As discussed later, our results indicate a possible molecular mechanism for the 5′-GGGA-3′ to 5′-GGA-3′ mutation induced by PhIP.

DNA Polymerases Involved in TLS through the dG-PhIP Adduct—TLS through many DNA lesions requires the action of two different polymerases, an "inserter" and an "extender," the former to perform nucleotide insertions opposite the lesion site and the latter for subsequent extensions (39). The catalytic efficiency of the dCTP-insertion reaction opposite the dG-PhIP adduct by REV1 was found to be more than 2,000-fold greater than that by pol κ (see Tables 2 and 3). This result strongly suggests that REV1 functions *in vivo* as an inserter polymerase for TLS through the dG-PhIP adduct. This insertion step by REV1 is also error free. REV1 has been reported previously to insert dCTP opposite abasic sites and various N2-dG adducts (26, 39–41). However, our current study is the first to show that REV1 inserts dCTP opposite a large size C8-dG adduct. We used a shorter (C-terminal deleted) form of pol κ in our current experiments and an intact pol κ may be more effective for this insertion reaction. As for pol η, a detailed kinetic analysis was not performed. Hence, the possibility cannot be excluded that pol κ and pol η also function as inserter polymerases.

In addition to the Y-family DNA polymerases, DNA polymerase ζ (pol ζ), belonging to the B-family DNA polymerases, is considered to be involved in TLS through various lesions as an extender DNA polymerase (39, 42, 43). We have not carried out a primer extension assay with pol ζ and thus the possibility cannot be completely excluded by our current data that pol ζ functions *in vivo* as an extender polymerase for TLS through the dG-PhIP adduct. In our present study, we provide evidence that pol κ can extend from dC opposite the dG-C8-PhIP adduct *in vitro*. It is, therefore, possible that pol κ, at least partially, functions as an extender polymerase *in vivo* for TLS through the dG-PhIP adduct. Further study about cooperation between two or more DNA polymerases, including pol ζ, is necessary to verify which DNA polymerases are involved in the bypass synthesis through the PhIP lesion.

The catalytic efficiency of pol κ for a dGTP insertion into substrate C-p28 was a little higher than that for dCTP insertions (see Table 2 and Fig. 6*D*). The former generates a single guanine deletion, and the latter is an error-free extension. Consequently, our data suggest that the extension reaction with pol κ from the nucleotide opposite the dG-C8-PhIP adduct causes frequent single-guanine deletions from the GGG stretch. It has been reported that one characteristic feature of pol κ homologs, from bacteria to humans, is their propensity to generate single-base deletions (44–47). The crystal structure of Dpo4, a thermophilic archaea homolog of pol κ, in ternary complexes with DNA and an incoming nucleotide supports the model that a single base deletion by pol κ is generated through a misaligned intermediate complex where the template dG forms an extrahelical looped out structure and the incoming dGTP skips this extrahelical base and pairs with the next template base dC (48) (see supplemental Fig. S6). It is reasonable to speculate therefore that, in the case of TLS through dG-C8-PhIP, mammalian pol κ generates the single guanine deletion via a similar intermediate where the PhIP-adducted dG is looped out and template-primer slippage occurs. However, further analyses for determining whether the one-base skipping of pol κ beyond the lesion observed by us is dependent on the nucleotide placed 5′ to the lesion or not, are necessary to clarify the detailed molecular mechanism underlying one base skipping of pol κ.

Molecular Mechanisms Underlying Mutation Induction by PhIP—We have demonstrated herein by *in vitro* DNA synthesis analyses using oligonucleotide templates containing dG-PhIP that: 1) replicative DNA polymerases stall at the PhIP adduct and cannot perform translesional DNA synthesis beyond this point; 2) REV1 inserts a dC opposite the dG-PhIP with a much higher efficiency than other TLS polymerases, including pol κ and pol η; and 3) pol κ has a potential ability to catalyze an extension reaction from the 5′-dC opposite the adduct and often skips over one dG in the template during this extension step. A working model for the induction of mutations at the PhIP adducts based on the results shown in the present study is illustrated in supplemental Fig. S6. This model could be adopted for other sequences containing a G repeat stretch longer than GGG.

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Mdmx enhances p53 ubiquitination by altering the substrate preference of the Mdm2 ubiquitin ligase

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ABSTRACT

***mdm2* and *mdmx* oncogenes play essential yet non-redundant roles in synergistic inactivation of the tumor suppressor, p53. While Mdm2 inhibits p53 activity mainly by augmenting its ubiquitination, the functional role of Mdmx on p53 ubiquitination remains obscure. In transfected H1299 cells, Mdmx augmented Mdm2-mediated ubiquitination of p53. In in vitro ubiquitination assays, the Mdmx/Mdm2 heteromeric complex, in comparison to the Mdm2 homomer, showed enhanced ubiquitinase activity toward p53 and the reduced auto-ubiquitination of Mdm2. Alteration of the substrate specificity via binding to Mdmx may contribute to efficient ubiquitination and inactivation of p53 by Mdm2.**

Structured summary:
MINT-7219995: *P53* (uniprotkb:P04637) *physically interacts* (MI:0914) with *Ubiquitin* (uniprotkb:P62988) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-7220023: *Ubiquitin* (uniprotkb:P62988) *physically interacts* (MI:0914) with *P53* (uniprotkb:P04637) by *pull down* (MI:0096)

1. Introduction

The p53 tumor suppressor protein plays a central role in preventing tumorigenesis. p53 functions as a sequence-specific transcriptional factor [1,2], and activated p53 exerts its function as a tumor suppressor by inducing numerous target genes [3–6]. In most cancer cells, its activity is lost via alteration of its gene or via other cellular events that inactivate p53 [7–9].

Mdm2 and Mdmx function as two major players in the suppression of p53 activity [10]. Accumulating reports indicate that the major function of Mdm2 in suppressing p53 is attributed to Mdm2-dependent p53 ubiquitination, which triggers proteasomal degradation or nuclear export of p53 [11], although it has been reported that Mdm2 inactivates p53 by other mechanisms [12–15]. Mdm2 possesses a RING finger domain, a protein–protein interaction motif that is found in many eukaryotic proteins and often possesses E3 ubiquitin ligase activity [16]. Indeed, Mdm2 functions as an E3 ubiquitin ligase, and the RING domain of Mdm2 is essential for its ubiquitin ligase activity toward p53 and Mdm2 itself [17–19].

Mdmx shares an extensive structural homology with Mdm2, and forms a heterodimer complex with Mdm2 through their RING finger domains [20,21], yet Mdmx in itself lacks the robust activity of an E3 ubiquitin ligase [22]. Thus, both genetic and biochemical evidence indicates that Mdmx and Mdm2 perform distinct yet co-operative functions in p53 inactivation.

Recent reports suggest that Mdmx may inactivate p53 by augmenting Mdm2-mediated ubiquitination of p53 [23–25]. However, precise mechanism by which Mdmx stimulates p53 ubiquitination by Mdm2 is not yet known.

In this paper, we demonstrated that wild-type Mdmx is capable of enhancing Mdm2-mediated p53 ubiquitination in vivo. Further, the in vitro study using purified Mdm2 or the Mdm2/Mdmx complex revealed that, when complexed with Mdmx, the extent of p53 ubiquitination by Mdm2 was enhanced while poly-ubiquitination of Mdm2 was significantly decreased. We propose that the effect of Mdmx on the preference of the substrate of the Mdm2 ubiquitin ligase plays an important role in effective ubiquitination of p53.

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2. Materials and methods

2.1. DNA transfection

In DNA transfection experiments using H1299 cells, 2 μg of DNA and 4 μl of Lipofectamine 2000 reagent (Invitrogen) were introduced per 2.0×10^5 cells according to manufacturer's protocol. Cells were then incubated for 20 h before harvesting.

2.2. In vivo ubiquitination assay

For detection of p53 conjugated with endogenous ubiquitin, in vivo ubiquitination assays were performed as previously described [26] with some modifications. Transfected H1299 cells were lysed in SDS lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1% SDS) supplemented with 1 mM DTT and protease inhibitor cocktail (PI) [27], boiled for 10 min, and diluted with ×4 volumes of dilution buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1.25% Triton X-100) supplemented with DTT and PI. After sonication of the lysates, p53 was immunoprecipitated with anti-p53 antibody (DO-1). Subsequently the immunoprecipitates were washed three times with 200-NP buffer [27], and analyzed by Western blotting with DO-1 and anti-ubiquitin antibody (FK2, MBL).

For detection of p53 conjugated with transfected $(His)_6$-ubiquitin, transfected H1299 cells were lysed in urea lysis buffer (100 mM NaH2PO4, 10 mM Tris–HCl, pH 8.0, 500 mM NaCl, 10% glycerol, 0.1% Triton X-100, 10 mM imidazole) supplemented with 10 mM β-mercaptoethanol, PI, 5 mM Iodoacetamide, and 1 mg/ml NEM. Proteins conjugated with His-tagged ubiquitin were purified as described before [28], and analyzed by Western blot analysis.

2.3. Protein expression and purification

Flag-tagged Human Mdm2 (Flag-Mdm2) or Human Mdmx RNA was transcribed from the corresponding cDNA using the Wheat Germ Expression Kit (Cell Free Science, Japan). Subsequently, the Flag-Mdm2 RNA alone or in combination with an excess amount of the Mdmx RNA was used for in vitro translation with wheat germ lysate (Cell Free Science) according to the manufacturer's instructions. Flag-Mdm2 or the Flag-Mdm2/Mdmx complex was then purified on agarose conjugated with anti-Flag antibody.

2.4. In vitro ubiquitination assay

In vitro ubiquitination assays were performed as previously described with some modifications [29]. Approximately 100 ng of Flag-Mdm2 or the Flag-Mdm2/Mdmx complex were mixed with the following purified components; 8 ng of GST-p53, 10 ng of E1 (Boston Biochem), 80 ng of E2 (UbcH5b, Boston Biochem), 3 μg of His-ubiquitin (Calbiochem), or methylated ubiquitin (Boston Biochem). In experiments shown in Fig. 4D, ^{125}I-ubiquitin (Perkin–Elmer) was included in the reaction mixture. These components were incubated in a reaction buffer (40 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM NaCl) in the presence of 2 mM Mg–ATP at 37 °C for the indicated times. After the reactions were terminated by adding SDS sample buffer, ubiquitinated proteins were separated in SDS–PAGE gels and detected by Western blot analyses or autoradiography.

3. Results

3.1. Wild-type Mdmx was capable of enhancing p53 ubiquitination in the presence of Mdm2 in vivo

Recently, we demonstrated that the non-phosphorylatable, active form of Mdmx augments p53 ubiquitination mediated by wild-type Mdm2 in transfected H1299 cells [30]. In order to determine whether wild-type Mdmx cooperates with Mdm2 to induce ubiquitination of p53 as well, wild-type Mdmx (Mdmx-wt) or the non-phosphorylated form of Mdmx (Mdmx-3A) was transfected together with Mdm2 into H1299 cells, and conjugation of p53 with endogenous ubiquitin was examined by Western blot analyses (Fig. 1). As expected from previous observation [30], Mdmx-3A, which is resistant to Mdm2-mediated ubiquitination and degradation, was expressed at higher levels than wild-type Mdmx (Fig. 1, lanes 2 and 3). p53 ubiquitination induced by Mdm2 was enhanced in the presence of co-transfected wild-type Mdmx (Fig. 1, lanes 5 and 8), indicating that wild-type Mdmx is capable of stimulating Mdm2-mediated ubiquitination of p53,

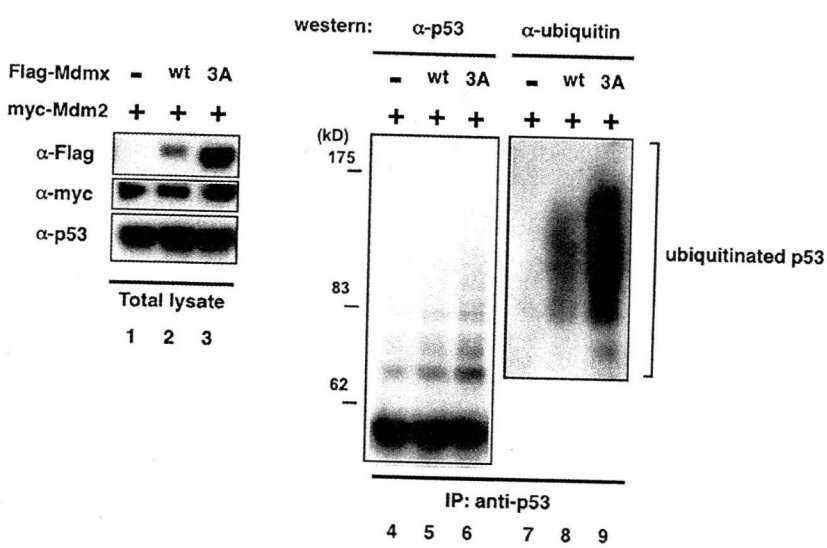

Fig. 1. Mdmx cooperates with Mdm2 to induce p53 ubiquitination. HA-p53 (0.15 mg) and either 0.4 mg of the control vector, wild-type Flag-Mdmx, or the Flag-Mdmx-3A mutant were transfected into H1299 cells in the presence of 0.2 mg of Myc-Mdm2. The total amount of transfected DNA was adjusted to 2 μg with pBluescript plasmid (Stratagene). Twenty hours after transfection, lysates prepared under denaturing conditions were used for immunoprecipitaiton with anti-p53 (DO-1) antibody. The immunoprecipitates were then used for Western blot analyses with DO-1 (left panel, and right bottom panel for low exposure) and with anti-ubiquitin antibody (right panel). Amounts of immunoprecipitates used for Western were normalized such that an equal amount of non-ubiquitinated p53 was loaded in each lane.

although the extent of the stimulation is less than that induced by the non-phosphorylatable mutant (Fig. 1, lanes 6 and 9).

3.2. Mutation at the C-terminal ubiquitinated lysines largely abolished p53 ubiquitination by Mdmx

It has been documented that Mdm2 ubiquitinates p53 at the six C-terminal lysines, the integrity of which are required for its nuclear export [31,32]. We created a mutant p53 in which all six lysines at the C-terminal domain (Fig. S1) were substituted by arginine (p53-K6R), and introduced wild-type p53 or the K6R mutant into H1299 cells together with Mdm2 in the presence or absence of Mdmx-3A. Examination of p53 ubiquitination in vivo revealed that the K6R mutation eliminates a majority of p53 ubiquitination enhanced by Mdmx (Fig. S2), indicating the six lysines were major sites for Mdmx-dependent ubiquitination.

3.3. Association of Mdmx with Mdm2 augments the ability of Mdm2 to ubiquitinate p53 and inhibits poly-ubiquitination of Mdm2 in vitro

In order to determine whether Mdmx enhances Mdm2-dependent ubiquitination of p53 via direct association with Mdm2, we next performed in vitro ubiquitination assays using purified recombinant proteins of Mdm2 or an Mdm2/Mdmx complex (see Section 2). Silver staining of the purified proteins indicated that the co-purified Mdmx formed a complex with Mdm2 at approximately a 1:1 molar ratio (Fig. 2A, right panel).

In order to determine the effect of the association with Mdmx on the activity of E3 ubiquitin ligase of Mdm2, homomeric Mdm2 or the Mdmx/Mdm2 complex was incubated with E1, E2 (UbcH5b), GST-p53, and ubiquitin, and time-course analyses of the ubiquitination of p53 and auto-ubiquitination of Mdm2 were simultaneously performed. The complex formation of Mdm2 with Mdmx-3A or with wild-type Mdmx resulted in an increase of p53 ubiquitination (Fig. 2B and C). In contrast, the Mdmx/Mdm2 complex showed a marked decrease in poly-ubiquitinated forms of Mdm2 in comparison to homomeric Mdm2 (Fig. 2B and C), indicating that the association with Mdmx-3A augments Mdm2-dependent p53 ubiquitination while it inhibits poly-ubiquitination of Mdm2.

3.4. Mdmx inhibits ubiquitination of the Mdm2-containing enzymatic complex

In order to confirm that Mdmx inhibits auto-ubiquitination of Mdm2, in vitro ubiquitination assays of the Mdm2 homomer or the Mdm2/Mdmx complex were performed in the presence of

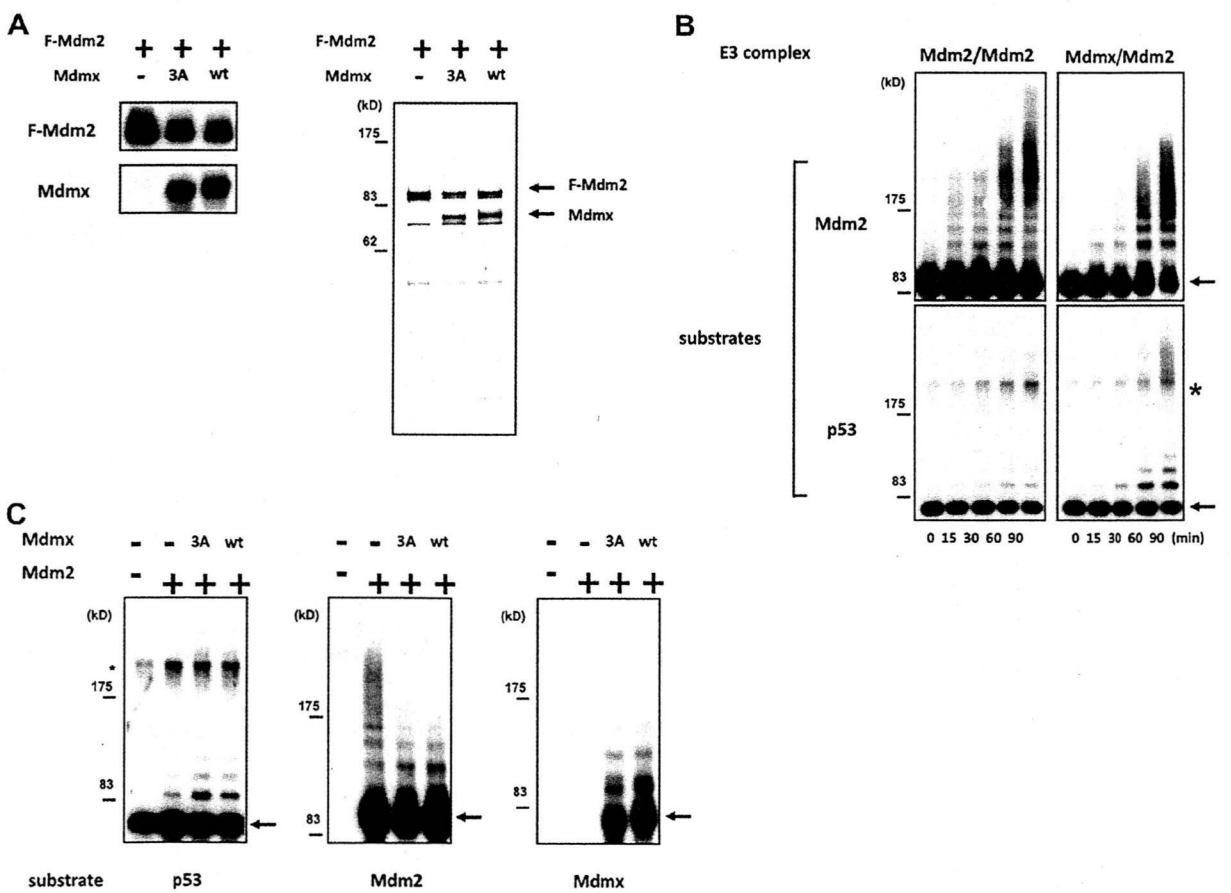

Fig. 2. Association of Mdmx with Mdm2 augments the activity of Mdm2 to ubiquitinate p53 and inhibits auto-ubiquitination of Mdm2 in vitro. (A) Purification of Mdm2 and the Mdm2/Mdmx complex. Flag-tagged Mdm2 was translated alone, or co-translated with Mdmx-3A or wild-type Mdmx in wheat germ lysates, as described in Section 2. The purified proteins were separated by10% SDS–PAGE, and detected by silver staining (right panel), or by Western blotting analyses with anti-Flag antibody (M2) or anti-Mdmx antibody (D-19) (left panel). (B) *In vitro* ubiquitination assays were performed with purified Mdm2 or Mdmx-3A/Mdm2. Ubiquitination reactions were terminated at the indicated times, and the extent of p53 ubiquitination and Mdm2 auto-ubiquitination was evaluated by Western blot analyses with anti-Flag antibody or anti-p53 antibody. The position of non-ubiquitinated substrates is designated by arrows. (C) In vitro ubiquitination assays were performed as described in (B), and the ubiquitination reactions were terminated after 30 min. Ubiquitination of Mdmx, p53, and Mdm2 was evaluated by Western blot analyses.

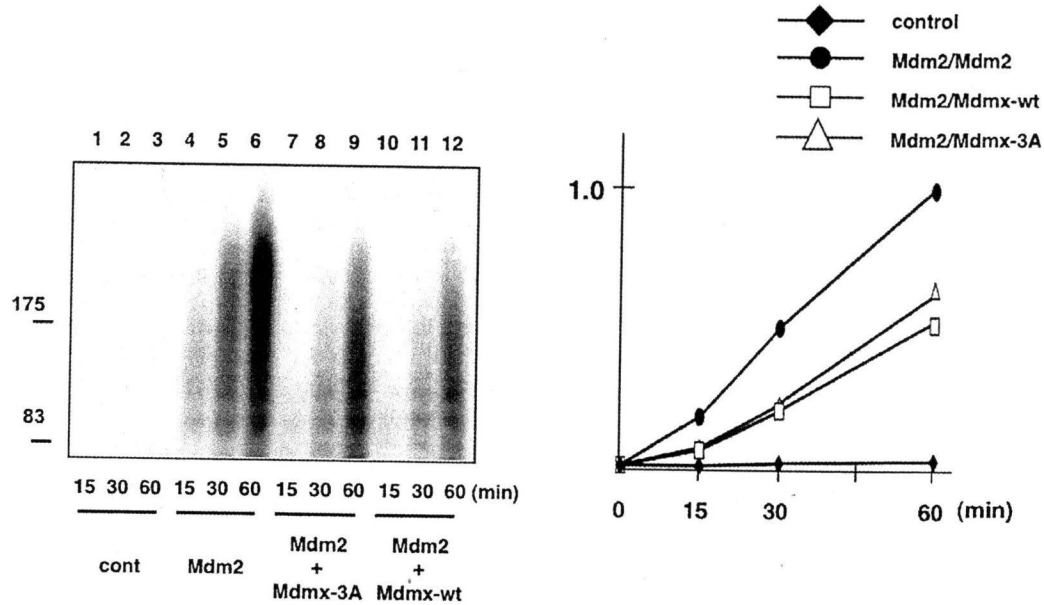

Fig. 3. In vitro ubiquitination reaction was performed as described in Fig. 2C, except that ^{125}I-labeled ubiquitin was included in the reaction. (left panel) Ubiquitinated Mdm2 or Mdm2/Mdmx was separated by 10% SDS–PAGE, and detected by autoradiography. Note that the ladder represents a mixture of ubiqutination of Mdm2 and Mdmx in lanes 7–12 (left panel). Levels of the ubiquitination were quantified and relative levels of ubiquitination were plotted (right panel).

^{125}I-labeled ubiquitin. Quantification of ubiquitin attached to the enzymatic complexes demonstrated that the auto-ubiquitination of the Mdm2 was indeed hindered by the complex formation with either wild-type Mdmx or Mdmx-3A (Fig. 3). Thus, the complex formation of Mdm2 with Mdmx affects the preference for the substrate of the Mdm2 ubiquitin ligase.

3.5. Mdmx stimulates Mdm2-dependent mono-ubiquitination of p53

It has been documented that poly-ubiquitination of p53 induces its degradation, while its mono-ubiquitnation stimulates nuclear export of p53 [33]. Because Mdmx does not significantly contribute to p53 degradation [34], we attempted to determine whether Mdmx stimulates mono-ubiquitination of p53 rather than its poly-ubiquitination. Mdm2 and p53 were introduced into H1299 cells together with His-Ub-K7R, $(His)_6$-tagged mutant ubiquitin which is not capable of forming a ladder of poly-ubiquitination due to arginine substitution in all seven lysine residues [29]. Subsequently, His-Ub-K7R was purified from lysates that were prepared from transfected cells, and p53 conjugated with His-Ub-7KR was detected by Western blot analyses with anti-p53 antibody. The introduction of wild-type Mdmx augmented mono-ubiquitination of p53 (Fig. 4, lane 2), and the Mdmx-3A mutation further enhanced the p53 mono-ubiquitination (Fig. 4, lane 3).

In order to determine whether Mdmx stimulates Mdm2-dependent mono-ubiquitination of p53 in vitro as well as in vivo, methylated ubiquitin was used instead of wild-type ubiquitin in in vitro ubiquitination assays. Indeed, the Mdmx/Mdm2 complex showed a stronger activity for p53 mono-ubiquitination than the homomeric Mdm2 (Fig. S3). Thus, the formation of a complex with Mdmx augments the activity of Mdm2 to mono-ubiquitinate p53.

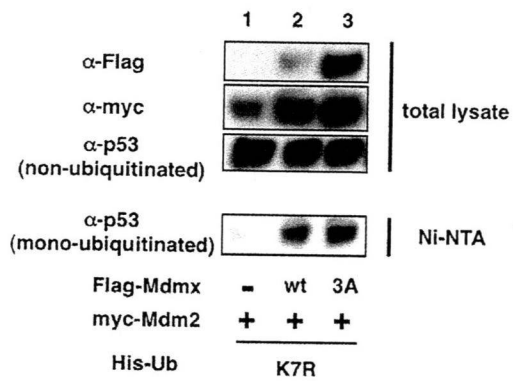

Fig. 4. Mdmx-3A or the control vector was transfected into H1299 cells together with Myc-Mdm2, HA-p53 and the indicated $(His)_6$-tagged ubiquitin K7R mutant. Twenty hours after transfection, cells were lysed with a buffer containing 6 M urea, and normalized lysates that contain equal amounts of non-ubiquitinated p53 were used to purify His-tagged ubiquitin on Ni-NTA agarose (QIAGEN). Ubiquitinated p53 was detected by Western blot analysis with anti-p53 antibody (DO-1).

4. Discussion

In this report, we demonstrated that wild-type Mdmx as well as its non-phosphorylatable mutant cooperates with Mdm2 to stimulate ubiquitination of p53 both in vivo and in vitro. In agreement with our observation, it was reported that Mdmx enhances the activity of Mdm2 as a ubiquitin ligase in vitro [35]. Mdmx complements the catalytic function of mutant Mdm2 proteins that are deficient in the enzymatic activity as a ubiquitin ligase [23–25] and Mdmx/Mdm2 hetero-RING complexes exhibit a greater E3 ligase activity than homomeric Mdm2 [36]. Such effects of Mdmx on Mdm2 should enhance Mdm2-dependent ubiquitination of p53, consistent with the role of Mdmx as an inhibitor of p53.

It was previously reported that Mdmx augments not only auto-ubiquitination of Mdm2 but also the ubiquitin ligase activity of Mdm2 toward p53 [35] in in vitro assays. However, auto-ubiquitination of the Mdm2 ubiquitin ligase negatively affects its activity because poly-ubiquitinated Mdm2 is targeted for proteasome-mediated degradation. Therefore, enhanced ubiquitinase activity of Mdm2 by Mdmx may not be translated into efficient stimulation of p53 ubiquitination if the association of Mdmx to Mdm2 simultaneously leads to stimulation of self-destruction of Mdm2. Our

observation that Mdmx inhibits poly-ubiquitination of Mdm2 while it stimulates p53 ubiquitination may attribute to a mechanism by which Mdmx stimulates Mdm2-dependent p53 ubiquitination without enhanced destruction of Mdm2, thus providing the molecular basis of how Mdmx cooperates with Mdm2 to inhibit p53 activity.

Recently Linke et al. reported the crystal structure of the heterodimer of Mdmx/Mdm2 RING domain, and proposed a model that favors transfer of ubiquitin to Mdmx that does not interact with E2 [37]. This can explain why Mdm2 is not extensively ubiquitinated in the Mdmx/Mdm2 heteromeric complex, thus providing mechanistic basis for reduced ubiquitination of Mdm2 in the Mdmx/Mdm2 complex (Fig. 2). It is noteworthy that, in in vitro ubiquitination assays, the levels of Mdm2 ubiquitination in the homomeric Mdm2 are higher than combined levels of ubiquitination of Mdm2 and Mdmx in the heteromeric complex (Fig. 3). Therefore, it is likely that Mdmx is relatively resistant to ubiquitination by bound Mdm2, unless Mdmx undergoes specific modification such as phosphorylation [27].

It is not clear at this moment how Mdmx stimulates Mdm2-mediated ubiquitination of p53. Mdm2 bound to Mdmx may position its catalytic domain more closer to the C-terminal domain of p53 than homomeric Mdm2, resulting in enhanced p53 ubiquitination. Alternatively, Mdm2 or Mdmx may compete with p53 as a substrate for Mdm2, and relative resistance of Mdmx against ubiquitination by bound Mdm2 may translate into facilitated p53 ubiquitination. Presumably, these two possibilities are not mutually exclusive, and combined effects of Mdmx on Mdm2-mediated ubiquitination may serve to stimulate ubiquitination and inactivation of p53.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.07.021.

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Involvement of JNK pathway in the promotion of the early stage of colorectal carcinogenesis under high-fat dietary conditions

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Involvement of JNK pathway in the promotion of the early stage of colorectal carcinogenesis under high-fat dietary conditions

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See Commentary, p 1575

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ABSTRACT

Background and aims: The molecular mechanisms underlying the promotion of colorectal carcinogenesis by a high-fat diet (HFD) remain unclear. We investigated the role of the insulin-signal pathway and the c-Jun N-terminal kinase (JNK) pathway, which reportedly play crucial roles in insulin resistance, during colorectal carcinogenesis in the presence of hyperinsulinaemia induced by a HFD.

Methods: Azoxymethane-induced aberrant crypt foci formation and cell proliferation in the colonic epithelium were compared between mice fed a normal diet (ND) and mice fed a HFD. A western blot analysis was performed to elucidate the mechanism affecting colorectal carcinogenesis by a HFD.

Results: The number of aberrant crypt foci and the colonic epithelial cell proliferative activity were significantly higher in the HFD group than in the ND group. While the plasma insulin level was significantly higher in the HFD group than in the ND group, a western blot analysis revealed the inactivation of Akt, which is located downstream of the insulin receptor, in the colonic epithelia of the HFD group. On the other hand, JNK activity was significantly higher in the HFD group than in the ND group. A JNK specific inhibitor significantly suppressed the increase in epithelial cell proliferation only under a HFD, but not under a ND.

Conclusions: Colonic cell proliferation was promoted via the JNK pathway in the presence of a HFD but not in the presence of a ND. This novel mechanism may explain the involvement of the JNK pathway in the effect of dietary fat intake on colon carcinogenesis.

Colorectal cancer is a major cause of morbidity and mortality worldwide.[1] Recently, associations between obesity and metabolic abnormalities, which are caused by a high intake of dietary fat and physical inactivity, and an elevated risk of colorectal cancer have been reported.[2 3] Many epidemiological studies have provided evidence of a relation between dietary fat intake and an increased risk of colorectal cancer.[4] Most animal experimental studies have shown that a high-fat diet (HFD) leads to an increased number of chemically induced aberrant crypt foci (ACF),[5] which are identifiable lesions in experimental colon carcinogenesis, and tumours[6] in the colon. The possibility that an elevated plasma insulin level promotes colorectal cancer has also been debated.[7] Some studies support the hypothesis that insulin, when exogenously administered, acts as an important growth factor for colonic epithelial cells.[8 9] However, whether an elevated plasma insulin level might enhance the proliferative state through the activation of an insulin signalling pathway downstream of the insulin receptor, such as the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway, in the colon – a non-classical insulin target tissue – remains unclear.[10] Furthermore, adipocytokines also act as positive or negative modulators of colonic epithelium and tumours.[11 12] We previously reported that adiponectin suppressed colorectal carcinogenesis only under a HFD condition.[12] Despite accumulating evidence, the molecular mechanisms underlying the influence of a high intake of dietary fat on the promotion of colorectal carcinogenesis are not fully understood. The identification and evaluation of the relation between colorectal cancer and a high intake of dietary fat will be critical for preventive strategies against colorectal cancer in the near future.

Recent studies have demonstrated that c-Jun N-terminal kinase (JNK) plays a crucial role in obesity and insulin resistance.[13] JNK is activated in obesity, in part because of lipotoxic stress.[14] Hirosumi *et al*[13] reported abnormally elevated JNK activity levels in the liver, muscle and adipose tissues of mice fed a HFD. Most of the molecular mechanisms of JNK activity and their relation to insulin resistance have been studied in the liver and adipose tissues of various models of obesity, but little is known about the action of JNK in colonic epithelial cells. We postulated that JNK might provide a link between dietary fat intake and colorectal cancer.

The JNK pathway represents one subgroup of mitogen-activated protein kinases (MAPK) that is activated primarily by cytokines and exposure to environmental stress.[15] A major target of the JNK signalling pathway is the activator protein-1 (AP-1) transcription factor, which is activated, in part, by the phosphorylation of c-Jun and related molecules.[16] JNK has been implicated in the pathogenesis of cancer in various tissues in oncogenic transformation and cell proliferation.[15] Genetic and pharmacological approaches have been used to evaluate the potential importance of JNK in tumour formation and growth.[17 18] Growth inhibition has been observed in response to the JNK inhibitor and antisense oligonucleotides in multiple myeloma cells[19] and breast cancer cells.[20] Nateri *et al*[21] used a mouse model of intestinal tumorigenesis to show that the ablation of the c-Jun gene or the mutation of the JNK phosphorylation sites on c-Jun reduced the tumour number and size. These oncogenic functions of c-Jun are dependent on the N-terminal phosphorylation of c-Jun by JNK, implicating JNK as a potential oncogene in the intestine. Several studies

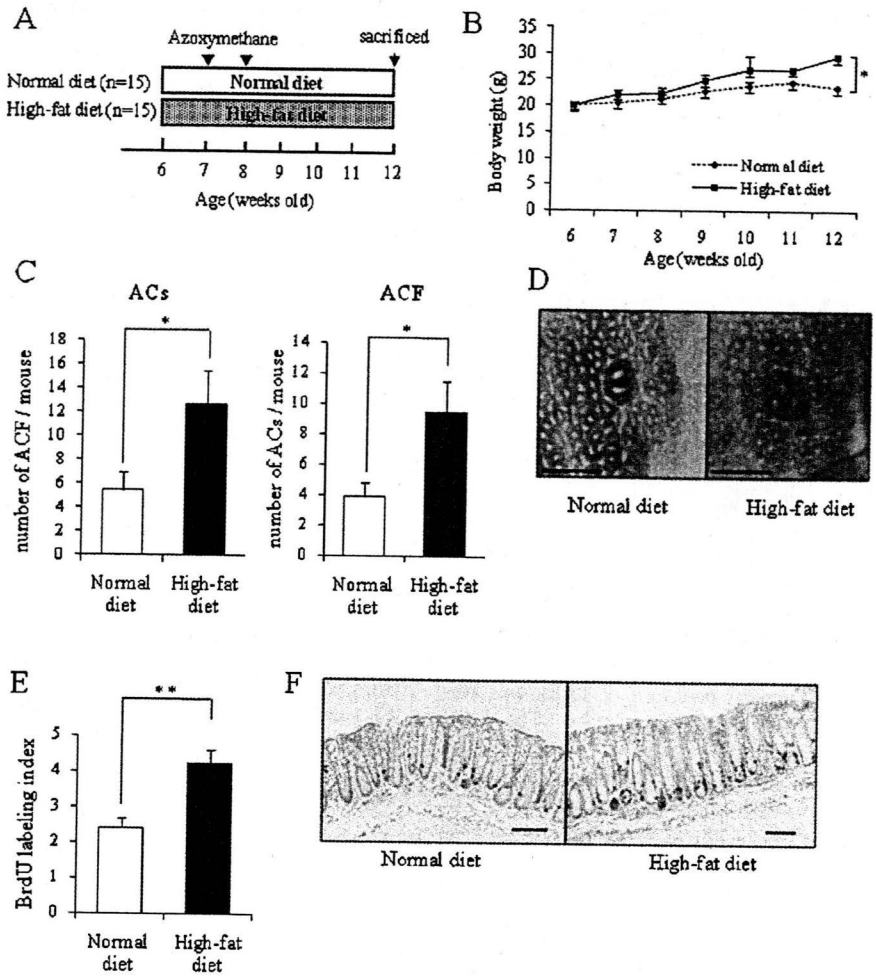

Figure 1 Enhanced proliferative activity of colonic epithelial cells in the presence of a high-fat diet. (A) ACF experiment protocol. Mice (6 weeks old) were divided into a normal diet group (n = 15) and a high-fat diet group (n = 15). Mice in each group were given two weekly intraperitoneal injections of 10 mg/kg of AOM. Six weeks after the start of the normal diet or high-fat diet, the mice were killed. (B) Changes in the body weights of mice fed a normal diet (broken line, n = 15) and mice fed a high-fat diet (solid line, n = 15) and treated with AOM. *Significant differences were observed between the normal diet and high-fat diet groups at all time points except for the initial measurement. *$p<0.01$. (C) Average numbers of ACs and ACF for mice fed a normal diet (n = 9) and mice fed a high-fat diet (n = 9). Each column represents the mean with the SEM; *$p<0.05$. (D) Stereomicroscopic observations of ACF in colon specimens from each group. Samples were stained with 0.2% methylene blue. Scale bar: 100 μm. (E) Average BrdU labelling index in each group in the ACF formation experiment. BrdU was administered intraperitoneally 1 h before the mice were killed. Both indices were expressed as the percentage of positively stained nuclei to the total number of nuclei counted in the crypts of the colon. Each bar represents the mean with the SEM of 9 mice/group. **$p<0.01$. (F) Representative immunohistochemical staining for BrdU in each group. Scale bar: 100 μm. ACs, aberrant crypts; ACF, aberrant crypt foci; AOM, azoxymethane; BrdU, 5-bromodeoxyuridine.

have demonstrated that colonic tumours of human origin exhibit an elevated expression of JNK/c-Jun.[22 23] However, the involvement of the JNK/c-Jun pathway in colon carcinogenesis under a HFD condition has not been previously reported.

Therefore, in this study, we investigated the activity of the insulin signalling pathway and the functional role of the JNK/c-Jun pathway in colorectal carcinogenesis and epithelial cell proliferation in the presence of hyperinsulinaemia induced by a HFD. This novel mechanism may explain the involvement of the JNK/c-Jun pathway in the effect of dietary fat intake on colon carcinogenesis.

MATERIALS AND METHODS

Animals and diets

C57Bl/6J mice were purchased from CLEA Japan (Tokyo, Japan). The animals were fed either a normal diet (ND) or a HFD until the end of the study. The compositions of the ND (MF; Oriental Yeast Co., Tokyo, Japan) and the HFD (High Fat Diet 32; CLEA Japan, Tokyo, Japan) have been previously described.[12] Three to five mice were housed per metallic cage, with sterilised softwood chips used as bedding, in a barrier-sustained animal room air-conditioned at 24 (SD 2)°C and 55% humidity under a 12 h light/dark cycle.

Analysis of aberrant crypt foci

Six-week-old male mice were divided into a ND group and a HFD group. Mice in each group were given two weekly intraperitoneal injections of 10 mg/kg of azoxymethane (AOM) (Sigma, St. Louis, Missouri, USA) and were killed at 6 weeks following the initiation of AOM injection (fig 1A). The entire colon was removed, gently flushed with saline to remove any faecal contents, opened longitudinally, and fixed in 10% neutralised formalin; the numbers of ACF and aberrant crypts

Table 1 Blood plasma levels of various metabolites in mice fed a normal diet or a high-fat diet

	Normal diet		High-fat diet	
	SP600125 (−)	SP600125 (+)	SP600125 (−)	SP600125 (+)
Glucose (mg/dl)	81.80 (2.20)	76.50 (2.93)	180.00 (9.63)**	143.00 (10.54)†
Insulin (ng/ml)	1.69 (0.32)	1.96 (0.44)	3.38 (0.52)*	1.54 (0.50)†
Triglycerides (mg/dl)	57.43 (5.21)	55.00 (6.21)	77.68 (10.31)	61.66 (5.81)
Cholesterol (mg/dl)	87.86 (5.96)	77.85 (2.94)	120.14 (5.62)*	92.97 (5.43)††
TNFα (pg/ml)	41.49 (7.19)	32.62 (0.64)	36.18 (0.75)	35.29 (2.03)

Data represent the mean (SEM) of 6–9 mice/group.
*p<0.05, **p<0.01 compared with mice fed a normal diet.
†p<0.05, ††p<0.01 compared with mice fed a HFD treated (−) with SP600125.
TNFα, tumour necrosis factor α

(ACs) were then counted as described previously.[24] To facilitate the counting, the colons were stained with 0.2% methylene blue solution and were observed using stereomicroscopy.

Assay for assessing the proliferative activity of colonic epithelial cells

We evaluated the 5-bromodeoxyuridine (BrdU) labelling index to determine the proliferative activity of the colonic epithelial cells. BrdU (BD Biosciences, New Jersey, USA) was diluted in phosphate-buffered saline at 1 mg/ml and was administered intraperitoneally at a dose of 50 mg/kg, 1 h before the mice were killed. The immunohistochemical detection of BrdU was performed using a commercial kit (BD Biosciences). The BrdU labelling index was expressed as the ratio of the number of positively stained nuclei to the total number of nuclei counted in the crypts of the colon. The criteria for crypt selection included the presence of a clearly visible and continuous cell column on each side of the crypt. Twenty crypts were evaluated in each mouse.

Plasma lipid levels and insulin resistance

The levels of plasma triglycerides, cholesterol, insulin, insulin-like growth factor-1 (IGF-1), tumour necrosis factor α (TNFα) and blood glucose were measured using a WAKO enzyme-linked immunosorbent assay (ELISA) kit (Wako Pure Chemical, Osaka, Japan) (n = 10 from each group). We measured the plasma concentrations of triglycerides and cholesterol according to the manufacturer's instructions.

Immunoblotting

The extracted protein was separated using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham, London, UK). The membranes were probed with primary antibodies specific for phospho-JNK, JNK, phospho-extracellular signal regulated kinase (ERK), ERK, phospho-p38 MAPK, p38 MAPK, phospho-c-Jun, c-Jun, phospho-insulin receptor substrate-1 (IRS-1) (Ser307), IRS-1, phospho-Akt (Ser473), Akt, phospho-mammalian target of rapamycin (mTOR), mTOR, phospho-p70 ribosomal S6 kinase (S6K), S6K (Cell Signalling Technology, Danvers, Massachusetts, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Trevigen, Gaithersburg, Maryland, USA). Horseradish-peroxidase-conjugated secondary antibodies and the ECL detection kit (Amersham) were used for the detection of specific proteins.

Gene expression analysis

Total RNA was extracted from the colonic epithelium using the RNeasy Mini Kit (Qiagen, Hilden, Germany). For real-time reverse transcription polymerase chain reaction, total RNA was reverse-transcribed into cDNA and amplified using real-time quantitative polymerase chain reaction using the ABI PRISM 7700 System (Applied Biosystems, Foster City California, USA). Probes and primer pairs specific for *cyclin D1* and *β-actin* were purchased from Applied Biosystems. The concentrations of the target genes were determined using the competitive computed tomography method and the values were normalised to the internal control.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were performed according to a previously described method.[25] Briefly, nuclear extracts from colonic tissue were prepared and gel shift assays using an AP-1 consensus oligonucleotide (Promega, Madison, Wisconin, USA) were performed. Samples were separated using 4% polyacrylamide gel electrophoresis (PAGE), and the gels were dried and exposed to radiograph film.

Effect of the JNK inhibitor SP600125 on the induction of ACF formation

The JNK inhibitor anthra[1,9-*cd*]pyrazol-6(2*H*)-one (SP600125) was purchased from Calbiochem (San Diego, Calfornia, USA). C57Bl/6J mice (6 weeks old) were intraperitoneally injected with SP600125 (10 or 50 mg/kg) or vehicle (5% dimethyl sulfoxide (DMSO), 20% Cremophor EL, 75% saline) daily until the end of the experiment. The mice were fed the ND or the HFD and received AOM injections according to the ACF protocol.

Statistical analysis

Statistical analyses of the number of ACF, the BrdU labelling index, and the blood test results were conducted using the Mann–Whitney U test. Other statistical analyses were performed using the Student t test. Values of $p<0.05$ were regarded as denoting statistical significance.

RESULTS

Enhanced formation of aberrant crypt foci and increase in the cell proliferative activity in mice fed a high-fat diet

To examine the effect of a HFD on the promotion of colonic epithelial cell proliferation, the formation of chemically induced ACF (defined as clusters of ACs) was examined in the colon specimens as a marker of early stage colorectal carcinogenesis.[26 27] The proliferative activity of the colonic epithelial cells was also determined using the BrdU labelling index. The ACF experimental protocol is shown in fig 1A. The numbers of ACF and ACs were significantly higher in mice fed a HFD than in those fed a ND (fig 1C). The macroscopic characteristics of the ACF in the ND and HFD groups are shown in fig 1D; no morphological differences in the ACF were observed between the ND and the HFD groups. Furthermore, a significant increase

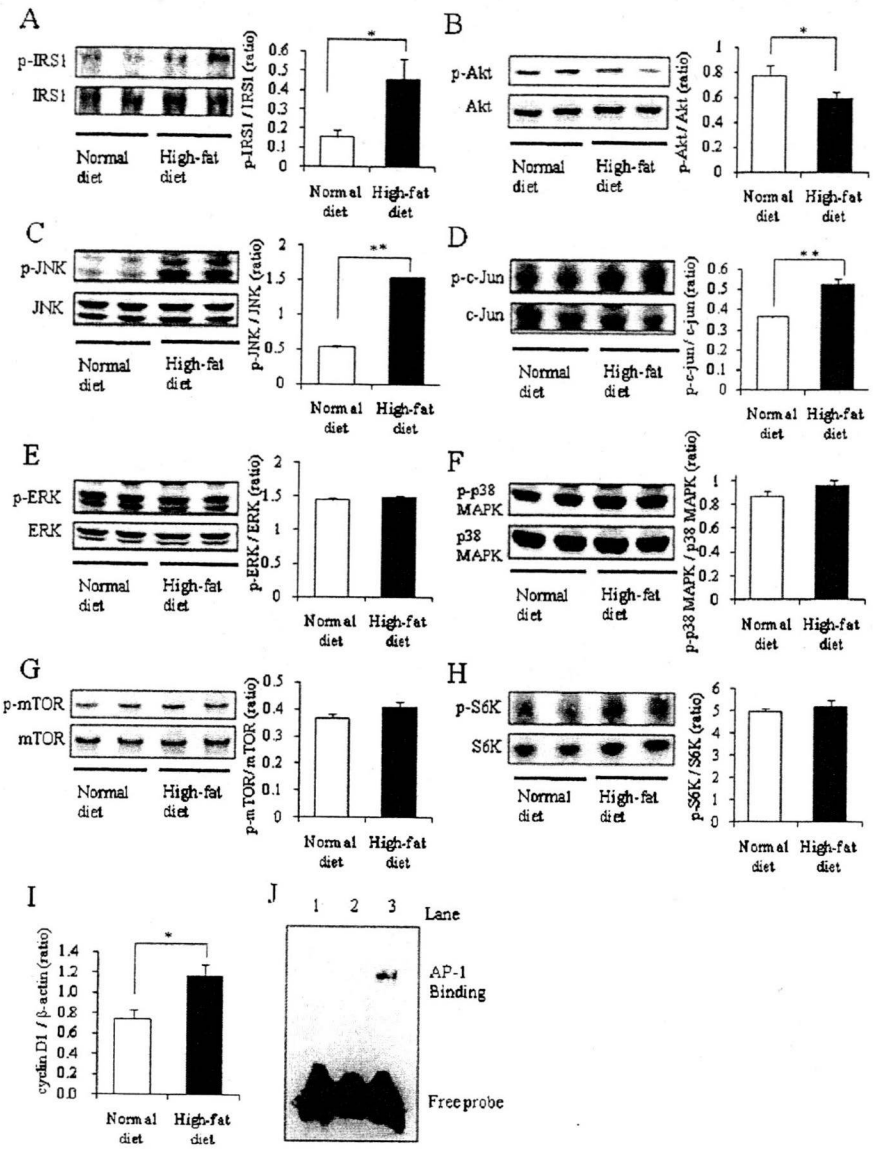

Figure 2 JNK activity and the signalling pathways for both insulin resistance and cell proliferation. A western blot analysis for phosphorylated and total IRS-1 (A), Akt (B), JNK (C), c-Jun (D), ERK (E), p38 MAPK (F), mTOR (G) and S6K (H) in colon specimens from mice fed a normal diet (n = 5) and mice fed a high-fat diet (n = 5). Representative western blotting images are shown. Right panel: graphs showing the ratios of the phosphorylated protein level to the total protein level. (I) Real time reverse transcription polymerase chain reaction analysis for the gene expression of *cyclin D1* in colon specimens from mice fed a normal diet (n = 6) and mice fed a high-fat diet (n = 6). Each column represents the mean with the SEM; *p<0.05, **p<0.01. (J) Electromobility shift assay autoradiogram shows the marked induction of AP-1 DNA binding under high-fat diet conditions. Lane 1, free probe alone (no nuclear extracts); lane 2, normal diet; lane 3, high-fat diet. ERK, extra-cellular signal-related kinase; IRS-1, insulin receptor substrate-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; S6K, p70 ribosmal S6 kinase.

in the BrdU index was observed in the HFD group, compared with in the ND group (fig 1E,F). These results indicate that the proliferative activity of the colonic epithelial cells was promoted by the HFD, resulting in an increase in the number of ACF.

The body weights were significantly higher in the HFD group than in the ND group after 1 week of treatment (that is, at 7 weeks of age), and this difference was maintained until the end of the study (fig 1B). We next investigated the blood plasma levels of various metabolites in the ND and HFD groups. The levels of fasting plasma glucose, insulin, TNFα, lipids and IGF-1 were measured and are shown in table 1 and supplementary fig 1. Compared with the ND group, the HFD group had significantly higher fasting plasma glucose, insulin cholesterol and IGF-1 levels. Meanwhile, the plasma TNFα and triglyceride levels were not significantly different between the two groups.

The JNK pathway is activated in the colonic epithelium of mice fed a high-fat diet

To clarify the mechanisms underlying the enhanced proliferative activity of the colonic epithelial cells in the HFD group, we investigated the expression levels of various potential target proteins in colon specimens prepared from the ND and HFD groups. Because the plasma insulin level was significantly increased in the HFD group, we first speculated that an insulin signalling pathway downstream of the insulin receptor, such as the PI3K/Akt signalling pathway, might be involved in the promotion of colonic epithelial cell proliferation in the presence of a HFD. Akt plays an important role in a variety of biological processes including cell survival, cell growth, and oncogenesis[28] and is activated by insulin via the phosphorylation of IRS-1.[29] Surprisingly, the results of a western blot analysis revealed that the amount of phosphorylated Akt was lower in the HFD group than in the ND group (fig 2B). However, a significant increase in the level of phosphorylated IRS-1 was observed in the HFD group, compared with in the ND group (fig 2A). These results imply the existence of insulin resistance in the colonic epithelium. The phosphorylation of IRS-1 has emerged as a key event in insulin resistance.[30] Indeed, a large number of protein kinases have been shown to cause the phosphorylation of IRS-1, including JNK,[31] ERK,[32] mTOR,[35] and S6K.[34] Therefore,

we next examined the expression levels of these proteins. No differences in the protein levels of phosphorylated ERK, p38 MAPK, mTOR and S6K were observed between the HFD and ND groups (fig 2E–H). On the other hand, significant increases in the levels of phosphorylated JNK and c-Jun were observed in the HFD group, compared with in the ND group (fig 2C,D). The expressions of JNK and c-Jun in the colonic epithelium were confirmed using a western blot analysis and an immunohistochemical analysis (supplementary fig 2). Some studies have reported that Akt suppresses the JNK pathway.[35 36] Moreover, the mRNA level of cyclin D1 was significantly higher in the HFD group than in the ND group (fig 2I). The JNK/c-Jun pathway is a critical component of the proliferative response and induces G0 to G1 cell cycle progression in many cell types;[37] furthermore, cyclin D1, which is a regulator of the G1 to S phase transition, has emerged as an important target for the JNK/c-Jun pathway in driving proliferation.[35] We next directed our attention to the AP-1 transcription factor, which is the target of the JNK signalling pathway and, as mentioned earlier, is an important transcription factor involved in oncogenic transformation and cell proliferation. Our data clearly indicated an impressive increase in the activation of AP-1 in the HFD group (fig 2J), indicating that the JNK/c-Jun pathway was activated in the colonic epithelia of these animals.

Inhibition of JNK suppresses colonic epithelial cell proliferation in mice fed a high-fat diet

To confirm the direct involvement of the JNK pathway in the promotion of colonic epithelial cell proliferation in the presence of a HFD, we used a specific JNK inhibitor, SP600125, in the ACF experiment. ACF formation and the BrdU labelling index were significantly suppressed by SP600125 in the HFD group in a dose dependent manner, but no effect was observed in the ND group (fig 3B–D). Furthermore, the JNK inhibitor attenuated the increase in the protein levels of phosphorylated c-Jun in the colons of mice in the HFD group (fig 3A). These results indicated that the activation of the JNK pathway played important roles in the increase in epithelial cell proliferation observed in the mice fed a HFD and might play an important role in promoting colonic epithelial cell proliferation in mice fed a HFD. The fasting plasma glucose, insulin and cholesterol

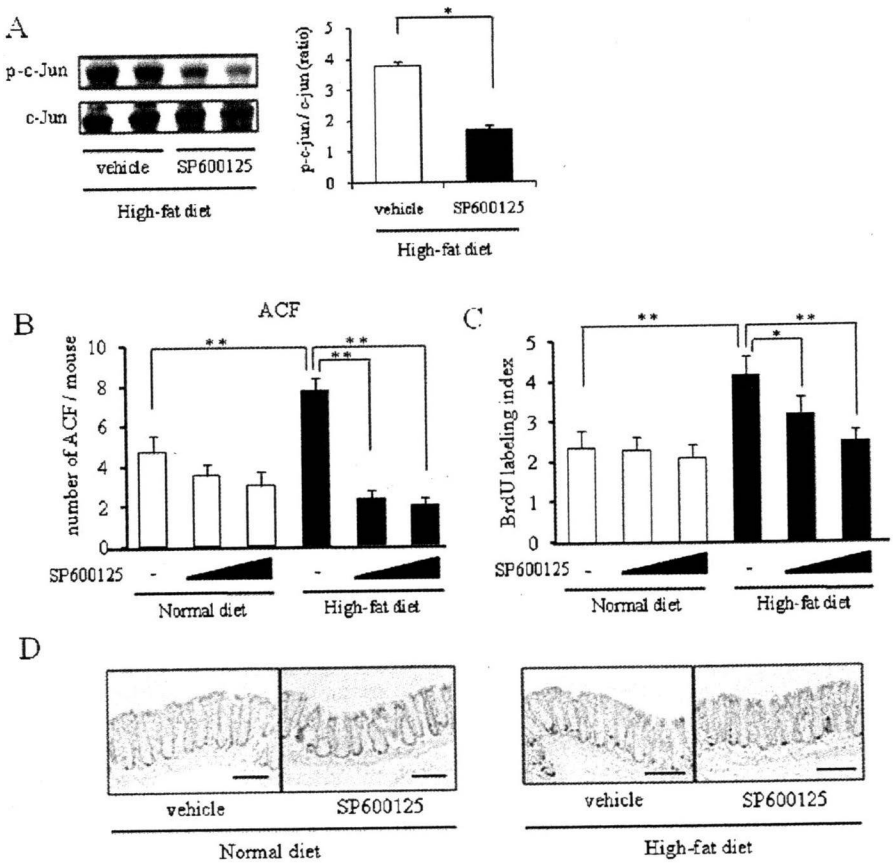

Figure 3 Suppression of colonic epithelial cell hyper-proliferation by JNK inhibitor in the presence of a high-fat diet. Mice (6 weeks old) were fed a normal diet or a high-fat diet and were injected intraperitoneally with the JNK inhibitor, SP600125 (10 or 50 mg/kg) or with the vehicle only daily until the end of the experiment. The mice in each group were also given two weekly intraperitoneal injections of 10 mg/kg of AOM. (A) Western blot analysis of phosphorylated and total c-Jun in colon specimens from mice treated (+) or not treated (−) with SP600125. Right panel: graphs showing the ratios of the phosphorylated protein levels to the total protein levels. Each column represents the mean with the SEM of 6 mice/group; *$p<0.05$, compared with mice treated (−) with SP600125. (B) Average numbers of ACF in the mice fed a normal diet and the mice fed a high-fat diet treated (+) or not treated (−) with SP600125. Each column represents the mean with the SEM of 6 mice/group; **$p<0.01$. (C) The average BrdU labelling index decreased in mice fed a high-fat diet in a dose dependent manner, but not in mice fed a normal diet. Each column represents the mean with the SEM of 6 mice/group; *$p<0.05$, **$p<0.01$. (D) Representative immunohistochemical staining for BrdU in each group. Scale bar: 100 μm. ACF, aberrant crypt foci; AOM, azoxymethane; BrdU, 5-bromodeoxyuridine; JNK, c-Jun N-terminal kinase.

levels were significantly reduced by SP600125 in the HFD group, but no effect was observed in the ND group (table 1). The plasma TNFα and triglyceride levels after treatment with SP600125 were similar between the two groups.

DISCUSSION

Previous studies have provided evidence of an association between dietary fat intake and an increased risk of colorectal carcinogenesis,[2 4] but the molecular mechanisms underlying the promotion of colorectal carcinogenesis by a HFD remain unclear. In the present study, we showed a significant enhancement in the formation of ACF and an increase in the proliferative activity of the colonic epithelial cells in the HFD group, compared with in the ND group. Additionally, we demonstrated the activation of the JNK/c-Jun pathway and the inactivation of Akt in colonic epithelial cells in the HFD group. Furthermore, a JNK specific inhibitor significantly suppressed the increase in epithelial cell proliferation only in the HFD group, suggesting that JNK/c-Jun may play an important role in promoting colorectal carcinogenesis and epithelial cell proliferation in the presence of a HFD.

Animal models exhibiting high-fat-induced hyperinsulinaemia provide a unique opportunity to explore the potential molecular mechanisms underlying the promotion of colorectal carcinogenesis and epithelial cell proliferation by a HFD. Insulin is now known to be an important growth factor for colonic epithelial cells,[7 8] and the insulin receptor has been detected in normal colorectal epithelium and cancer tissue.[58] The insulin-signal transduction pathway can be mediated by the activation of IRS-1/PI3K/Akt signalling and is involved in the regulation of gene expression and mitogenicity. Therefore, we first speculated that PI3K/Akt activation might be involved in the promotion of colonic cell proliferation in the presence of a HFD because the plasma insulin level was elevated in the HFD group. However, our results surprisingly showed that the Akt activity was decreased in the HFD group compared with in the ND group, whereas a significant increase in the level of phosphorylated IRS-1 was observed in the HFD group, compared with in the ND group. These results suggested that, in vivo, HFD induced insulin resistance in the colonic epithelium, causing the inhibition of PI3K/Akt signalling (supplementary fig 3). These molecular mechanisms of insulin resistance have been studied in the liver and adipose tissues of various models of diabetes,[13] with results that are compatible with the presently reported results. Thus, we next examined signalling pathways involved in both insulin resistance and proliferation, including the JNK pathway.

We observed an increased in JNK/c-Jun activity in colonic epithelial cells from the HFD group. Therefore, we investigated the effect of SP600125, a specific JNK inhibitor, on the colonic epithelium to determine the direct involvement of JNK activation in colon carcinogenesis and epithelial proliferation under a HFD condition. The inhibition of JNK activation reportedly improves insulin resistance.[59] Our results indeed demonstrated that SP600125 attenuated the blood glucose and plasma insulin levels, suggesting that this inhibitor might suppress colonic epithelial cell proliferation in the presence of a HFD via some indirect effects, such as the amelioration of insulin resistance. However, a western blot analysis for colonic epithelial protein in the HFD group clearly showed the attenuation of JNK activity after treatment with SP600125. Furthermore, the increase in ACF formation and the BrdU labelling index were distinctly restored by SP600125 in the HFD. Therefore, these results imply that insulin resistance and the promotion of colonic epithelial proliferation in the presence of a HFD occur via independent mechanisms. Our data strongly suggest that a HFD promotes the JNK/c-Jun pathway, resulting in the promotion of cell proliferative activity and, consequently, the promotion of colon carcinogenesis.

We also investigated the activation of the mTOR/S6K pathway in colonic epithelial cells to elucidate the involvement of this pathway in cell proliferation in the presence of a HFD. The important role of mTOR in mammalian cells is related to its control of mRNA translation. The targets for mTOR signalling are proteins involved in the control of the translational machinery; these targets include S6K, which regulates the initiation and elongation phases of translation.[40] Regarding upstream control, mTOR is regulated by signalling pathways linked to several oncoproteins or tumour suppressors.[41] Recently, the mTOR/S6K pathway, which is similar to the JNK pathway, has emerged as a critical signalling pathway in the development of insulin resistance.[42] Although the excess nutrient levels associated with an obese state can lead to the activation of mTOR/S6K and the desensitisation of insulin signalling,[42] we found no differences in the colonic epithelial protein levels of phosphorylated mTOR and S6K between the HFD and ND groups in the present study. Furthermore, we previously reported that treatment with rapamycin, an mTOR specific inhibitor, did not reduce the BrdU labelling index or ACF formation in mice fed a HFD.[12] These results indicate that the activation of the mTOR pathway may not play an important role in the increase in colonic epithelial cell proliferation in the presence of a HFD condition (supplementary fig 4).

Adipocytokines, such as free fatty acid and tumour necrosis factor α, have been reported to be potent JNK activators,[13 43] although the molecular pathways involved in their action remain unclear. Several studies have demonstrated that transforming growth factor β (TGFβ) regulates the JNK signalling pathway.[44] TGFβs are known to act as inhibitors of cell proliferation;[45] recently, however, the elevated expression of TGFβs has been suggested to be responsible for oncogenesis.[46] Raju *et al*[47] showed that TGFβs were upregulated in colonic tumours and a select subset of ACF and that dietary fat modulated TGFβ expression in colonic tumours and mucosae. In the present study, however, the expression levels of TGFβs in the colonic epithelium were not elevated in the HFD group (data not shown). Further studies are warranted to investigate whether the JNK signalling pathway via TGFβ might play an important role in colon carcinogenesis and epithelial proliferation under a HFD condition.

This study presents a novel mechanism explaining the involvement of the JNK pathway in the effect of dietary fat intake on colorectal carcinogenesis and epithelial cell proliferation. Importantly, JNK activation was associated with the promotion of colonic cell proliferative activity only in the presence of a HFD, but not in the presence of a ND. At present, an elevated plasma insulin level is thought to possibly enhance the proliferative state through the activation of an insulin signalling pathway downstream of the insulin receptor, such as the PI3K/Akt signalling pathway, in the colon.[10] Currently, however, in vivo mechanistic evidence has confirmed this hypothesis to be insufficient.[48] Our in vivo results suggested that a HFD induced insulin resistance and an abnormally elevated level of JNK activity in the colonic epithelial and that the activated JNK pathway promoted colorectal epithelial cell proliferation. This study therefore demonstrated that JNK activation is one of several possible mechanisms underlying the promotion of colonic cell proliferative activity and the early

stage of colon carcinogenesis in the presence of a HFD. We propose that JNK/c-Jun may be a novel therapeutic target for the prevention of colorectal cancer in obese populations consuming a HFD. In the future, continued investigations are needed to elucidate the JNK activators and the precise role of JNK activation in the process of colorectal carcinogenesis under the HFD conditions.

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Cytoplasmic tethering is involved in synergistic inhibition of p53 by Mdmx and Mdm2

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The *mdm2* and *mdmx* oncogenes play essential yet nonredundant roles in synergistic inactivation of p53. However, the biochemical mechanism by which Mdmx synergizes with Mdm2 to inhibit p53 function remains obscure. Here we demonstrate that, using nonphosphorylatable mutants of Mdmx, the cooperative inhibition of p53 by Mdmx and Mdm2 was associated with cytoplasmic localization of p53, and with an increase of the interaction of Mdmx to p53 and Mdm2 in the cytoplasm. In addition, the Mdmx mutant cooperates with Mdm2 to induce ubiquitination of p53 at C-terminal lysine residues, and the integrity of the C-terminal lysines was partly required for the cooperative inhibition. The expression of subcellular localization mutants of Mdmx revealed that subcellular localization of Mdmx dictated p53 localization, and that cytoplasmic Mdmx tethered p53 in the cytoplasm and efficiently inhibited p53 activity. RNAi-mediated inhibition of Mdmx or introduction of the nuclear localization mutant of Mdmx reduced cytoplasmic retention of p53 in neuroblastoma cells, in which cytoplasmic sequestration of p53 is involved in its inactivation. Our data indicate that cytoplasmic tethering of p53 mediated by Mdmx contributes to p53 inactivation in some types of cancer cells. (*Cancer Sci* 2009; 100: 1291–1299)

The p53 tumor suppressor plays a central role in the prevention of tumorigenesis.[1,2] p53 exerts its function as a tumor suppressor by transcriptionally activating numerous target genes that are involved in inducing a variety of biological outcomes.[3–5] It is increasingly becoming evident that two related oncogenes, *mdm2* and *mdmx*, play central roles in the regulation of p53 activity.[6,7]

Analyses of knockout mice revealed that *mdmx* and *mdm2* suppress p53 in a nonredundant yet synergistic manner.[8] Mdmx and Mdm2 functionally cooperate to inhibit p53[9,10] and these inhibitors form a heterodimer complex through their RING finger domains.[11,12] Thus, Mdmx and Mdm2 play distinct yet cooperative functions for p53 inactivation, presumably via their physical interaction.

Mdm2 inactivates p53 by targeting it for ubiquitin-mediated proteasomal degradation and by promoting its transport from the nucleus into the cytoplasm,[13] and it is likely that inhibition of p53 by Mdm2 is attributed to these functions. Both functions of Mdm2 require the RING finger domain, which possesses E3 ubiquitin ligase activity. Indeed, Mdm2 functions as an E3 ubiquitin ligase for p53[14] although it has been reported that Mdm2 inhibits p53 via other mechanisms.[15]

In contrast to Mdm2, Mdmx lacks robust activity of an E3 ubiquitin ligase for p53[16] although Mdmx possesses a RING finger domain with high sequence similarity to that of Mdm2. In accordance with its inability to ubiquitinate p53 by itself, Mdmx-dependent inhibition of the transcriptional activity of p53 is independent of p53 degradation.[17] Recently, it was reported that Mdmx can complement the E3 activity of C-terminal mutants of Mdm2, suggesting that Mdmx contributes to p53 suppression in a manner distinct from Mdm2.[18,19]

In the present paper, by using nonphosphorylatable Mdmx mutants that are resistant to degradation by Mdm2, we showed that Mdmx and Mdm2 synergistically induce the cytoplasmic retention of p53 in DNA transfection assays. We demonstrated that cytoplasmic Mdmx, but not nuclear Mdmx, efficiently cooperates with Mdm2 to keep p53 in the cytoplasm and inhibits p53 activity. Further, RNAi-mediated inhibition of Mdmx or introduction of nuclear localization mutants of Mdmx reduced cytoplasmic retention of p53 in neuroblastoma cells. It has been documented that p53 is sequestered in the cytoplasm in some types of cancer, such as neuroblastoma, and the sequestration of p53 is likely to contribute to its inactivation. We will discuss how Mdmx and Mdm2 contribute to cytoplasmic sequestration of p53, and its implication during development of some types of cancer.

Materials and Methods

Cell lines. H1299 and U2OS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Antibodies. Anti-Flag antibody (M2) was purchased from Sigma. Anti-p53 monoclonal antibody (DO-1) was purchased from Calbiochem. Anti-HA antibody was purchased from Roche (F Hoffmam-La Roche Ltd, Basel, Switzerland). Anti-myc-tag antibody (9E10), anti-GFP antibody (B-2), anti-topoisomerase I antibody (C-2), anti-γ tubulin antibody (D-10), and anti-Mdmx antibody (D-19) were purchased from Santa Cruz (Santa Cruz, CA).

DNA transfection. In DNA transfection experiments, 2 μg DNA and 4 μL Lipofectamine 2000 reagent (Invitrogen) were introduced per 2.0×10^5 cells. Transfected cells were then incubated for 20 h before harvesting. In experiments in which the subcellular localization mutants of Mdmx were transfected to determine localization of endogenous p53, Lipofectamine LTX (Invitrogen, Carlsbad, CA) was used instead according to the manufacturer's protocol.

Luciferase assay. Twenty hours after transfection, cells were lysed and luciferase activity was measured using the Dual-Luciferase Assay System (Promega, Madison, WI). Mean values (±SD) from three independent experiments were determined. Basal promoter activity expressed in the absence of HA-p53 was measured and subtracted in each experiment.

Immunostaining. Cells were fixed in 4% paraformaldehyde in PBS for 10 min, washed with 1× PBS, and permeabilized in 100% methanol for 30 min at −20°C. The fixed cells were then used for immunostaining as previously described.[20]

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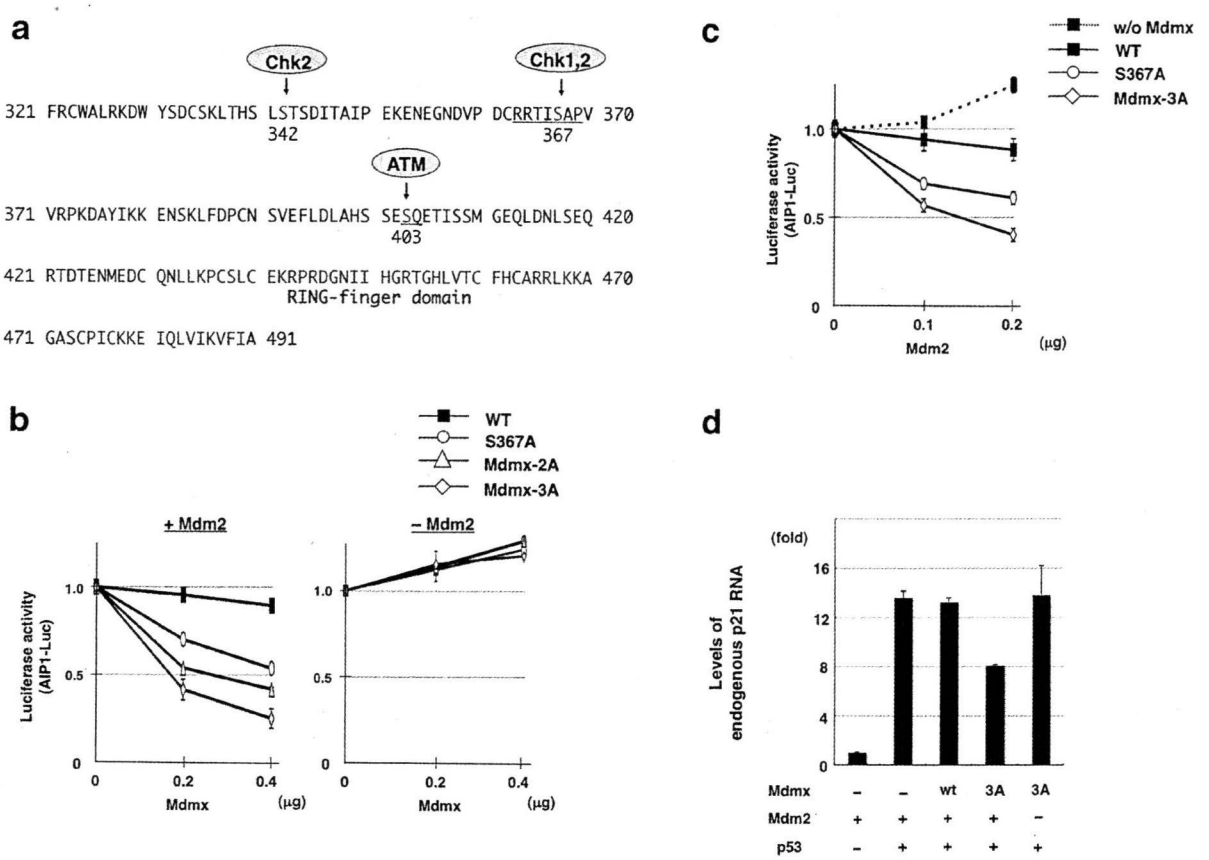

Fig. 1. Non-phosphorylatable Mdmx cooperates with Mdm2 to suppress p53. (a) Schematic representation of the positions of the Mdmx mutations. The serine residues phosphorylated after DNA damage are shown in red. The RING finger domain is shown in blue. (b,c) Inhibition of the transcriptional activity of p53 by the nonphosphorylatable mutants of Mdmx. (b) The indicated amounts of the wild-type Flag-Mdmx or Mdmx mutants were transfected into H1299 cells together with 0.15 μg HA-p53, 0.1 μg AIP-luc, and *Renilla* luciferase in the presence (left panel) or absence (right panel) of 0.2 μg myc-Mdm2. The total amount of transfected DNA was adjusted to 2 μg with pBluescript. Luciferase activity was measured 20 h after transfection. The numbers represent mean values ± standard deviations from experiments carried out in triplicate. The presented values were calculated as follows: value of cells transfected with the indicated amount of Mdmx/value of cells transfected without Mdmx. (c) The indicated amounts of myc-Mdm2 were transfected into H1299 cells together with 0.15 μg HA-p53, AIP-luc, *Renilla* luciferase, in the presence of 0.4 μg control vector, wild-type Flag-Mdmx, or the indicated Mdmx mutant. Luciferase assays were carried out as described in (b). (d) H1299 cells were cotransfected as described in (b). Total RNA prepared from transfected cells was used to measure the levels of endogenous p21 RNA by real-time RT-PCR using Taqman probe (Applied Biosciences, Foster City, CA). Levels of p21 were normalized with those of β-Actin.

shRNA infection. SH-SY5Y cells or IMR-32 cells were infected with lentiviruses as previously described.[21] Cells were infected with the control lentiviruses or the viruses that expressed the specific Mdmx shRNA overnight, incubated for an additional 2 days, and used for western blot analyses or immunostaining.

Additional information on Materials and Methods is provided in the Supporting Information.

Results

Non-phosphorylatable Mdmx effectively cooperates with Mdm2 to suppress p53 activity in H1299. Cellular stresses such as DNA damage cause degradation of Mdmx, via its phosphorylation by damage-induced kinases.[22] Serine 367 (S367) of Mdmx is phosphorylated after DNA damage, and alanine substitution of S367 (S367A), which mimics the nonphosphorylated form, promotes the cooperation between Mdmx and Mdm2 to inhibit p53 activity.[23] In addition to S367, two other serine residues comprise the major phosphorylation sites of Mdmx after DNA damage.[22] One of these sites, serine 403 (S403), is phosphorylated by ATM kinase,[22] whereas its downstream kinases, Chk1 or Chk2, phosphorylate serine 342 (S342) and S367, and facilitate the binding of 14-3-3 to Mdmx[22,24–26] (Fig. 1a). Phosphorylation of each site stimulates the proteasome-mediated degradation of Mdmx via its ubiquitination by Mdm2.[22,23,25]

Assuming that the phosphorylation of S342 and S403, in addition to S367, also compromises p53 suppression by Mdmx, we speculated that additional alanine substitution of S342 and S403 would allow Mdmx to inhibit p53 more effectively. We created the Mdmx mutants with the alanine substitution at S342 (Mdmx-2A) or at S342 plus S403 (Mdmx-3A) in addition to S367A, and introduced each mutant into p53-deficient H1299 cells together with p53 and the p53-responsive luciferase reporter (AIP-luc), in the presence or absence of the transfected Mdm2. Subsequently, the inhibitory effect of each Mdmx mutant on p53 activity was examined (Fig. 1b,c). Low amounts of Mdm2 were transfected so that introduction of Mdm2 alone did not inhibit p53 activity (Fig. 1c). As we reported previously,[23] the S367A mutation augmented the inhibition of p53 activity by Mdmx in the presence of transfected Mdm2 (Fig. 1b,c). The additional alanine substitution at S342 and S403 enhanced the ability of Mdmx to suppress p53 (Fig. 1b,c). In contrast, none of these mutants showed an inhibitory effect on p53 activity in the absence of the transfected Mdm2 (Fig. 1b). We observed similar Mdm2-dependent inhibition

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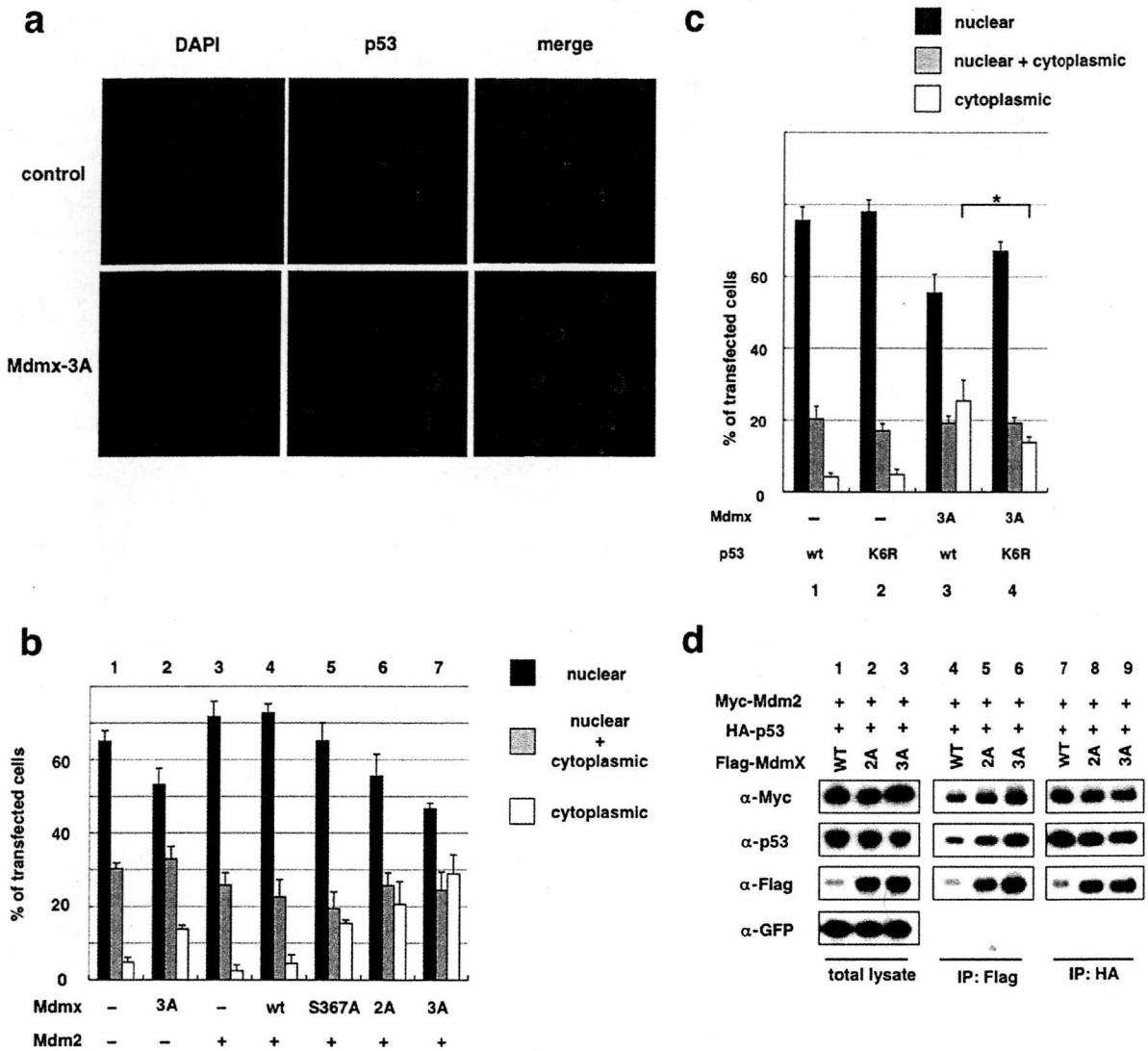

Fig. 2. Non-phosphorylatable Mdmx cooperates with Mdm2 to induce cytoplasmic localization of p53 in H1299. (a) H1299 cells were cotransfected with HA-p53 and myc-Mdm2, in the presence or absence of Mdmx-3A, and used for staining with DAPI and anti-HA antibody. Representative staining of the transfected cells is shown. (b) H1299 cells were cotransfected with the indicated Flag-Mdmx mutants and HA-p53 in the absence (columns 1 and 2) or presence (columns 3–7) of myc-Mdm2, and used for staining with anti-HA antibody. Subcellular localization of p53 of 100 transfected cells was evaluated in triplicate, and the average percentage of cells with the indicated staining pattern of p53 is shown. (c) Wild-type HA-p53 or HA-p53-K6R was transfected into H1299 cells together with myc-Mdm2 in the presence or absence of Flag-Mdmx-3A. Immunostaining analyses were carried out as described in (b). Asterisks indicate statistically significant differences ($P < 0.05$) as given by a one-way ANOVA followed by Tukey post-test. (d) HA-p53, myc-Mdm2, and GFP were transfected into H1299 together with the indicated Flag-tagged Mdmx as described in (b), and lysates prepared from transfected cells were used for immunoprecipitation (IP) with anti-Flag antibody (lanes 4–6) or anti-p53 (DO-1) antibody (lanes 7–9). The total lysates (lanes 1–3) and the immunoprecipitates were analysed by western blot analyses with the indicated antibodies.

of p53 activity by Mdmx-3A on another p53-responsive promoter (Bax-luc) (Supporting Information Fig. S1a). Wild-type Mdmx had an inhibitory effect that was comparable to that of Mdmx-3A in the presence of a chk2 inhibitor (Supporting Information Fig. S1b), suggesting that wild-type Mdmx is capable of inhibiting p53 in the absence of inhibitory phosphorylation.

Cotransfection of Mdm2 with these mutants suppressed the inhibitory effects of p53 on cell growth (Supporting Information Fig. S1c). In accordance with the inhibition of cell growth, Mdmx-3A, but not wild-type Mdmx, inhibits RNA expression of endogenous p21, which is a crucial target of p53 and inhibits cell cycle progression (Fig. 1d). Taken together, these data suggest that non-phosphorylated forms of Mdmx effectively cooperate with Mdm2 to inhibit p53 function.

Non-phosphorylatable Mdmx cooperates with Mdm2 to induce cytoplasmic localization of p53 in H1299. It has been demonstrated that low levels of Mdm2 inhibit p53 by inducing nuclear export.[27] In order to determine whether the nonphosphorylatable mutants of Mdmx cooperate with Mdm2 to inhibit p53 activity by stimulating cytoplasmic localization of p53, we next examined the subcellular localization of p53 after cotransfection of Mdmx, Mdm2, and p53 under the same conditions described in Figure 1(b). Introduction of Mdm2 alone did not significantly affect nuclear localization of p53 (Fig. 2a,b). Although cointroduction of Mdm2 and wild-type Mdmx had only a marginal effect on enhancement of cytoplasmic localization of p53 (Fig. 2b), cointroduction of Mdm2 and Mdmx-3A markedly enhanced a fraction of transfected cells with cytoplasmic p53 staining (Fig. 2a,b). Cytoplasmic

localization of p53 induced by Mdmx-3A alone was much less striking if compared to that induced by Mdm2 and Mdmx-3A (Fig. 2b), indicating that the effect of Mdmx-3A on the subcellular localization is largely dependent on the cointroduced Mdm2. Of note, there was a gradual enhancement of the cytoplasmic localization of p53 as Mdmx harbored an increasing number of alanine mutations at the phosphorylation sites (i.e. Mdmx-wt < S367A < 2A < 3A) (Fig. 2b), indicating that the extent of the stimulation of the cytoplasmic localization by the nonphosphorylatable mutations parallels their inhibitory effect on p53 activity (Fig. 1b). The cooperative effect of Mdmx-3A and Mdm2 to stimulate cytoplasmic localization of p53 was also observed in U2OS cells (Supporting Information Fig. S2a).

Cellular stresses such as DNA damage cause degradation of Mdmx via its phosphorylation by damage-induced kinases.[22,28] Mdmx was highly phosphorylated at S367 in transfected H1299[23] (K. Okamoto, unpublished data). In the presence of a chk2 inhibitor, wild-type Mdmx is capable of inducing cytoplasmic localization of p53 to an extent comparable to that of Mdmx-3A (Supporting Information Fig. S2b), indicating that in the absence of the inhibitory kinase, wild-type Mdmx is capable of inhibiting p53 activity (Supporting Information Fig. S1b) and inducing cytoplasmic localization of p53. These observations suggest that Mdmx phosphorylation may occur during the procedure of DNA transfection, and that the nonphosphorylatable Mdmx mutation facilitates clear observation of the cooperative effects of Mdmx and Mdm2 on p53 inhibition, by negating the inhibitory effects of Mdmx phosphorylation.

Mutation at the C-terminal lysines of p53 partially compromises the inhibitory effects of Mdmx-mediated enhancement of ubiquitination and inhibition of p53. It has been documented that Mdm2 ubiquitinates p53 at the six C-terminal lysines, the integrity of which are required for its nuclear export.[29,30] In addition to ubiquitination, some of these lysines are targeted for other types of modification, including neddylation, acetylation, and methylation.[31,32] Recent publications have indicated that Mdmx rescues the catalytic activity of Mdm2 mutants for ubiquitination and neddylation of p53 *in vivo*.[18,19,33] In order to determine whether Mdmx-3A enhances Mdm2-dependent p53 ubiquitination, we examined whether Mdmx enhances Mdm2-mediated ubiquitination in transfected H1299. Indeed, Mdmx-3A synergized with Mdm2 to induce p53 ubiquitination (Supporting Information Fig. S2c). In order to determine whether cooperative ubiquitination targets the C-terminal lysines of p53 by Mdmx and Mdm2, we created a mutant p53 in which all six lysines at the C-terminal domain were substituted with arginine (p53-K6R). *In vivo* ubiquitination assays confirmed that the K6R mutation eliminates the majority of p53 ubiquitination in transfected H1299 (data not shown). The K6R mutation partially inhibited Mdmx-3A-mediated cytoplasmic localization of p53 (Fig. 2c) and transcriptional inhibition of p53 (Supporting Information Fig. S2d). Thus, modification of the six lysines is partly required for Mdmx-dependent cytoplasmic localization and inactivation of p53, yet there exist other mechanisms by which Mdmx and Mdm2 cooperate to suppress p53 function.

Non-phosphorylatable mutations of Mdmx increase levels of the association of Mdmx to Mdm2 and p53. Next we determined whether the nonphosphorylatable mutations of Mdmx affect the levels of transfected p53, Mdm2, and Mdmx as well as the interaction among them (Fig. 2d). Mdmx-2A or Mdmx-3A expression did not markedly decrease the levels of p53 (Fig. 2d). In contrast, both the Mdmx-2A and Mdmx-3A mutations clearly increased the levels of introduced Mdmx (Fig. 2d). The levels of wild-type Mdmx and the Mdmx mutants were comparable in the presence of a proteasomal inhibitor MG132 (Supporting Information Fig. S2e), suggesting that the nonphosphorylatable mutations render Mdmx less sensitive to Mdm2-dependent proteasomal degradation.[22] In accordance with increased levels of Mdmx-2A and Mdmx-3A, the Mdmx mutations led to increased levels of the association of Mdmx to Mdm2 and p53 (Fig. 2d). These results indicate that the nonphosphorylatable mutations, by protecting Mdmx from Mdm2-dependent degradation, increase levels of the association of Mdmx to Mdm2 and p53.

Mdmx-3A mutation stimulates the association of Mdmx with Mdm2 and p53 predominantly in the cytoplasm. In order to examine whether the Mdmx-3A mutation affects subcellular localization of Mdmx and/or Mdm2 as well as p53, we next carried out immunostaining analyses of transfected Mdm2 and Mdmx. In agreement with a previous report,[10] transfected wild-type Mdmx was predominantly localized to the cytoplasm (Fig. 3a). Both wild-type Mdmx and Mdmx-3A mainly remained in the cytoplasm either in the presence or absence of cotransfected Mdm2 (Fig. 3a). Mdm2 predominantly localized to the nucleus in the absence of transfected Mdmx (Fig. 3b). Cotranfection of wild-type Mdmx mildly enhanced cytoplasmic localization of introduced Mdm2, and the extent of the cytoplasmic localization was markedly augmented by the Mdmx-3A mutation (Fig. 3b). Thus, the Mdmx-3A mutation facilitates cytoplasmic localization of cointroduced Mdm2.

The positive effects of Mdmx-3A mutation on the levels of the Mdmx–Mdm2 complex (Fig. 2d) and on the cytoplasmic localization of Mdm2 (Fig. 3b) suggest that the mutation leads to an increase of the Mdmx–Mdm2 complex in cytoplasm. Therefore, we next examined the extent of their interaction in each subcellular compartment after subcellular fractionation. In agreement with the results of the immunostaining (Fig. 3a,b), both Mdmx-3A and Mdm2 were mainly localized to the cytoplasm, and the Mdmx-3A–Mdm2 complex was predominantly formed in the cytoplasm (Fig. 3c). Cytoplasmic Mdmx-3A clearly colocalized with not only Mdm2 (data not shown) but also with p53 (Fig. 3d). Analyses of the subcellular localization of Mdmx-3A and p53 or of Mdmx-3A and Mdm2 in individual cells revealed that localization of Mdmx-3A in the cytoplasm was clearly associated with cytoplasmic localization of p53 (Supporting Information Fig. S3a) and Mdm2 (Supporting Information Fig. S3b). These data indicate that the Mdmx-3A mutation leads to an increase in the association of Mdmx with p53 and Mdm2 in the cytoplasm.

Cytoplasmic Mdmx is responsible for p53 localization in cytoplasm. In order to determine whether cytoplasmic Mdmx-3A induces localization of p53 to the cytoplasm, we generated Mdmx mutants in which either a peptide that corresponds to a nuclear localization signal of SV40 large T antigen (PKKKRKV) or a nuclear export signal of Rev of human immunodeficiency virus type-1 (LQLPPLERLTL) was connected to Mdmx-3A (NLS-Mdmx-3A or NES-Mdmx-3A). Subsequently, we introduced these Mdmx mutants together with Mdm2 and p53, and evaluated the effect of subcellular localization of Mdmx-3A on Mdm2 and p53. As expected, NLS-Mdmx-3A and NES-Mdmx-3A showed predominant localization to nuclei and cytoplasm respectively (Fig. 4a,b). Clear cytoplasmic localization of Mdm2 (Fig. 4c) and p53 (Fig. 4d) was induced by NES-Mdmx-3A, but not by NLS-Mdmx-3A. Inhibition of transcriptional activity of p53 by Mdmx-3A was enhanced by NES-Mdmx-3A and rather reduced by NLS-Mdmx-3A (Fig. 4e). Thus, cytoplasmic Mdmx-3A tethers p53 to the cytoplasm, whereas it effectively inhibits p53 activity in transfected H1299 cells.

Mdmx in the cytoplasm promotes cytoplasmic retention of endogenous p53. Next we examined whether subcellular localization of Mdmx-3A dictates localization of endogenous p53. Wild-type Mdmx, Mdmx-3A, NES-Mdmx-3A, or NLS-Mdmx-3A was introduced into U2OS cells, in which wild-type p53 is expressed predominantly in nuclei,[34] and we determined whether the mutants affect the subcellular localization of endogenous p53. The Mdmx-3A mutants were expressed at comparable levels (Fig. 4f). As we observed in H1299, NLS-Mdmx-3A and NES-Mdmx-3A predominantly localized to nuclei and cytoplasm respectively (data not shown). Introduction of wild-type Mdmx did not significantly affect nuclear localization of p53. In contrast, introduction of the

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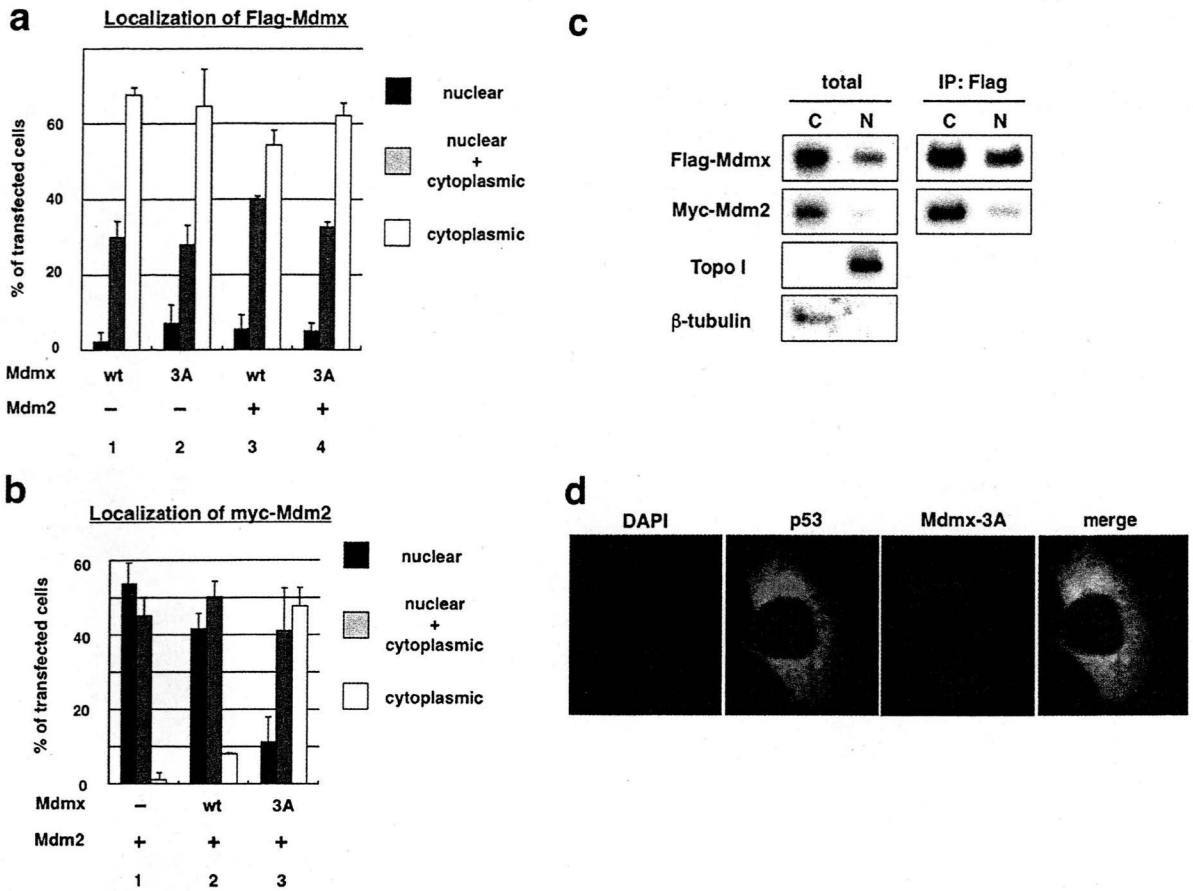

Fig. 3. The Mdmx-3A mutation stimulates the localization of Mdm2 and p53 predominantly to the cytoplasm. (a,b) HA-p53 was transfected into H1299 cells together with the indicated Flag-Mdmx in the presence or absence of myc-Mdm2 as described in Figure 2(b). The transfected cells were sequentially immunostained with (a) anti-Flag (M2) antibody or (b) antimyc antibody, antimouse IgG antibody conjugated with Alexa 595, and anti-HA antibody conjugated with Alexa 488 (Molecular Probe). Subcellular localization of (a) Flag-Mdmx or (b) myc-Mdm2 in cells that express HA-p53 was evaluated as described in Figure 2(b). (c) H1299 cells were transfected with HA-p53 together with Flag-Mdmx-3A and myc-Mdm2. The transfected cells were subjected to subcellular fractionation. The total lysates and the Flag-immunoprecipitates were then used for western blot analyses with the indicated antibodies. Topoisomerase I and γ tubulin are shown as nuclear and cytoplasmic markers respectively. (d) Representative staining of cells that express cytoplasmic p53 and Mdmx.

Mdmx-3A mutants induced localization of p53 to the cytoplasm, and a striking enhancement of cytoplasmic localization of p53 was observed in the presence of NES-Mdmx-3A (Fig. 4f). Taken together, these data indicate that cytoplasmically located Mdmx, presumably by tethering p53, induces localization of endogenous p53 to the cytoplasm.

Both Mdmx and Mdm2 predominantly localize to the cytoplasm of neuroblastoma cells. Inactivation of p53 via its cytoplasmic localization is frequently observed in some types of cancer such as neuroblastoma,[35] and yet the precise mechanism by which p53 is sequestered in cytoplasm remains obscure. It was reported that Mdm2 mediates the cytoplasmic retention of p53 in neuroblastoma.[36,37] In order to examine whether Mdmx as well as Mdm2 is involved in p53 inactivation via cytoplasmic sequestration in neuroblastoma, we analyzed SH-SY5Y and IMR-32 cells that, like most other neuroblastoma cells, harbor wild-type p53 with cytoplasmic localization (Fig. 5a; Supporting Information Fig. S4a). Expression levels of Mdmx in SH-SY5Y were much higher than those in normal human fibroblasts, and even higher than those in MCF-7 (data not shown), breast cancer cells in which the *mdmx* gene is amplified and Mdmx is expressed at high levels.[38] Both Mdmx and Mdm2 predominantly localized to the cytoplasm in SH-SY5Y cells (Supporting Information Fig. S4a). The extent of S367 phosphorylation in SH-5YSY cells was much lower than that in the transfected H1299 cells (Supporting Information Fig. S4c). These observations suggest that Mdmx is expressed, probably in nonphosphorylated forms, at high levels in the cytoplasm in unstressed SH-SY5Y cells.

Nuclear Mdmx inhibits cytoplasmic retention of p53 in SH-SY5Y. In order to determine whether subcellular localization of Mdmx-3A dictates localization of endogenous p53 in neuroblastoma cells as well as in U2OS cells, the effects of subcellular localization of wild-type Mdmx or the Mdmx mutants on endogenous p53 localization were evaluated as described in Figure 4(f). The Mdmx-3A mutants were expressed at comparable levels (Fig. 5b). In accordance with cytoplasmic localization of endogenous Mdmx (Supporting Information Fig. S4a), Mdmx-3A and wild-type Mdmx exclusively localized to the cytoplasm (Fig. 5b). As expected, the majority of NLS-Mdmx-3A localized to nuclei (87%) and NES-Mdmx-3A totally localized to the cytoplasm. Immunostaining of transfected SH-SY5Y cells revealed that the expression of NLS-Mdmx-3A, but not NES-Mdmx-3A, reduced cytoplasmic localization of p53 (Fig. 5b), indicating that nuclear expression of Mdmx-3A inhibits cytoplasmic retention of p53 in SH-SY5Y.

Mdmx is required for inactivation of p53 in neuroblastoma cells. In order to further examine the role of Mdmx in p53 inactivation