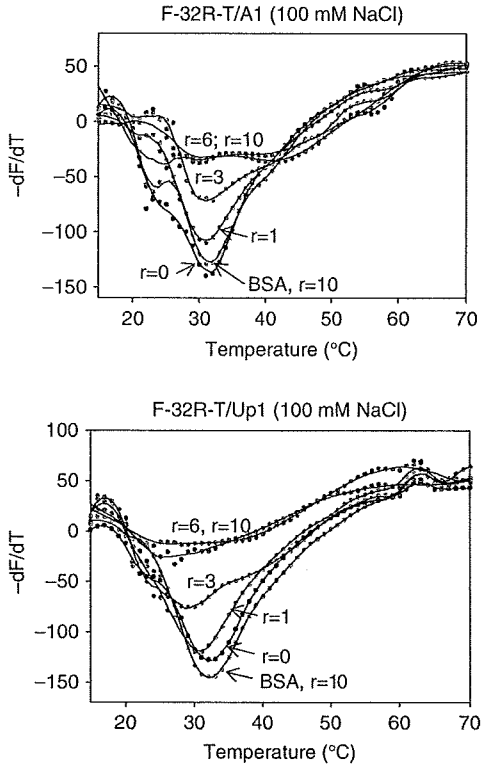

Figure 6. $-dF_{525}/dT$ versus T curves in 50 mM Tris pH 7.4, 100 mM NaCl of F-32R-T in the absence or presence of A1 (**a**), Up1(**b**), BSA at various [protein]/[DNA] ratios.

$P = 0.62$) of Up1 and A1 at $r = 1, 2, 3, 6, 10$. It can be seen that, compared to complementary 32Y, Up1 does not promote a significant increase of the donor emission, a behaviour that might suggest that Up1 has little effect on the quadruplex conformation [for instance the P-value is 0.62 at $r = 0$ (spectrum 2), 0.67 at $r = 10$ (spectrum 8), $\Delta P = 0.05$]. If we assume that $\Delta P = 0.23$ reflects total opening of the G-quadruplex, $\Delta P = 0.05$ suggests that F-32R-T bound to Up1 is partially opened (20%). Alternatively, it is possible that F-32R-T in the DNA–protein complex is completely opened but with the 5′ and 3′ ends brought close to one another so that FRET takes place. To gain insight into this possibility we performed melting experiments. We reasoned that in case the quadruplex is partially unfolded, its T_m would be lowered, whereas in case it is completely opened by A1/Up1, the quadruplex-to-ssDNA transition should be abrogated. Figure 5b shows typical melting curves for quadruplex F-32R-T in 50 mM KCl, obtained with a real-time PCR machine, after the DNA was incubated for 30 min with A1/Up1 ($r = 1, 2, 4, 10$) or BSA ($r = 10$) just before melting. It can be seen that an excess of BSA does not change the T_m of the G-quadruplex, as one expects with an unspecific protein which does not interact with DNA. In contrast, when quadruplex F-32R-T is incubated with A1/Up1, a strong change of the melting curves is observed. The cooperative transition relative to the denaturation of the G-quadruplex (T_m of 48°C) is completely abrogated and replaced with a broad and non-cooperative curve, which reflects the disruption of the DNA–protein complex. The abrogation of the quadruplex-to-ssDNA transition is clearly observed with both F versus T and $-dF/dT$ versus T curves. A similar behaviour has been reported for the UV-melting of the virus type 1 nucleocapsid protein bound to the quadruplex formed by d(GGGTTGGTGTG GTTGG) (35). In 100 mM NaCl, where quadruplex F-32R-T shows a cooperative transition with a T_m of 32°C, we also observed the abrogation of the cooperative transition by A1/Up1 (Figure 6). These data suggest that when F-32R-T is bound to A1/Up1, its secondary structure is completely disrupted and F-32R-T in the DNA–protein complex is in the single-stranded form. The fact that the opening of the quadruplex by Up1 is accompanied by a ΔP which is 20% of that observed with 32Y (0.05 against 0.23) can be rationalized on the basis of the crystal structure between Up1 and the telomeric repeat (T TAGGG)$_2$ (18). In the crystal, the two RRM elements within a Up1 molecule bind to two separate 12mer oligonucleotides, which are antiparallel and separated by an *inter*strand distance of 25–50 Å. Thus, we expect that F-32R-T bound to A1/Up1 adopts a U-shape with the two fluorophores close enough to promote energy transfer (18) (Figures 5c).

It is well known that the cationic porphyrin TMPyP4 stabilizes quadruplex DNA by stacking externally to the G-tetrads and interacting with the loop nucleotides (36). We therefore tested whether TMPyP4 reduces the quadruplex destabilizing action of A1/Up1. Quadruplex F-32R-T (200 nM) was incubated for 12 h in 50 mM KCl, in the presence of 200 and 600 nM TMPyP4. Figure 7a shows that TMPyP4 enhances the T_m of quadruplex F-32R-T from 48°C (curve 1) to 68 (curve 4) and 76°C (curve 5). The mixtures were treated for 30 min with 1 μM A1 ($r = 5$) and then melted. While A1 at $r = 5$ is able to completely disrupt the *KRAS* quadruplex in 50 mM KCl (see Figure 5b), in the presence of the porphyrin it promotes only a partial destabilization of the G-quadruplex: the T_m is reduced from 68°C to 58°C (in the presence of 200 nM TMPyP4, curve 2, Figure 7a) or from 76°C to 63°C (600 nM TMPyP4, curve 3, Figure 7a). So, the stabilizing effect of the porphyrin partially inhibits the capacity of the protein to unfold the G-quadruplex. To exclude the possibility that TMPyP4 directly interacts with and inhibits A1, we performed a control experiment with TMPyP2, the positional isomer of TMPyP4 showing little affinity for quadruplex DNA (Figure 7b). As expected, TMPyP2 neither stabilizes appreciably quadruplex 32R, nor impairs the unfolding of the quadruplex structure by A1. These experiments provide a possible molecular mechanism that explains how TMPyP4 is found to repress the activity of the *KRAS* promoter (12,13).

Krainer and co-workers (23) showed that A1/Up1 binds to the single-stranded and structured human telomeric repeat $(TTAGGG)_{n=2,4}$. They suggest that A1 is likely to function as an auxiliary factor of the telomerase holoenzyme and propose that the protein stimulates telomerase elongation through unwinding of the G-quadruplex structures formed during the

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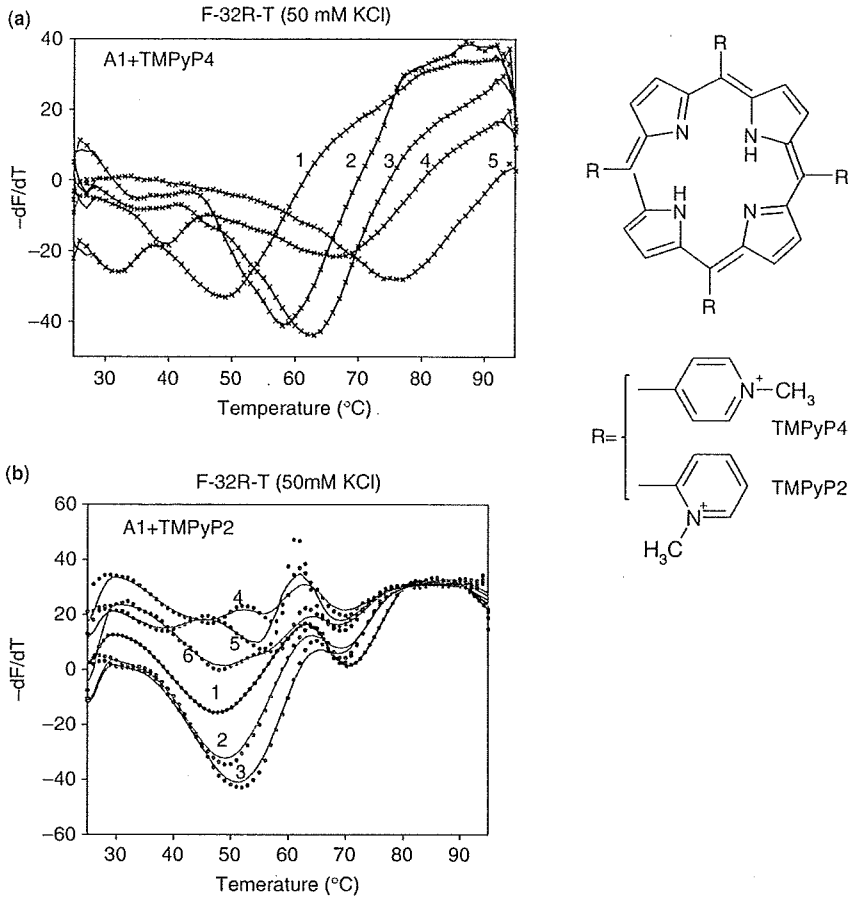

Figure 7. (a) $-dF_{525}/dT$ versus T melting curves of 200 nM F-32R-T in 50 mM Tris pH 7.4, 50 mM KCl (curve 1), in the presence of 200 nM (curve 4) or 600 nM (curve 5) porphyrin TMPyP4. Curves 2 and 3 show the melting curves obtained by F-32R-T treated with 200 nM TMPyP4 + A1 ($r = 5$) or 600 nM TMPyP4 + A1 ($r = 5$), respectively; (b) dF_{525}/dT versus T melting curves of 200 nM F-32R-T in 50 mM Tris pH 7.4, 50 mM KCl (curve 1), in the presence of 200 nM (curve 2) or 600 nM (curve 3) porphyrin TMPyP2. Curves 4, 5 and 6 show the melting curves obtained by F-32R-T treated with A1 ($r = 5$); 200 nM TMPyP2 + A1 ($r = 5$); 600 nM TMPyP2 + A1 ($r = 5$), respectively. Exc 475 nm, Em 525 nm; (c) Structures of TMPyP2 and TMPyP4.

translocation steps. Our study suggests that protein A1, being a component of a multiprotein complex formed within NHE (13), may have a similar function for the *KRAS* promoter: i.e. to resolve the folded quadruplex conformations. The destabilizing activity of A1 should facilitate a quadruplex-to-duplex transformation, that seems to be necessary to activate transcription (12,13). To test this hypothesis, we investigated whether the kinetic of hybridization between quadruplex F-32R-T and the complementary 32Y strand becomes faster in the presence of Up1. When quadruplex F-32R-T in 100 mM KCl ($T_m = 70°C$) is mixed at 25°C with the 32Y strand the quadruplex sequence is transformed into the more stable duplex ($T_m = 78°C$) and the fluorescence of the donor increase as in the duplex it is separated from the acceptor (Figure 8a, from A to C). This assembly process can be monitored by measuring the increase of donor (FAM) fluorescence, ΔF, as a function of time ($\Delta F = F - F_0$, where F_0 is the FAM fluorescence at 525 nm at $t = 0$ and F the fluorescence at time t). The ΔF versus t curve shows an exponential shape that was best-fitted to a double-exponential equation (37). For the slow phase a constant k_{slow} of $1.56 \times 10^{-3} \pm 6 \times 10^{-5}\ s^{-1}$ was obtained (Figure 8b). The hybridization performed in the presence of Up1 occurs with a faster kinetic which was nicely best-fitted to a single-exponential equation: $k = 5.2 \times 10^{-3}\ s^{-1}$. In this case the assembly occurs between F-32R-T bound to Up1 and 32Y, the fluorescence increases from B to C (Figure 8b). The half-life $t_{1/2}$ for the hybridization of 32R to 32Y in the presence of Up1 is 133 s, while in the absence of Up1 is 444 s, i.e. more than 3 times higher. This demonstrates that Up1 is indeed a G4-DNA destabilizing protein that facilitates the quadruplex-to-duplex transformation within NHE.

Finally, by a primer extension assay using a template containing the *KRAS* G-rich element we tested whether A1/Up1 is able to remove the block to Taq polymerase caused by quadruplex formation (12,13,24). Figure 9 shows that when the template is incubated in 100 mM KCl prior to primer extension, Taq polymerase is arrested

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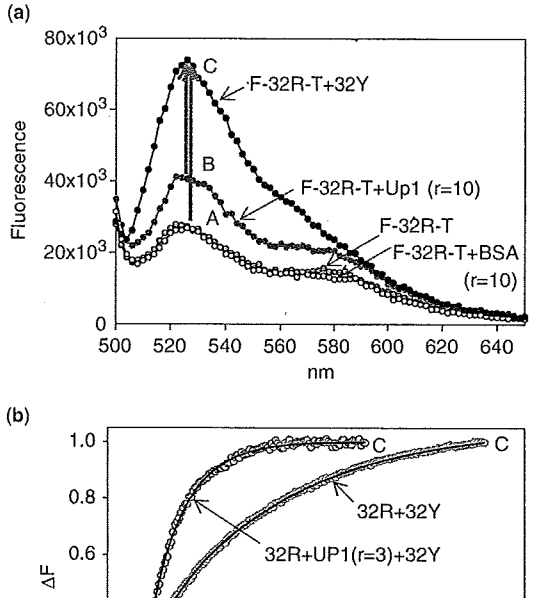

Figure 8. (a) FRET spectra of 200 nM F-32R-T in 50 mM KCl, in the presence of BSA ($r = 10$), Up1 ($r = 10$) and 6-fold complementary 32Y strand. (b) Increase of fluorescence ΔF as a function of time following the addition to 200 nM F-32R-T of 6-fold complementary 32Y in 50 mM Tris pH 7.4, 100 mM KCl. Exc 475 nm; Em 525 nm. The experiment has been conducted in the absence and presence of Up1 ($r = 3$). The solid lines are the best-fits of the experimental points with an exponential equation (SigmaPlot 11, Systat Software Inc).

at the 3′ end of the G-rich tract, as this element folds into a G-quadruplex structure. Contrarily to what we expected, the addition of increasing amounts of protein A1/Up1 strengthened the pause of Taq polymerase. The precise points at which Taq polymerase was arrested were determined by Sanger sequencing reactions and are indicated with arrows in the template sequence. This suggests that A1/Up1 forms with the DNA template a complex which is sufficiently stable to arrest the processivity of Taq polymerase. That's why A1/Up1 enhances the block of Taq polymerase at the G-rich element. However, to corroborate this hypothesis DNA footprinting experiments should be done to demonstrate direct binding of A1/Up1 to the site of arrest. The complex between A1/Up1 and 32R is destabilized when the G-rich strand hybridizes to its complementary sequence to afford a B-DNA duplex for which A1/Up1 has no affinity (see EMSA). Finally, in keeping with the results in Figure 3, the primer-extension assay shows that A1/Up1 binds to the G-rich tract of NHE with a high selectivity, since significant arrests of polymerase at other points of the template are not observed.

DISCUSSION

This work describes the ability of A1, and its derivative Up1, to destabilize the quadruplexes of the *KRAS* promoter and to facilitate their hybridization to the complementary polypyrimidine strand. In accord with pull-down experiments (13), EMSA confirmed that recombinant Up1 and A1 bind to the *KRAS* quadruplex with a high affinity and sequence-specificity, as the binding to other G-quadruplex structures such as *HRAS1*, *HRAS2*, *CMYC*, *VEGF* appeared either weak or inconsistent. Only the quadruplex from the *CKIT* sequence (Table 1) is recognised by Up1. The association of A1 to the *KRAS* promoter is restricted to the polypurine strand, as EMSA shows that A1 does not bind to the complementary polypyrimidine strand, nor to NHE in duplex conformation. Considering that the minimum length for strong binding to Up1 is a stretch of 12 nucleotides (18), 32R, being composed by 32 nucleotides, has potentially two binding sites. In fact, EMSA shows that 32R forms two DNA–protein complexes that are expected to have a stoichiometry of 1:1 and 1:2 (DNA:Up1). This is in accord with the results of Zhang *et al.* (23) showing that Up1 forms with the telomeric repeats $(TTAGGG)_4$ two DNA–protein complexes.

In accord with previous observations (24,25), A1 and Up1 promote a significant reduction of the 260 nm ellipticity, typical of G4-DNA in the parallel conformation. This demonstrates that both proteins are able to unfold the quadruplex structures of the *KRAS* promoter. This conclusion is further supported by FRET-melting experiments showing that the quadruplex formed by F-32R-T is completely disrupted by A1 or Up1. When the *KRAS* G-quadruplex is incubated for 30 min with A1/Up1 before melting, the cooperative transition of the G-quadruplex is replaced by a non-cooperative transition. This suggests that when the *KRAS* sequence is bound to A1/Up1, it is open and in a single-stranded conformation, as shown by the crystal of Up1 with the telomeric repeat (18). In contrast, when a 10-fold excess BSA is added to the G-quadruplex, no change in the quadruplex transition is observed. We interestingly found that protein A1/Up1 facilitates the assembly into a duplex of the two complementary NHE strands. In fact, the half-life of renaturation is reduced from 444 to 133 s in the presence of Up1, 100 mM KCl. This is in accord with earlier studies reporting that A1 promotes a rapid renaturation of nucleic-acid strands, probably by melting the secondary structures that are formed transiently during the annealing process (38). The finding that A1 resolves the *KRAS* quadruplexes has an important biological significance because previous studies supported the notion that the *KRAS* G4-DNA might behave as a transcription suppressor (12,13,28)

The role of A1 *in vivo* has been investigated in the context of the telomere biogenesis (21–23). One possible function of the protein would be to disrupt the G4-DNA structures of the telomere G-repeats, allowing proper elongation by the telomerase (23). The data of our study suggest that A1 could have a similar function in the transcription of *KRAS*. This is in keeping with the fact that A1: (i) binds to the folded G4-DNA conformations of

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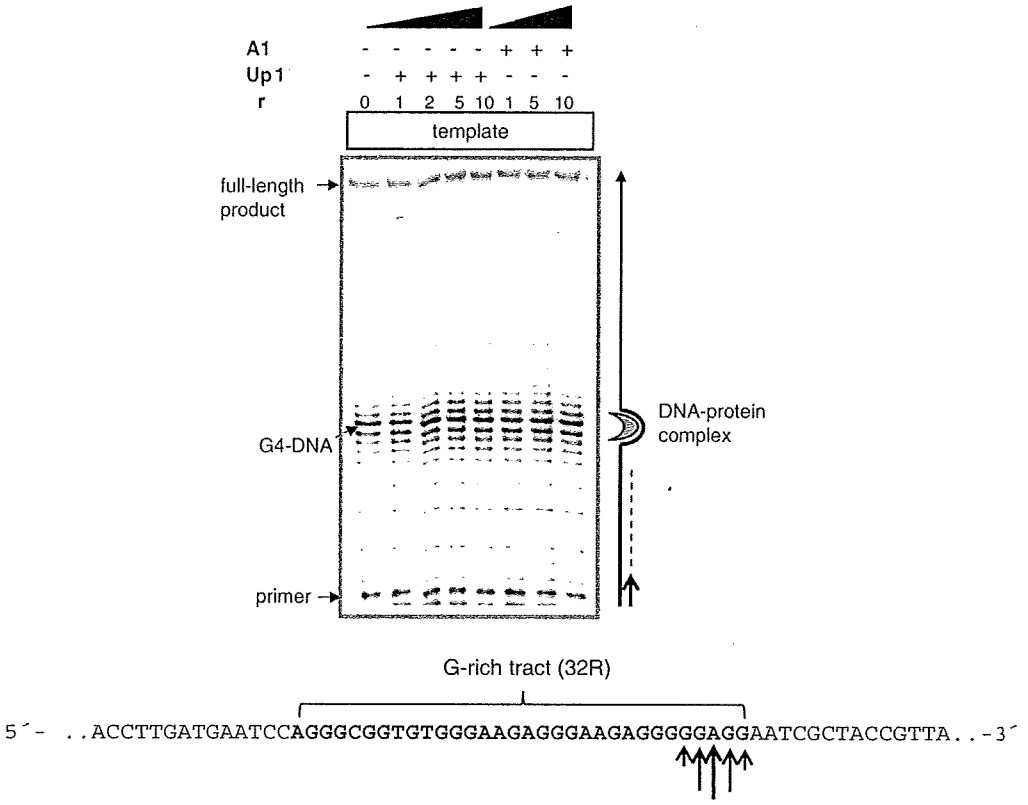

Figure 9. Primer extension assay showing that Taq polymerase pauses at the G-rich element of *KRAS* where the template forms G-quadruplex structures in the presence of KCl. The 87-mer DNA template (100 nM) was mixed with ^{33}P-labelled primer (50 nM) (Table 1) and incubated for 24 h in 140 mM KCl to allow quadruplex formation by the G-rich element. The mixtures were added with increasing amounts of Up1 (lanes 2–5) or A1 (lanes 6–8). r = [protein]/[DNA] as specified, and incubated for 30 min prior to primer extension. Taq polymerase is arrested at the G-rich element due to quadruplex formation. In the presence of Up1 or A1 the polymerase arrest is stronger. The points in which Taq polymerase is arrested, have been identified by standard Sanger sequencing reactions. Primer extension reaction performed at 37°C for 1 h. Reaction products separated in a 12% Urea-TBE denaturing gel.

NHE but not to the complementary pyrimidinic strand or duplex NHE; (ii) disrupts G4-DNA and (iii) facilitates the assembly of the NHE strands into a duplex. A possible model for transcription regulation of *KRAS* is the following. NHE should exist in equilibrium between a folded (quadruplex) and a double-stranded conformation. In the folded form the promoter is locked into a form that might inhibit transcription (12,13). To activate transcription, the folded form of NHE should hybridize to the complementary strand in order to restore the duplex. As the quadruplex-to-duplex transformation is likely to be kinetically slow, the functions of A1 would be of destabilizing the quadruplex and allow the G-rich strand to hybridize to its complementary within a time compatible with a response of the cell to molecular stimuli. There are a number of genes with C+G-rich elements in the region surrounding the transcription start site that seems to be characterized by a transcription regulation mechanism involving G-quadruplex structures (12,13,39–45).

Several proteins from different organisms that interact with quadruplex DNA have been reported (46). They can be classified by function into five major groups: (i) proteins that increase the stability of DNA quadruplexes; (ii) proteins that destabilize quadruplex DNA in a non catalytic way; (iii) proteins that unwind catalytically quadruplex DNA in an ATP-dependent fashion; (iv) proteins that promote the formation of quadruplex DNA; (v) Nucleases that specifically cleave DNA at or adjacently to a quadruplex domain. Like other members of the hnRNP family such as hnRNP A2 (20) and CBF-A (20,47) that destabilise the G-quadruplex formed by the d(CGG)n fragile X expanded sequence, protein A1 acts on DNA in a non-catalytic way, i.e. remaining bound to the DNA substrate. Another protein with a similar property is POT-1 which binds to the telomere G-rich DNA overhangs and disrupts G4-DNA structures (48,49). However, contrarily to A1/Up1, POT-1 causes a significant increase of the *P*-value of the quadruplex from the human telomeric repeat, because in the DNA-protein complex the telomeric repeat assumes an extended conformation in which the donor–acceptor are separated by a distance that is too long for FRET (26). Similarly, A1 disrupts the G4-DNA structures assumed by NHE and its remaining bound to the G-rich sequence prevents the DNA from assuming again the folded conformation. We were indeed surprised to observe by primer extension experiments that

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at 37°C, A1/Up1 did not remove the block to Taq polymerase and the protein even enhanced the polymerase arrest. This clearly indicates that after interaction the protein remains bound to the template, and the resulting DNA–protein complex is sufficiently strong to arrest the processivity of the polymerase.

Finally, the proposed transcription regulation model suggests two strategies to downregulate the *KRAS* oncogene and sensitize pancreatic cancer cells, which are refractory to conventional treatment, to chemotherapy. First, use of G4-ligands that lock the promoter in the non-transcriptable form by stabilizing the G-quadruplexes; second, use of decoy molecules specific for the proteins that recognize the G4-DNA structure of NHE (28). Work is in progress in our laboratory along this direction.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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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)

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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,

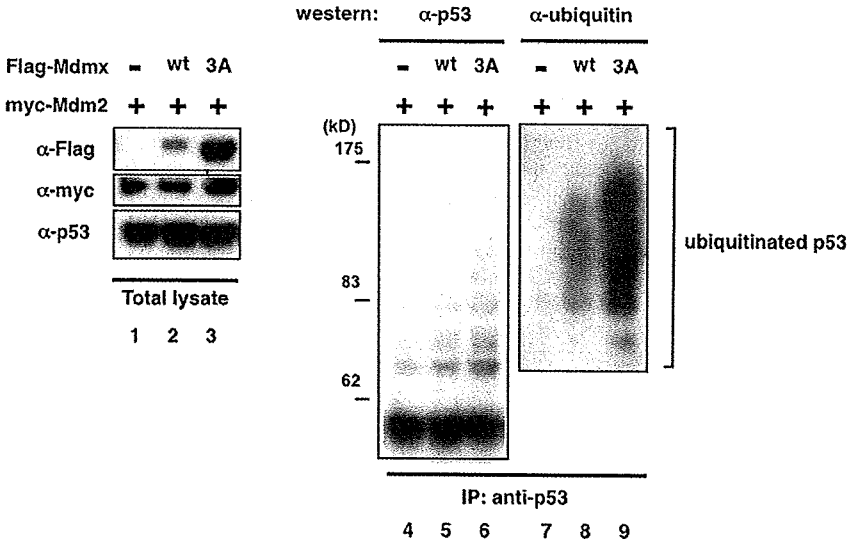

Fig. 1. Mdmx cooperates with Mdm2 to induce p53 ubiquitination. HA-p53 (0.15 mg) and either 0.4 mg of the control vector, wild-type Flag-Mdmx, or the Flag-Mdmx-3A mutant were transfected into H1299 cells in the presence of 0.2 mg of Myc-Mdm2. The total amount of transfected DNA was adjusted to 2 μg with 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

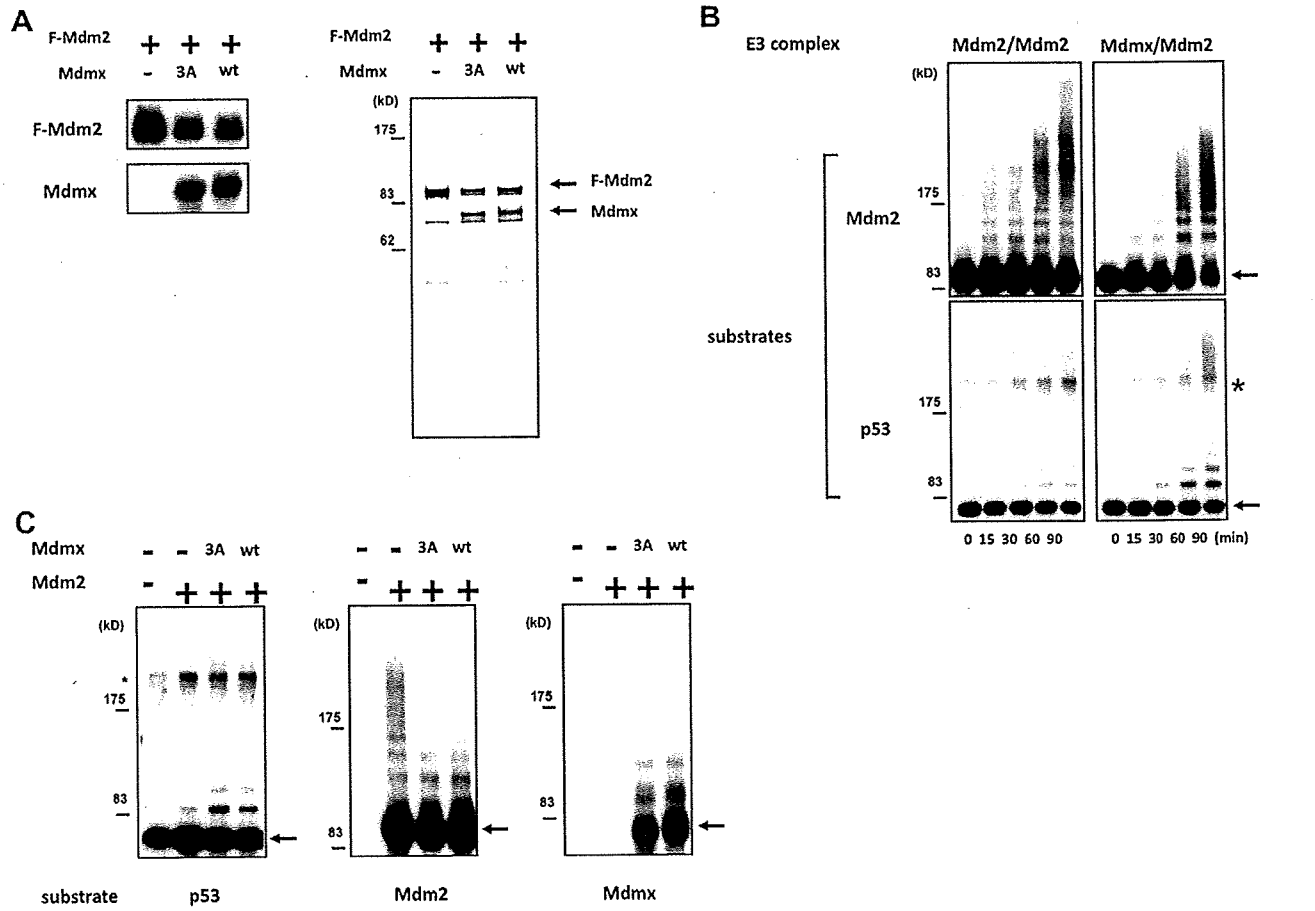

Fig. 2. Association of Mdmx with Mdm2 augments the activity of Mdm2 to ubiquitinate p53 and inhibits auto-ubiquitination of Mdm2 in vitro. (A) Purification of Mdm2 and the Mdm2/Mdmx complex. Flag-tagged Mdm2 was translated alone, or co-translated with Mdmx-3A or wild-type Mdmx in wheat germ lysates, 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.

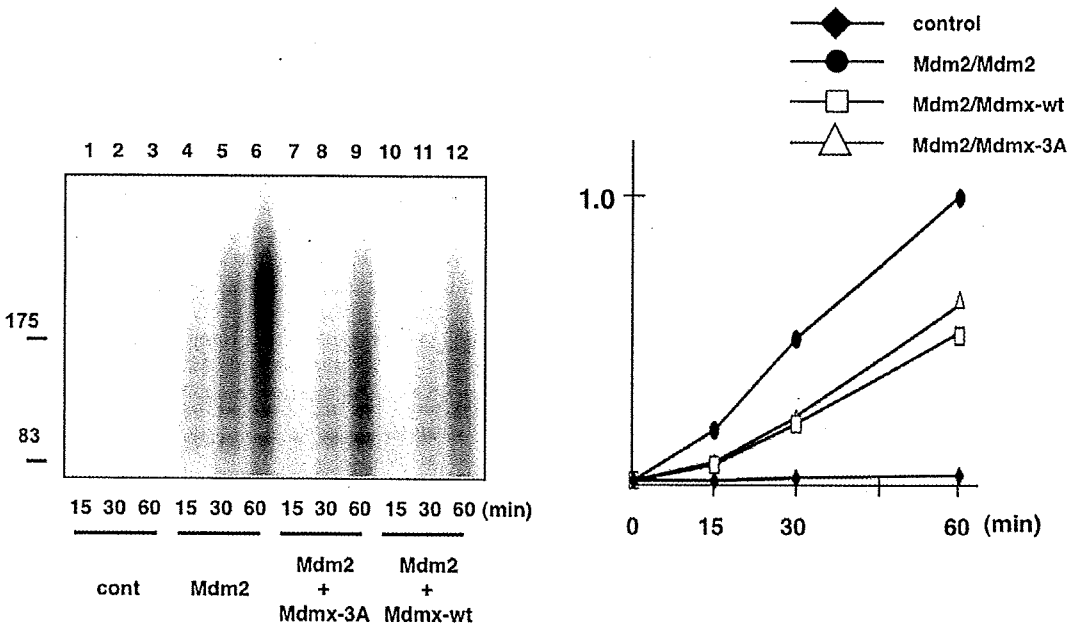

Fig. 3. In vitro ubiquitination reaction was performed as described in Fig. 2C, except that ^{125}I-labeled ubiquitin was included in the reaction. (left panel) Ubiquitinated Mdm2 or Mdm2/Mdmx was separated by 10% SDS–PAGE, and detected by autoradiography. Note that the ladder represents a mixture of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

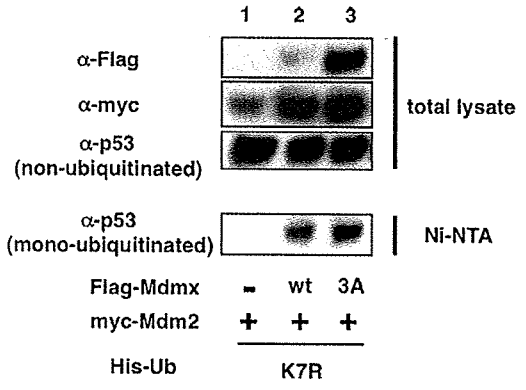

Fig. 4. Mdmx-3A or the control vector was transfected into H1299 cells together with Myc-Mdm2, HA-p53 and the indicated $(His)_6$-tagged ubiquitin K7R mutant. Twenty hours after transfection, cells were lysed with a buffer containing 6 M urea, and normalized lysates that contain equal amounts of non-ubiquitinated 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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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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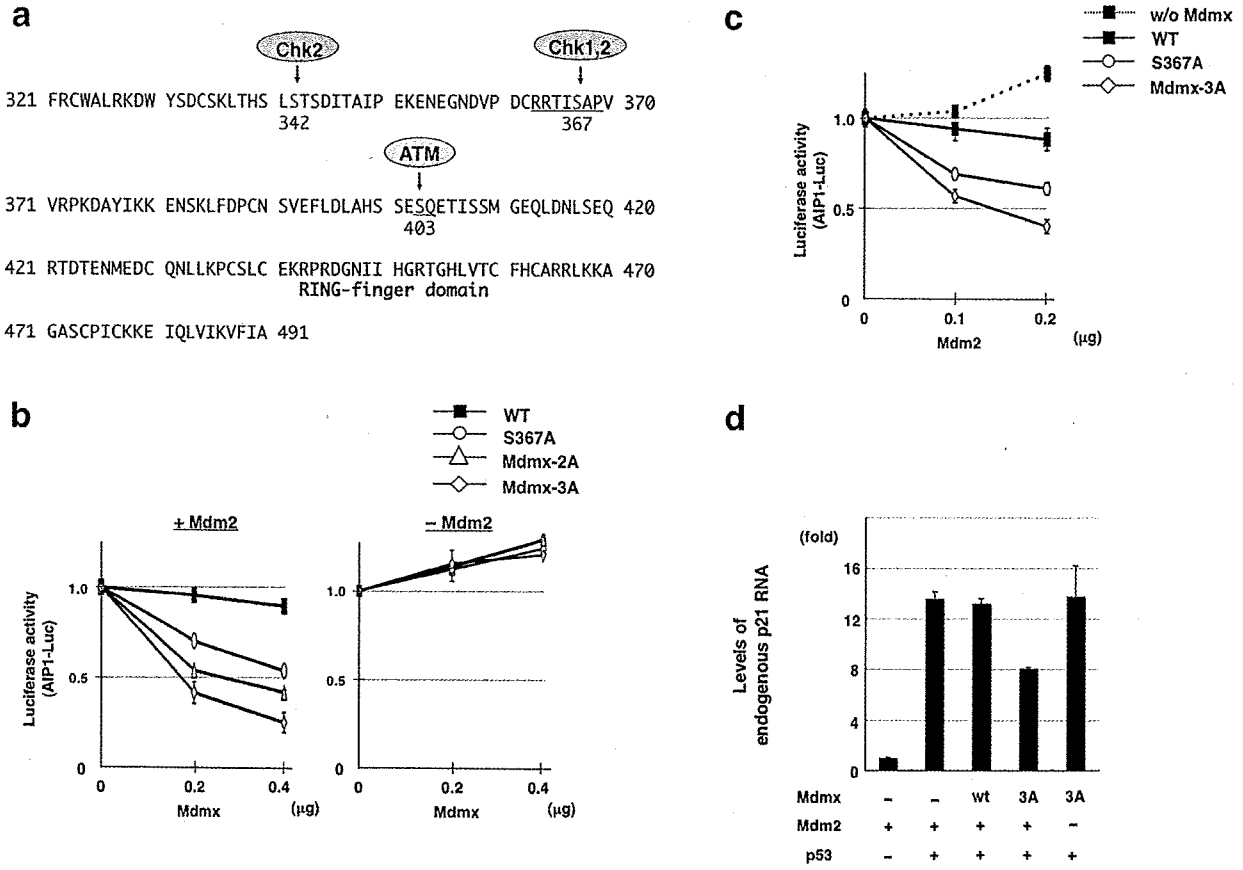

Fig. 1. Non-phosphorylatable Mdmx cooperates with Mdm2 to suppress p53. (a) Schematic representation of the positions of the Mdmx mutations. The serine residues phosphorylated after DNA damage are shown in red. The RING finger domain is shown in blue. (b,c) Inhibition of the transcriptional activity 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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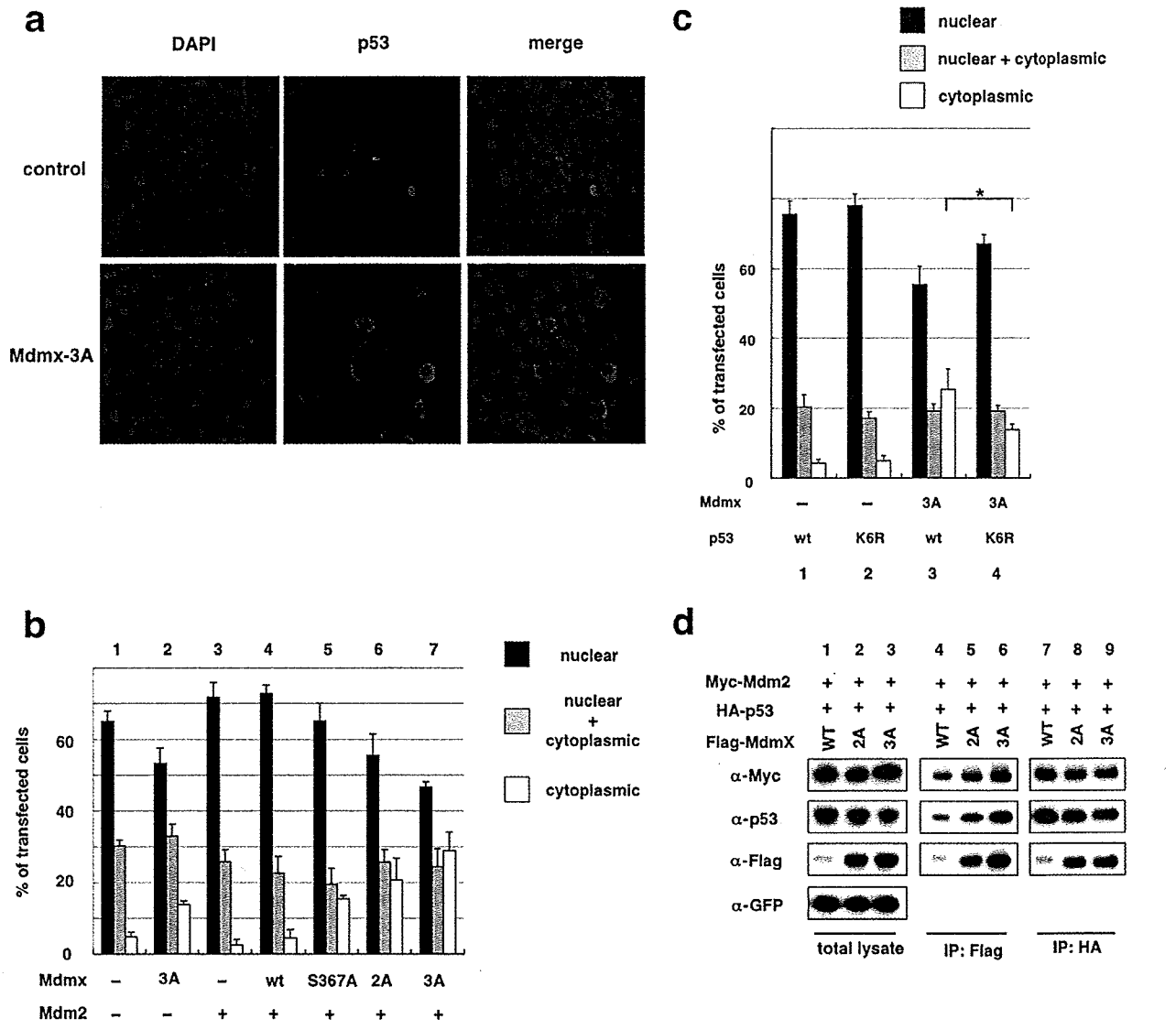

Fig. 2. Non-phosphorylatable Mdmx cooperates with Mdm2 to induce cytoplasmic localization of p53 in H1299. (a) H1299 cells were cotransfected with HA-p53 and myc-Mdm2, in the presence or absence of Mdmx-3A, and used for staining with DAPI and anti-HA antibody. Representative staining of the transfected 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

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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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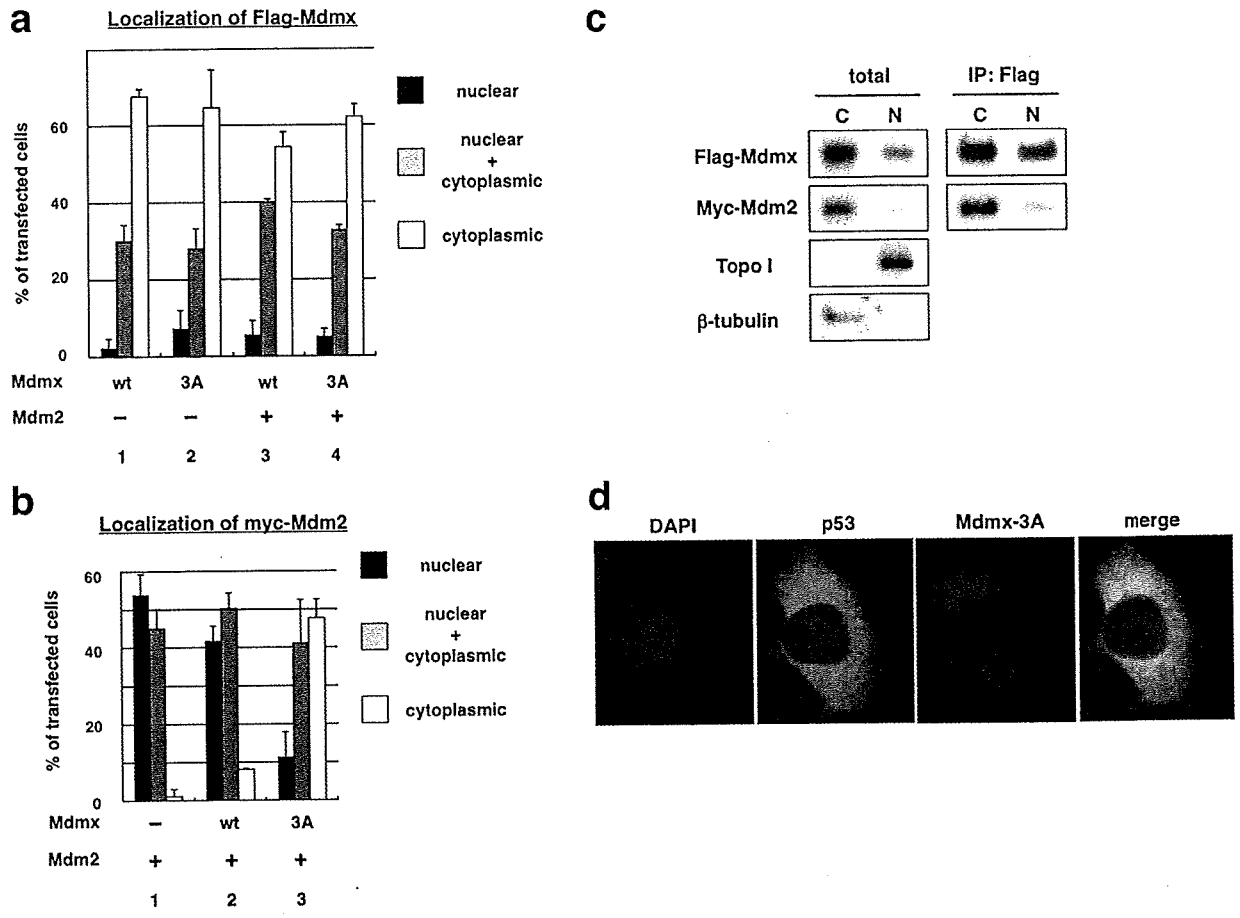

Fig. 3. The Mdmx-3A mutation stimulates the localization of Mdm2 and p53 predominantly to the cytoplasm. (a,b) HA-p53 was transfected into H1299 cells together with the indicated Flag-Mdmx in the presence or absence of myc-Mdm2 as described in Figure 2(b). The transfected cells were sequentially immunostained 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

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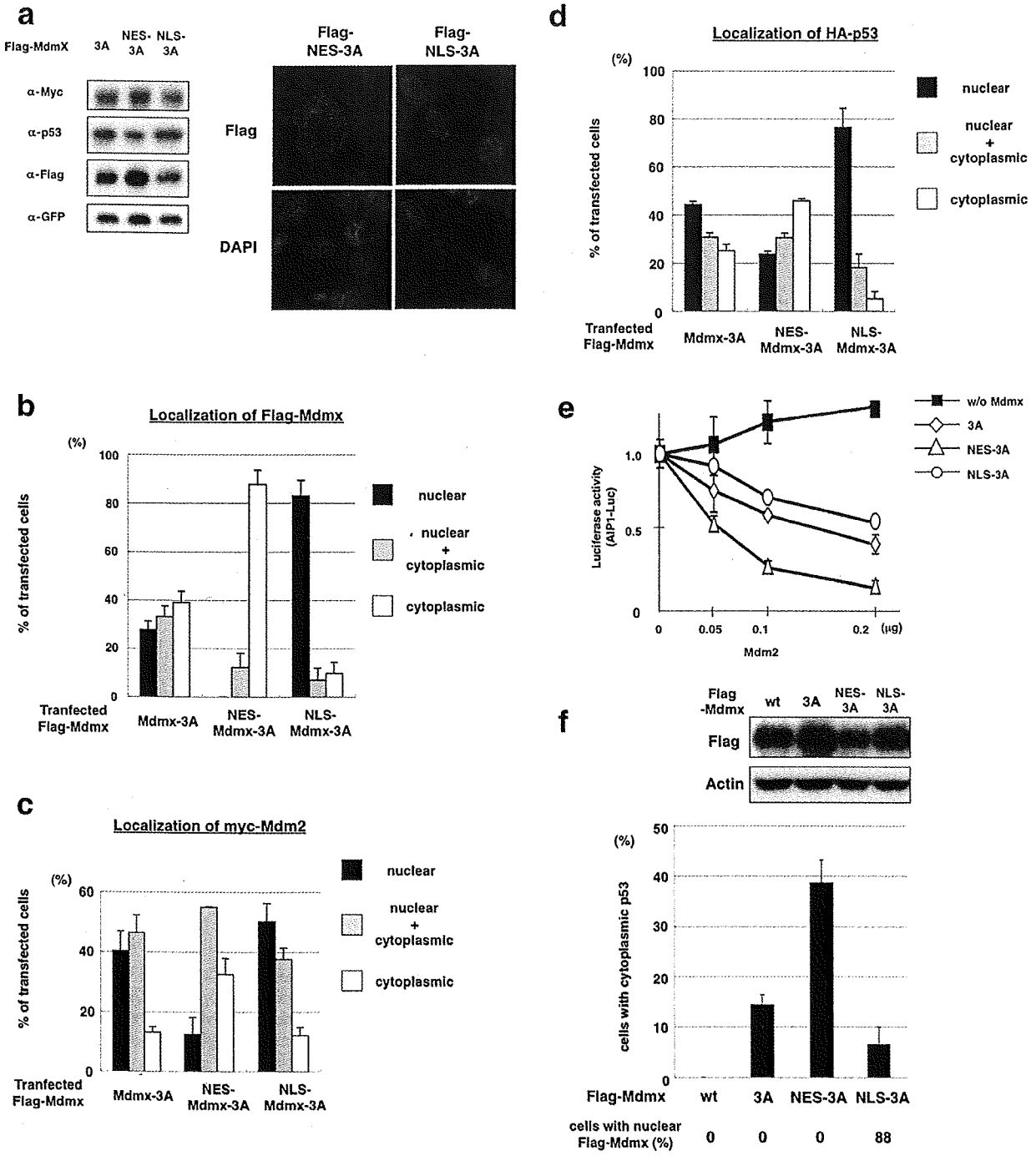

Fig. 4. Cytoplasmic Mdmx tethers p53 and Mdm2 to the cytoplasm and stimulates p53 inhibition. (a) H1299 cells were cotransfected with HA-p53, myc-Mdm2, GFP, and the indicated Flag-tagged Mdmx. Left panel, western blot analyses with the indicated antibodies. Right panel, representative staining with anti-Flag antibody and DAPI. (b–d) H1299 cells were transfected with the indicated Mdmx, together with myc-Mdm2, and immunostained with (b) anti-Flag antibody, (c) anti-Mdm2 antibody, or (d) anti-HA antibody. Subcellular localization of transfected (b) Mdmx, (c) Mdm2, or (d) p53 was represented as described in Figure 2(b). (e) Flag-Mdmx mutant or the control vector was transfected into H1299 cells together with HA-p53, in the presence of the indicated amounts of myc-Mdm2, and luciferase assays were carried out as described in Figure 1(c). (f) U2OS cells were transfected with the indicated Flag-Mdmx. Upper panel, western blot analyses with the anti-Flag or the anti-actin antibodies. Lower panel, cells transfected with the indicated plasmids were immunostained with the anti-Flag and antip53 (CM1) antibodies, and a fraction of the transfected cells with cytoplasmic p53 staining was quantified.

in neuroblastoma cells, we inhibited Mdmx expression by infecting cells with the lentiviruses expressing Mdmx shRNA. Mdmx inhibition by the specific shRNA, while not significantly affecting levels of p53, induced expression of p21, a crucial p53 target (Fig. 5c) and reduced the cytoplasmic localization of p53 (Fig. 5d,e). The positive role of Mdmx in cytoplasmic localization of p53 was confirmed by western blot analyses of nuclear and cytoplasmic lysates prepared from the infected cells. Depletion of Mdmx

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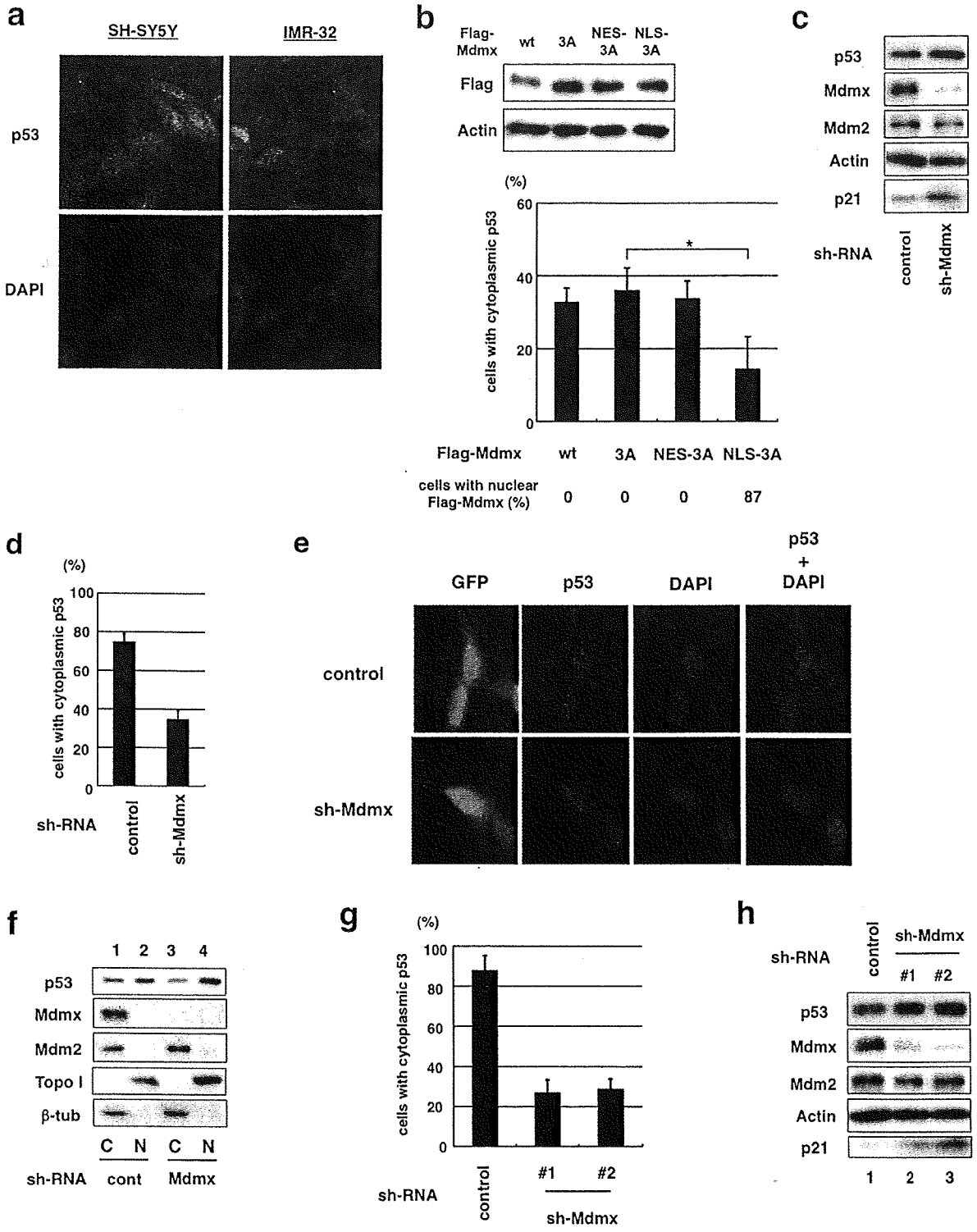

Fig. 5. Mdmx is required for cytoplasmic retention of p53 in neuroblastoma cells. (a) Cytoplasmic retention of p53 in neuroblastoma. SH-SY5Y or IMR-32 cells were stained with antip53 antibody (CM1) and DAPI. (b) SH-SY5Y cells were transfected with the indicated Flag-tagged Mdmx. Western blot analyses and quantification of a fraction of cells with cytoplasmic p53 were carried out as described in Figure 4(f). (c–f) SH-SY5Y cells were infected with the control lentivirus or the viruses expressing Mdmx shRNA. (c) Lysates prepared from the infected cells were used for western blot analyses with the indicated antibodies. (d) The infected cells were immunostained with antip53 polyclonal antibody (CM1) and DAPI. The average percentage of the infected cells with cytoplasmic staining of p53 was presented after evaluating subcellular localization of p53 of 100 cells in triplicate. (e) Representative pictures of staining of cells that were infected with the control lentiviruses or the viruses that express Mdmx shRNA. Note that the viruses express GFP, and infection efficiency is ~100% judging from GFP expression. (f) Subcellular fractionation and western blot analyses were carried out with the indicated antibodies. (g,h) IMR-32 cells were infected with the control lentiviruses or the viruses that express Mdmx shRNA, and (g) western blot analyses or (h) the quantification of subcellular distribution of p53 was carried out as described in (c) and (d) respectively.

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decreased p53 levels in the cytoplasm and increased those in nuclei, while the depletion did not significantly affect cytoplasmic localization of Mdm2 (Fig. 5f).

Similarly, inhibition of Mdmx by the specific shRNA led to induction of p21 expression and inhibition of cytoplasmic localization of p53 in IMR-32, another neuroblastoma cell line (Fig. 5g,h; Supporting Information Fig. S4c). Thus, Mdmx contributes to cytoplasmic retention of p53 in neuroblastoma cells.

Discussion

Genetic evidence indicates that *mdmx* is a crucial inhibitor of p53 and that *mdmx* and *mdm2* cooperatively function to inhibit p53. However, the mechanical basis of the cooperation of the oncogenes is not clearly established. In an attempt to recapitulate synergistic inhibition of p53 by Mdmx and Mdm2, we took advantage of our observation that the nonphosphorylatable mutations confer Mdmx resistance against Mdm2-mediated degradation. We demonstrated that nonphosphorylatable mutations of Mdmx markedly enhance the ability of Mdmx to cooperate with Mdm2 for inhibition of p53, suggesting that the stress-induced phosphorylation of Mdmx is important for its ability to suppress p53. The importance of the Mdmx phosphorylation was further supported by the functionality of wild-type Mdmx on p53 suppression in the presence of a chk2 inhibitor (Supporting Information Figs S1b and 2b).

Through the analyses of the function of the Mdmx mutants, we found that the nonphosphorylatable mutant of Mdmx effectively cooperates with Mdm2 to induce p53 ubiquitination. The ability of the nonphosphorylatable mutations of Mdmx to inhibit p53 activity was associated with enhanced cytoplasmic retention of p53 and with increased levels of the interaction of Mdmx to p53 and Mdm2 in cytoplasm. A causal role of cytoplasmic Mdmx to induce localization of p53 in the cytoplasm was demonstrated using the Mdmx mutants that harbor autonomous subcellular localization signals.

p53 is sequestered in the cytoplasm in some types of cancer, and it is assumed that the sequestration of p53 contributes to p53 inactivation.[35,39] Mdm2 is essential for inhibition and cytoplasmic sequestration of p53 in neuroblastoma cells,[36,37] and the cooperative function of Mdmx and Mdm2 to induce p53 retention in the cytoplasm may contribute to its inactivation in some of cancer cells. We found that, in addition to Mdm2, Mdmx is also required for cytoplasmic sequestration of p53 in neuroblastoma cells. Considering that Mdm2 enhances the interaction between p53 and Mdmx in the transfected H1299 cells, Mdmx and Mdm2 may cooperate by stimulating the formation of a complex with p53. Of note, Mdmx stabilizes p53 via a formation of a complex with Mdm2,[16] and formation of such a stable complex may account for cytoplasmic sequestration of p53.

In addition to the cytoplasmic tethering via physical interaction, regulation of post-translational modification of the C-terminal of p53 is likely to contribute to the cooperative inhibition of p53 by Mdm2 and Mdmx, because mutations in the six C-terminal lysines, which are targets for the regulatory modification, partly abolished the cooperative inhibition of p53 (Supporting Information Fig. S2d). Mdm2 promotes cytoplasmic translocation of p53 via its ubiquitination at the same lysine residues,[27,40] and accumulating data[9,18,19] as well as ours (Supporting Information Fig. 2c) indicate that Mdmx promotes Mdm2-dependent p53 ubiquitination. Hence, it is likely that enhancement of Mdm2-dependent ubiquitination of p53 by Mdmx also contributes to the cooperative inhibition of p53 activity by these oncoproteins. In fact, the cytoplasmic retention of p53 in neuroblastoma is in part attributed to multimono-ubiquitination of p53 due to defective function of HAUSP, a de-ubiquitinating enzyme for p53 and Mdmx and Mdm2.[34,41,42] However, we did not observe a significant change in the pattern of p53 laddering, which presumably represents ubiquitinated p53, in neuroblastoma cells after knock down of Mdmx (data not shown). The two mechanisms that mediate cytoplasmic localization of p53, namely cytoplasmic tethering and ubiquitin-dependent translocation, are not mutually exclusive, and presumably contribute to cytoplasmic retention of p53 by Mdmx.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supporting Information Materials and Methods

Fig. S1. Non-phosphorylatable Mdmx cooperates with Mdm2 to suppress p53.

Fig. S2. Non-phosphorylatable Mdmx cooperates with Mdm2 to induce cytoplasmic localization of p53 in H1299.

Fig. S3. The Mdmx-3A mutation stimulates the localization of Mdm2 and p53 predominantly to the cytoplasm.

Fig. S4. Mdmx is required for cytoplasmic retention of p53 in neuroblastoma cells.

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