabilization of MDM2 (60). Phosphorylation of MDM2 and p53 during DNA damage inhibits the interaction of MDM2 with p53 and is associated with increased self-ubiquitination and degradation of MDM2 (47, 61). Activation of the p38 MAPK/MK2 pathway results in phosphorylation of Hdm2 Ser166, and this phosphorylation is a common response to mitogenic activation and UV-induced damage. However, these two treatments do not have the same effect on p53 stability (62). These findings therefore suggest that the MDM2/p53 pathway may be in part regulated in a phosphorylation-independent manner. We found here that serum or HGF can decrease p53 levels through the SRF/Enigma/MDM2 pathway (Figures 4 and 5). Moreover, a specific MEK inhibitor, PD98059, or depletion of SRF or Enigma diminished the HGF effects on MDM2 and p53 levels (Figure 4). Thus, the SRF/Enigma/MDM2 pathway we identified here may represent a novel mechanism by which the p53 level is regulated in a manner independent of phosphorylation of MDM2 and p53. This possibility is supported by the findings that MDM2 overexpression exhibits a more profound effect on cellular transformation (59) and phosphorylation of p53 plays only very subtle, fine-tuning roles in regulating its function in mouse models (14).

MDM2 was shown to shuttle between the nucleus and the cytoplasm constantly and p53-independently (63). It was shown that MDM2 mono- and poly-ubiquitinates p53-dependent on MDM2 levels and monoubiquitinated p53 is MDM2-independently accumulated in the cytosol (64). Cocompartmentalization of MDM2 and p53 was shown to be a major determinant for MDM2-mediated degradation of p53 (65). Enigma is localized mainly in the cytosol and moderately in the nucleus and forms a trimeric complex with MDM2 and p53 in both compartments (Supplemental Figure 8). Thus, an Enigma-mediated increase in the level of MDM2 activity may promote polyubiquitination of p53 for degradation in both the cytosol and nucleus, whereas the 26S proteasomes are evenly distributed (66).

Enigma and MDM2 levels are increased in proliferating cells, and Enigma is colocalized with MDM2 mostly in the cytosol (Figure 6C and Supplemental Figure 8). MDM2 is mainly cytoplasmic in exponentially growing cells, whereas p53 is evenly distributed between the cytoplasm and nucleus (67). Enigma and MDM2 are colocalized at apical cytoplasm of human gastric and colorectal tumor cells (Figure 6B). Collectively, these findings suggest that Enigma and MDM2 are colocalized in the cytoplasm of actively growing cells to efficiently target p53 for degradation. Presumably, these tumor cells may represent rapidly proliferating cells in a tumor mass, where the antiproliferative activities of p53 have to be attenuated.

MDM2 is highly expressed in tumors that retain wild-type p53 (38). A slight reduction in Mdm2 levels leads to a significant reduction in intestinal adenoma formation in the mouse (68). A single nucleotide polymorphism identified in the MDM2 promoter that slightly increases MDM2 levels is associated with increased tumor incidence (69). These results suggest that even minor changes in MDM2 levels induce significant phenotypes (46). Enigma has been shown to be highly expressed in gastric tumor cells and bone metastasis (70, 71). Thus, Enigma may function as an oncoprotein by stabilizing and activating MDM2 E3 ligase and thereby ablatting p53. Moreover, Enigma may be a new therapeutic target for selective activation of wild-type p53 in tumor cells without much affecting normal cells.

Methods

Plasmids. Human Enigma and MDM2 cDNAs were supplied by 21C Frontier Human Gene Bank (KRIBB). We generated pEBG-Enigma, pHis-Enigma, pHA-Enigma, pGEX-53, pEBG-MDM2, pGEX-MDM2, pHis-MDM2, or pHis-PCAF by PCR-cloning Enigma, Mdm2, p53, or PCAF cDNAs into pCMV-Taq2 (Stratagene), pET28a (Novagen), pCDNA (Invitrogen), pEBG, pGEX-FT-1 (GE Life Sciences), or pColdI (Takara). We constructed an MDM2(C464A) mutant by PCR-cloning Hdm2 cDNA and inserted it into pEBG, pGEX-1, or pColdI. We generated deletion constructs of pF-Enigma, pEBG-Enigma, pHis-Enigma, or pEBG-MDM2 by PCR-cloning each of them by a standard cloning procedure. His-Ub, p53, and F-SRF vectors were previously described (33, 37, 72). F-Enigma, F-ENH, and F-PCAF constructs were supplied by W.K. Cho (KRIBB), A. Iavarone (Columbia University Medical Center, New York, New York, USA), and S.-B. Seo (Chung-Ang University, Seoul, South Korea), respectively. We synthesized an Enigma-specific siRNA (5′-AAAGACCTTCTACTCCAAGAA-3′) oligonucleotide duplex and cloned it into pSuper vector to generate siEnigma vector. The SRF-specific siRNA (siSRF) and a scrambled control siRNA (siControl) vectors were previously described (33, 37).

Recombinant proteins. We induced expression of pColdI vector-based His-proteins with 1 mM IPTG for 16 hours at 18–20°C and expression of pET28a vector-based ones for 4 hours at 25°C. GST fusion and His-tagged proteins were expressed in Escherichia coli BL21DE3 and were affinity purified as previously reported (37). His-tag from His-proteins was removed by incubation with 100 U/ml thrombin for 16 hours at 16°C.

Adenoviral vectors. We inserted the BamHI–XhoI fragment of F-Enigma vector or the Xhol–HindIII fragment of siEnigma vector into pCMV-Shuttle vector. Generation, amplification, purification, titer determination, and

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transduction of adenoviral vectors have been previously described (25). Ad-p53 and Ad-LaCZ viruses were previously described (25, 72).

Antibodies and chemicals. We purchased antibodies specific for ubiquitin (Cell Signaling Technology), Flag, actin, GST (Sigma-Aldrich), p53 (DO-1), SRF, His, HA (Santa Cruz Biotechnology Inc.), p21 (BD Biosciences – Pharmingen), and MDM2 (BD Biosciences – Pharmingen SMP14). We generated Enigma-specific antibody by immunizing mice with His-Enigma.

We purchased HGF (R&D), PD98059 (Cell Signaling Technology), Flag- or His-ubiquitin, rabbit E1, ADR, MG132, PMSF (Sigma-Aldrich), Lipofectamine (Invitrogen), thrombin (Merck), and proteinase inhibitor cocktail (Roche).

Cell culture. Standard conditions and procedures were used for culturing cell lines. We obtained Mdm2+/p53− and Mdm2−/p53+/− MEFs (73) from G. Lazo-no (The University of Texas MD Anderson Cancer Center, Houston, Texas, USA). We constructed the 293–F-Enigma cell line by transfection of F-Enigma plasmid into 293 cells and selection of the cell using neomycin (1 mg/ml).

Immunoprecipitation and immunoblotting. We transfected cells with calcium phosphate or Lipofectamine, treated them for the indicated times, lysed them with RIPA buffer for immunoblotting (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5 mM EDTA, 1% NP40, 0.1% SDS, 0.1% sodium deoxycholate, 0.5 mM PMSF), or with IP buffer for immunoprecipitation (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 0.5 mM EDTA, 1% NP40, 0.5 mM PMSF, one tablet proteinase inhibitor cocktail/100 ml). To remove IgG proteins in immunoprecipitates, we incubated IP samples with 50 μl of 0.1 M glycine buffer (pH 2) for 1–2 minutes, filtered them through a microspin column (QiAprep Spin Miniprep Kit, QiAGEN), immediately neutralized the filtrates with 5 μl of 1 M Tris (pH 10), and added them to 2x SDS-PAGE sample buffer. We lysed human tissues in a lysis buffer (iNtRON) containing each labeled probe but not formamide at 55°C for 16 hours.

In vitro binding assay. We mixed relevant proteins in 500 μl IP buffer, adjusted the reaction volume up to 1 ml by the addition of IP buffer, incubated the mixtures at 4°C for 1 hour, and performed the binding assay.

Ubiquitination assay. To detect Ub conjugates in cells, we lysed cells in a buffer (6 M guanidine-HCl, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris-Cl [pH 8.0], 5 mM imidazole, 10 mM β-mercaptoethanol) and incubated cell lysates with Ni2+–NTA agarose beads for 4 hours at room temperature. The beads were washed with buffer A (8 mM urea, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris-Cl [pH 8.0], 10 mM β-mercaptoethanol) and buffer B (8 mM urea, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris-Cl [pH 6.3], 10 mM β-mercaptoethanol). The bead-bound proteins were eluted with a buffer (200 mM imidazole, 0.15 M Tris-Cl [pH 6.7], 30% glycerol, 0.72 M β-mercaptoethanol, 5% SDS) and were analyzed with relevant antibodies.

We performed the in vitro ubiquitination assay with E1 (0.2 μg), GST-UbcH5C (E2, 0.2 μg), and His-Ub or Flag-Ub (1 μg) as previously reported (37). For detection of in vitro ubiquitinated proteins by GST pull-down/IB assay, we added 10% SDS to the reaction mixtures for a final concentration of 1% SDS. One-tenth of reaction mixtures were used for IB to identify input proteins. The rest was diluted up to 1 ml with IP buffer and incubated with GST-Sepharose beads (Amersham) at 4°C for 16 hours. Bead-bound proteins were analyzed by IB. For detection of in vitro ubiquitinated proteins by IP/IB assay, we boiled 1% SDS of reaction mixtures for 5 minutes, diluted them up to 1 ml with IP buffer, and incubated them with relevant antibodies at 4°C for 16 hours and then with protein A–Sepharose beads for another 2 hours. Bead-bound proteins were eluted by the addition of 0.1 M glycine buffer (pH 2), and the eluates were neutralized with 1 M Tris (pH 10) and then analyzed by IB.

Immunofluorescence staining. Cells were grown in glass coverslips in a 6-well dish, fixed with ice-cold methanol for 15 minutes at room temperature, blocked with PBS containing 5% normal bovine serum (NBS) for 20 minutes, and then incubated with a mixture of rabbit anti-MDM2 (1:100 dilution) and mouse anti-Enigma (1:100) in PBS containing 0.5% NBS for 90 minutes at room temperature. Cells were washed with PBST, incubated with FITC-conjugated goat anti-rabbit IgG plus rhodamine-conjugated goat anti-mouse IgG plus DAPI (0.1 μg/ml) in PBST containing 1% NBS for 1 hour at room temperature, washed with PBST, and mounted with VECTA-SHIELD HardSet. Images were taken with a microscope (Olympus).

Cell viability. We transfected cells with relevant expression vectors. At 16 hours after transfection, 5 × 104 cells per 60-mm dish were seeded and grown for 12 hours and treated with or without 20 μg/ml ADR for 48 hours. We counted viable cells with a hemocytometer. We repeated the experiments 3 times, each in triplicate. Cell viability data are mean ± SD.

Cell cycle analysis. Cells were washed with PBS, fixed in 70% ethanol at −20°C for 16 hours, incubated in PBST with 100 μg/ml propidium iodide (PI, Sigma-Aldrich) plus 100 μg/ml RNase A at 37°C for 30 minutes or 50 μg/ml PI plus 100 μg/ml RNase A plus FITC-conjugated Annexin V (1:100, Santa Cruz Biotechnology Inc.) in a binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2). The DNA content of the cells was then assessed by flow cytometry with a FACS caliber (BD).

TUNEL assay. Cells were fixed in 4% paraformaldehyde at 4°C for 20 minutes, washed 3 times in PBS for more than 30 minutes, and treated with permeation solution (0.1% sodium citrate and 0.1% Triton X-100) for 5 minutes. After being washed with PBS, cells were analyzed using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s instructions.

Human samples. All samples from humans were obtained with informed consent. The use of stomach and liver tumors and their normal counterparts was approved by the Research Ethics Committee of Chonbuk National University Medical School and Hospital, Jeonju, Korea; Keimyung University School of Medicine, Daegu, Korea; and Chungnam National University Hospital, Daejeon, Korea.

Mouse experiments. We maintained 5- to 6-week-old BALB/c mice (Taconic) and nude mice (Japan SLC Inc.) in accordance with the guidelines and under approval of the Institutional Review Committee for Animal Care and Use, KRIBB.

Liver extracts. We injected mice with PBS with or without HGF or relevant adenoviral vectors through the tail vein. Adenoviral vectors expressed transgenes in more than 90% hepatocytes. We prepared protein extracts and total RNAs from mouse liver as previously reported (33, 37).

Tissue array. We performed tissue arrays as previously described (25). Tissue arrays were supplied by W.H. Kim (Seoul National University College of Medicine, Seoul, Korea).

Tumor growth. We transduced HLK3 cells with or without Ad-F-Enigma or Ad-LaCZ at an MOI of 100, or Ad-siEnigma or Ad-siControl at an MOI of 200, incubated them for 24 hours, and generated tumors in nude mice by subcutaneous inoculation of 105 cells with or without adenoviral transduction in 0.1 ml PBS. At day 14 after cell inoculation, we injected mice with or without 4 mg/kg ADR intravenously, monitored tumor growth for 28 days after ADR injection, and photographed excised tumors from mice. We calculated tumor size as previously described (37).
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