The tumour suppressor RASSF1A promotes MDM2 self-ubiquitination by disrupting the MDM2–DAXX–HAUSP complex

Min Sup Song¹, Su Jung Song¹,
So Yeon Kim, Hyun Jung Oh
and Dae-Sik Lim*

National Research Laboratory for Genomic Stability, Department
of Biological Sciences, Korea Advanced Institute of Science
and Technology, Daejeon, Republic of Korea

The tumour suppressor p53, which accumulates in response to DNA damage and induces cell-cycle arrest and apoptosis, has a key function in the maintenance of genome integrity. Under normal conditions, the antiproliferative effects of p53 are inhibited by MDM2, a ubiquitin ligase that promotes p53 ubiquitination and degradation. MDM2 is also self-ubiquitinated and degraded. Here, we show that the tumour suppressor RASSF1A regulates G₁–S cell-cycle progression in a p53-dependent manner by promoting MDM2 self-ubiquitination and preventing p53 degradation. Importantly, RASSF1A associates with MDM2 and death-domain-associated protein (DAXX) in the nucleus, thereby disrupting the interactions between MDM2, DAXX, and the deubiquitinase, HAUSP, and enhancing the self-ubiquitin ligase activity of MDM2. Moreover, RASSF1A partially contributes to p53-dependent checkpoint activation at early time points in response to DNA damage. These findings reveal a new and important function for RASSF1A in regulating the p53–MDM2 pathway.

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Introduction

The tumour suppressor p53 has an important function in protecting the integrity of the genome. In response to various types of stress, p53 is stabilized and activated, resulting in cell-cycle arrest, apoptosis, or senescence (Vogelstein *et al*, 2000; Michael and Oren, 2003). The stability of p53 is mainly regulated by the RING domain-containing E3 ubiquitin ligase, MDM2 (Haupt *et al*, 1997; Kubbutat *et al*, 1997), and in normal unstressed cells p53 has a short half-life. The *MDM2* gene is also transactivated by p53; thus, MDM2 and p53

together form a negative feedback loop (Prives, 1998). Several pathways leading to inhibition of MDM2 action have been identified, including the phosphorylation of MDM2 by DNA-damage-induced kinases (Khosravi *et al*, 1999; Maya *et al*, 2001; Stommel and Wahl, 2004) and the interaction of MDM2 with other proteins (Brooks and Gu, 2006; Toledo and Wahl, 2006), including ARF, which disrupts MDM2 regulation of p53 by inhibiting MDM2 ubiquitin ligase activity (Kamijo *et al*, 1998; Pomerantz *et al*, 1998; Zhang *et al*, 1998; Sherr and Weber, 2000). Ribosomal proteins, such as L11, L23, and L5, also inhibit MDM2 activity and disrupt p53 regulation (Lohrum *et al*, 2003; Zhang *et al*, 2003; Dai and Lu, 2004; Dai *et al*, 2004; Jin *et al*, 2004). Protein YY1 and the oncoprotein gankyrin have also been suggested as potentiating cofactors for MDM2, facilitating MDM2–p53 interaction and p53 ubiquitination (Sui *et al*, 2004; Higashitsuji *et al*, 2005). Interestingly, MDM2 is an unstable protein that is ubiquitinated in an autocatalytic manner (Fang *et al*, 2000; Stommel and Wahl, 2004). MDM2 distinguishes between self-ubiquitination and target p53 ubiquitination, and thus mechanisms that regulate these respective activities are important for regulation of p53 activity.

Recently, it has been demonstrated that death-domain-associated protein (DAXX), together with the deubiquitinating enzyme, HAUSP, can prevent MDM2 self-ubiquitination and enhance the activity of MDM2 towards p53, thereby promoting p53 degradation (Tang *et al*, 2006). DAXX is also an apoptosis regulatory protein that links the death receptor Fas to c-Jun NH₂-terminal kinase (JNK) (Yang *et al*, 1997). However, *Daxx*-null mice die at early embryonic stages because of extensive cell death, indicating an additional function for Daxx in regulating cell survival during development (Michaelson *et al*, 1999). HAUSP (also known as USP7) interacts with p53, leading to p53 deubiquitination and stabilization (Li *et al*, 2002). However, the ablation of HAUSP expression results in an increase in p53 and p53-dependent G₁ arrest, as well as a marked reduction in MDM2, indicating that HAUSP also has a key function in regulating MDM2 stability (Cummins *et al*, 2004; Li *et al*, 2004; Meulmeester *et al*, 2005). Furthermore, the MDM2-related protein, MDMX, also regulates MDM2 activity, with implications for MDM2-dependent regulation of p53 (Parant *et al*, 2001). MDMX does not directly mediate the ubiquitination and destruction of p53, but rather appears to be required for MDM2 stability and MDM2-mediated p53 degradation. However, an excess of MDMX stabilizes p53 (Linares *et al*, 2003; Marine and Jochemsen, 2004).

RASSF1A is a tumour suppressor gene that is inactivated in many human cancers (Dammann *et al*, 2000). Mice with homozygous deletions of *RASSF1A* are prone to tumour development (Tommasi *et al*, 2005; van der Weyden *et al*, 2005). Both *RASSF1A* and *RASSF1C* contain C-terminal SARAH protein–protein interaction and RA domains, but

*Corresponding author. Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), 373-1 Guseong-D, Yuseong-G, Daejeon 305-701, Republic of Korea.
Tel.: +82 42 869 2635; Fax: +82 42 869 2610;
E-mail: daesiklim@kaist.ac.kr

¹These authors contributed equally to this work

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differ in their N-terminal sequences. Interestingly, only RASSF1A, which serves multiple functions in a diverse series of critical cellular processes by interacting with other proteins, has been shown to function as a tumour suppressor (Agathangelou *et al*, 2005; Donninger *et al*, 2007). RASSF1A regulates mitotic progression by influencing microtubule dynamics and the activity of APC-Cdc20 (Liu *et al*, 2003; Rong *et al*, 2004; Song *et al*, 2004; Vos *et al*, 2004). RASSF1A also binds the p120 E4F transcription factor, which forms a complex with both the Rb and p53 tumour suppressors (Fenton *et al*, 2004). In addition, RASSF1A induces a G₁ arrest through inhibition of cyclin D1, thereby suggesting a function for RASSF1A in the G₁-S transition (Shivakumar *et al*, 2002). In fact, RASSF1A undergoes ubiquitin-mediated degradation by Skp2 at the G₁-S transition (Song *et al*, 2008). RASSF1A has also been implicated in the regulation of apoptosis, acting to link death receptor signaling to the apoptosis machinery and serving as a part of the mammalian Hippo complex, which is involved in apoptosis and cell proliferation (Baksh *et al*, 2005; Oh *et al*, 2006; Guo *et al*, 2007). Moreover, RASSF1A induces apoptosis by promoting the dissociation of MST2 from Raf and facilitating MST2 interaction with LATS1 after Fas treatment (Matallanas *et al*, 2007). RASSF1A and RASSF1C bind to DAXX, and RASSF1C is released from DAXX after DNA damage, subsequently translocating to cytoplasmic microtubules and activating SAPK/JNK (Kitagawa *et al*, 2006). However, the full spectrum of RASSF1A functions, particularly in DNA-damaged cells, remains unclear. Here, we show that RASSF1A disrupts interactions among MDM2, DAXX, and HAUSP, thus enhancing MDM2 self-ubiquitination and stabilizing p53 during an early stage in response to DNA damage.

Results

Overexpression of RASSF1A induces a p53-dependent delay in G₁-S cell-cycle progression

Several studies have suggested a growth-inhibitory function of RASSF1A at G₁ and during mitosis (Shivakumar *et al*, 2002; Liu *et al*, 2003; Rong *et al*, 2004; Song *et al*, 2004) that is cell line context-dependent. Previously, we reported that ectopic expression of RASSF1A induced a delay in G₁-S cell-cycle progression in cell lines that express wild-type p53 (U2OS, A549, and MCF7) but not in those (SaoS2, H1299, HeLa, and 293T) in which p53 is inactive (Song *et al*, 2008), suggesting that RASSF1A likely modulates G₁-S progression in a p53-dependent manner. To exclude the possibility that this difference in the effect of RASSF1A might reflect something other than p53 status, we first examined parental p53 wild-type HCT116 cells and isogenic HCT116 cells in which both p53 alleles had been subjected to targeted deletion. Exogenous expression of RASSF1A in p53^{+/+} HCT116 cells increased the proportion of cells in G₁ from 29.84 to 43.48%, whereas RASSF1A expression in p53^{-/-} HCT116 cells increased the fraction of cells in G₂-M (Figure 1A). Exogenous expression of RASSF1A with restoration of p53 expression in p53^{-/-} cells also slightly increased the number of cells at G₁. Notably, RASSF1A also induced the accumulation of both p53 and p21 in p53 wild-type cells, but not in p53^{-/-} HCT116 cells (Figure 1B). By contrast, RASSF1C failed to increase the number of cells at G₁ or the abundance or activity of p53 (Supplementary Figure S1A and

B). In addition, the level of 5-bromo-2-deoxyuridine (BrdU) incorporation into DNA was reduced in U2OS cells expressing hemagglutinin (HA)-RASSF1A (Figure 1C). To determine whether the apparent increase in p53 levels induced by RASSF1A was associated with an increase in p53 activity, we examined the effects of RASSF1A on p53 transcriptional activity in U2OS cells transfected with a p53-responsive promoter-reporter construct. RASSF1A, but not RASSF1C, caused a marked increase in both the abundance and activity of wild-type p53 (Figure 1D and Supplementary Figure S1C). Collectively, these results suggest a functional link between RASSF1A and p53 in cell-cycle control.

RASSF1A promotes the stability and activity of p53 by antagonizing MDM2

The role of p53 in cell-cycle control is modulated by mechanisms that affect p53 stability and activity. To determine whether RASSF1A affects the stability of p53 directly or through MDM2, we first determined whether RASSF1A affects MDM2 ubiquitin ligase activity by measuring the stability of MDM2 and p53 in U2OS cells overexpressing RASSF1A. Interestingly, overexpression of RASSF1A decreased MDM2 half-life and increased the levels of ubiquitinated MDM2 (Figure 2A and B). Consequently, the half-life of endogenous p53 was prolonged and ubiquitination of p53 was inhibited by RASSF1A but not RASSF1C (Figure 2A and B and Supplementary Figure S2). We next examined the half-lives of endogenous Mdm2 and p53 in *Rassf1a* wild-type and null mouse embryo fibroblasts (MEFs). Compared with wild-type MEFs, the half-life of p53 was reduced in *Rassf1a*^{-/-} MEFs in association with an increase in the half-life of Mdm2 (Figure 2C). Consistent with this observation, Mdm2 ubiquitination was reduced in *Rassf1a*^{-/-} MEFs, whereas ubiquitination of p53 was increased; these changes were reversed in *Rassf1a*^{-/-} MEFs complemented with RASSF1A (Figure 2D). Moreover, exogenous expression of RASSF1A stabilized ectopically expressed p53 in *Mdm2*^{+/+}p53^{-/-} MEFs compared with vector-only controls (Supplementary Figure S3A, left). By contrast, the stability of ectopic p53 in *Mdm2*^{-/-}p53^{-/-} MEFs was prolonged with or without exogenous expression of RASSF1A (Supplementary Figure S3A, right), indicating that excess RASSF1A likely stabilized p53, even in the presence of Mdm2.

Finally, given that MDM2 targets p53 for degradation and that MDM2 overexpression abolishes p53-mediated G₁ arrest (Zhang *et al*, 1998), we examined whether RASSF1A might functionally antagonize MDM2 activity. Ectopic expression of p53 induced G₁ arrest in U2OS cells, an effect that was prevented by overexpression of MDM2 (Figure 3A, left). However, co-transfection of cells with expression plasmids for RASSF1A, p53, and MDM2 restored the p53-induced G₁ arrest. This effect of RASSF1A was accompanied by restoration of the levels of p53 and p21 to those in cells transfected with only the p53 expression construct, and by a marked reduction in the abundance of MDM2 (Figure 3A, right). By contrast, RASSF1C failed to restore p53-induced G₁ arrest and the levels of p53 and p21 in the presence of MDM2 (Figure 3B). The effect of RASSF1A on p53 abundance was also evidenced by nuclear accumulation of p53 in the presence of ectopically expressed MDM2 (data not shown). We next examined the levels of ubiquitinated p53 and MDM2 in *Mdm2*^{-/-}p53^{-/-} MEFs exogenously expressing p53, MDM2,

Figure 1 Overexpression of RASSF1A delays G₁-S progression in a p53-dependent manner. **(A)** HCT116 cells ($p53^{+/+}$, $p53^{-/-}$ and $p53^{-/-}$ complemented with HA-p53) were transfected with pcDNA-CD4 and HA-RASSF1A. The cell-cycle profile of CD4-positive cells was determined 36 h after transfection. Data are expressed as means $\pm$ standard errors of values from three independent experiments. **(B)** Cells in **(A)** were analysed by immunoblotting for the indicated proteins. Asterisk indicates nonspecific bands. **(C)** U2OS cells incubated for the indicated times after transfection with HA-RASSF1A were exposed during the final 1 h to 10 μ M BrdU and then analysed by flow cytometry to determine the percentage of BrdU-positive cells. Data are expressed as means $\pm$ standard errors of values from three independent experiments. **(D)** U2OS cells were co-transfected with pGL3-p53-responsive reporter and pcDNA3- β -Gal reporter constructs and the indicated combinations of pcDNA3-p53 (wt), pcDNA3-p53-R248W (mt) and HA-RASSF1A. Cell lysates were subjected to immunoblot and luciferase assays. Luciferase activity was normalized to β -galactosidase activity and expressed relative to that in cells transfected with an empty vector. Data are expressed as means $\pm$ standard errors of values from three independent experiments.

and His-ubiquitin, and either RASSF1A or RASSF1C (Figure 3C). Consistent with our previous observations, RASSF1A, but not RASSF1C, increased MDM2 ubiquitination and reduced MDM2-mediated p53 ubiquitination. Collectively, these data indicate that RASSF1A promotes the stability and activity of p53, most likely by antagonizing MDM2.

RASSF1A associates with both MDM2 and DAXX

To investigate the mechanism by which RASSF1A regulates MDM2-mediated p53 degradation, we first tested whether RASSF1A might bind to MDM2. A co-immunoprecipitation assay detected interaction between endogenous RASSF1A and MDM2 in several cell lines even in the absence of p53 (Figure 4A and data not shown). *In vitro* binding experiments also revealed that RASSF1A interacted with MDM2 (Figure 4B and C), but did not interact directly with p53 (Supplementary Figure S3B). Although RASSF1A did not associate with ectopically expressed p53 in $Mdm2^{-/-}p53^{-/-}$ MEFs, RASSF1A formed a complex with p53 in the presence of ectopic MDM2 (Supplementary Figure S3C). Thus, RASSF1A does not interact directly with p53 but is able to form a complex with MDM2 and p53 *in vivo*. To determine whether endogenous RASSF1A colocalizes with MDM2, we used an immunocytological approach. Although RASSF1 is well known to localize to microtubules, RASSF1A localization to the nucleus may be underestimated. Under the conditions used (see Materials and methods), immunostaining clearly showed that endogenous RASSF1A was localized to cytoplasmic microtubules and colocalized with MDM2 in the

nucleus (Supplementary Figure S4A). These results were also confirmed by western blotting and co-immunoprecipitation assays, which detected a significant amount of endogenous RASSF1A in the nuclear fraction and demonstrated an interaction between nuclear RASSF1A and MDM2 (Supplementary Figure S4B).

We next determined which functional domains of MDM2 and RASSF1A were required for their interaction. An *in vitro* binding assay using deletion mutants of both proteins revealed that the N-terminal region (residues 1–119, RASSF1A-N) of RASSF1A (Figure 4B), but not the C-terminal region (residues 120–340, RASSF1A-C), associated with the C-terminal region (residues 375–491) of MDM2, which contains the RING domain. No specific binding was detected with the MDM2 N-terminal region (residues 1–154), containing the p53-interacting domain, or the central region (residues 155–374), containing the acidic domain (Figure 4C). Taken together, these *in vitro* binding data and the failure of RASSF1C to enhance MDM2 ubiquitination activity (Figure 3C) indicate that the N-terminal region of RASSF1A is likely necessary for MDM2 to physically interact with and stabilize p53. As no major difference of RASSF1A level was detected with MDM2 overexpression in $Mdm2^{-/-}p53^{-/-}$ MEFs (Supplementary Figure S5A), RASSF1A may not be a substrate for MDM2. In addition, neither the N-terminal region alone nor the C-terminal part of RASSF1A was sufficient to affect the MDM2 and p53 stability, suggesting that full length of RASSF1A is required for the regulation of the MDM2 and p53 stability (Supplementary Figure S5B and C). Finally, an *in vitro* gel-based assay using ³²P-labelled ubiquitin revealed that recombinant RASSF1A increased the

Figure 2 RASSF1A regulates the half-life and stability of MDM2 and p53. (A) U2OS cells transfected with HA-RASSF1A were incubated for the indicated times with cycloheximide (CHX; 50 μ g/ml) and analysed by immunoblotting. Relative levels of MDM2 and p53 were quantified and normalized to actin. Error bars indicate standard errors. (B) U2OS cells transfected with increasing amounts of HA-RASSF1A were treated with MG132 for 6 h and then cell lysates were immunoprecipitated with anti-MDM2 or anti-p53 antibodies. The resulting immunoprecipitates (IPs) were analysed by immunoblotting (IB) using an anti-ubiquitin antibody. Molecular weights are in kDa. (C) *Rassf1a*^{+/+} or *Rassf1a*^{-/-} MEFs were incubated with cycloheximide (50 μ g/ml) for the indicated times and analysed by immunoblotting. Relative levels of Mdm2 and p53 were quantified and normalized to actin. Error bars indicate standard errors. (D) *Rassf1a*^{+/+} or *Rassf1a*^{-/-} MEFs were treated with MG132 for 6 h and cell lysates were analysed for Mdm2 and p53 ubiquitination by immunoblotting. Molecular weights are in kDa.

self-ubiquitination of wild-type MDM2 but not that of an MDM2 mutant (MDM2(Δ RING)) lacking the RING domain (Figure 4D). These results suggest that RASSF1A regulates the intrinsic self-ubiquitination ligase activity of MDM2 *in vitro*.

It has recently been shown that DAXX binds to MDM2 and is required for MDM2 activity towards p53 (Tang *et al*, 2006). DAXX also associates with RASSF1A and RASSF1C *in vivo* (Kitagawa *et al*, 2006). To determine whether RASSF1A might regulate MDM2 stability by interacting with DAXX, we employed a co-immunoprecipitation assay and found that both exogenous and endogenous RASSF1A interacted with DAXX (Figure 5A and B). This interaction of RASSF1A with Daxx was independent of Mdm2 and p53 (Figure 5B). Immunofluorescence analysis also revealed that RASSF1A

and DAXX colocalized in the nucleus of Triton X-100-permeabilized, fixed cells (Supplementary Figure S4C). Consistent with previous findings (Kitagawa *et al*, 2006), we found that this association was mediated by an interaction between the RASSF1A C-terminal region (residues 120–340, RASSF1A-C) and the DAXX N-terminal region (residues 1–240, DAXX-N) containing the MDM2- and HAUSP-interacting domains (Figure 5C and D). Collectively, these data indicate that RASSF1A associates with both MDM2 and DAXX in the nucleus.

RASSF1A increases MDM2 self-ubiquitination by disrupting MDM2-DAXX-HAUSP interactions

DAXX-N is able to associate with MDM2 or HAUSP as well as with RASSF1A, and the effects of RASSF1A on MDM2 stability are opposite to those of DAXX and HAUSP (Tang *et al*, 2006,

Figure 3 RASSF1A regulates p53 stability by inhibiting MDM2-mediated p53 degradation. (A, B) U2OS cells were co-transfected with pcDNA-CD4 and expression vectors for HA-p53, pCMV-MDM2, HA-RASSF1A, or HA-RASSF1C as indicated. The cell-cycle profile of CD4-positive cells was analysed 36 h after transfection (left) and the cell lysates were analysed by immunoblotting (right). Asterisk indicates nonspecific bands. (C) *Mdm2*^{-/-} *p53*^{-/-} MEFs were co-transfected with MDM2, HA-p53, and His₆-tagged ubiquitin, with or without co-transfection of HA-RASSF1A or HA-RASSF1C. Cells were treated with MG132 for 6 h prior to harvesting. The His₆-purified fraction was analysed for ubiquitinated MDM2 and p53. Molecular weights are in kDa.

and this study). These observations suggest that RASSF1A might affect MDM2-DAXX-HAUSP interactions, thus preventing DAXX and HAUSP from stabilizing MDM2. Co-transfection and co-immunoprecipitation assays showed that RASSF1A interfered with the interaction of MDM2 and the deubiquitinating enzyme, HAUSP, and also inhibited the interactions between MDM2 and DAXX or HAUSP, and DAXX and HAUSP (Figure 6A and Supplementary Figure S6). By contrast, the association of MDM2 with DAXX or HAUSP was increased in RASSF1A-depleted U2OS cells (Figure 6B). Restoration of RASSF1A expression in *Rassf1a*^{-/-} MEFs reversed the observed increases in Mdm2-Hausp and Mdm2-Daxx associations (Figure 6C). Taken together, these data suggest that RASSF1A prevents the association of DAXX-HAUSP with MDM2. Because DAXX also binds to RASSF1C (Figure 5C and Kitagawa *et al*, 2006), we sought

to determine whether RASSF1C was also able to inhibit DAXX-HAUSP-MDM2 interactions. Co-transfection and co-immunoprecipitation assays suggested that RASSF1C did not significantly reduce DAXX-MDM2 interactions *in vivo* (Supplementary Figure S7A). Most importantly, the interaction of the deubiquitinating enzyme HAUSP with either DAXX or MDM2 was not affected by exogenous RASSF1C (Supplementary Figure S7B and C). An *in vitro* competitive binding assay revealed that RASSF1A, but not RASSF1C, interfered with MDM2-DAXX interactions (Figure 6D). These results suggest that the ability of RASSF1A to prevent MDM2-DAXX interactions requires both MDM2 binding to the N terminus of RASSF1A and DAXX binding to the C terminus of RASSF1A.

We next investigated the function of RASSF1A in DAXX- and HAUSP-mediated MDM2 stabilization and

Figure 4 RASSF1A interacts with MDM2 *in vivo* and *in vitro*. (A) Lysates from *p53*^{+/+}, *p53*^{-/-} and *Mdm2*^{-/-}*p53*^{-/-} MEFs were immunoprecipitated with anti-Mdm2 antibodies and the resulting immunoprecipitates were analysed by immunoblotting. Asterisk indicates nonspecific bands. (B, C) The indicated fragments of RASSF1A and MDM2 were produced by *in vitro* translation in the presence of [³⁵S]methionine. Fragments were mixed, immunoprecipitated with anti-Flag (B) or anti-HA (C) antibodies and analysed by SDS-PAGE and autoradiography. (D) Self-ubiquitylation of GST-MDM2 and GST-MDM2(ΔRING) with or without GST-RASSF1A was detected using ³²P ubiquitin or immunoblot analysis (IB) with α-GST. Molecular weights are in kDa.

Figure 5 RASSF1A interacts with DAXX *in vivo* and *in vitro*. (A) 293T cells transfected with Flag-DAXX and HA-RASSF1A expression plasmids were immunoprecipitated (IP) with anti-Flag or immunoglobulin G (IgG) antibodies and the resulting precipitates and cell lysates were subjected to immunoblot analysis. (B) Lysates from *p53*^{+/+}, *p53*^{-/-} and *Mdm2*^{-/-}*p53*^{-/-} MEFs were immunoprecipitated with an anti-Daxx antibody, and the resulting immunoprecipitates (IPs) were analysed by immunoblotting. The indicated fragments of RASSF1A (C) or Daxx (D) were produced by *in vitro* translation in the presence of [³⁵S]methionine. Fragments were mixed, immunoprecipitated (IP) with an anti-Flag antibody and analysed by SDS-PAGE and autoradiography.

deubiquitination. Ectopic expression of DAXX or HAUSP in U2OS cells strongly stabilized endogenous MDM2. However, in cells that expressed RASSF1A in addition to DAXX or

HAUSP, the stabilizing effect of DAXX or HAUSP on MDM2 was significantly reduced (Figure 6E and Supplementary Figure S8A). Consistent with this, exogenous RASSF1A

Figure 6 RASSF1A increases MDM2 self-ubiquitination by disrupting MDM2-DAXX-HAUSP interactions. (A) *Mdm2*^{-/-}*p53*^{-/-} MEFs transfected with MDM2 and Flag-DAXX or Myc-HAUSP and increasing amounts of HA-RASSF1A were treated with MG132 for 6 h. Cell lysates were then immunoprecipitated (IP) with anti-MDM2 or anti-Flag antibodies, and the resulting precipitates were analysed by immunoblotting. (B) U2OS cells transfected with GFP siRNA or RASSF1A siRNA were immunoprecipitated (IP) with an anti-MDM2 antibody and the resulting precipitates were analysed by immunoblotting. (C) *Rassf1a*^{+/+}, *Rassf1a*^{-/-}, and *Rassf1a*^{-/-} MEFs complemented with pcDNA-RASSF1A were immunoprecipitated (IP) with anti-Mdm2 and the resulting precipitates were analysed by immunoblotting. (D) GST-RASSF1A (70, 140, and 210 ng) or GST-RASSF1C (70, 140, and 210 ng) were added to a mixture of *in vitro*-translated (IVT) HA-MDM2 and Flag-DAXX and incubated for 2 h at room temperature, followed by immunoprecipitation (IP) with an anti-Flag antibody and immunoblotting. (E) U2OS cells transfected with Flag-DAXX with or without HA-RASSF1A were incubated for the indicated times with cycloheximide (50 µg/ml) and analysed by immunoblotting. (F) U2OS cells were transfected with a combination of Flag-DAXX and Flag-RASSF1A in the presence of a His₆-tagged ubiquitin expression plasmid. Cells were treated with MG132 for 6 h prior to harvesting. The His₆-purified fractions were analysed for ubiquitinated MDM2.

enhanced MDM2 ubiquitination even in the presence of ectopically expressed DAXX or HAUSP (Figure 6F and Supplementary Figure S8B). Collectively, these data suggest that RASSF1A interferes with the interaction of MDM2 with HAUSP and the adaptor protein DAXX in the regulation of MDM2 stability, which in turn, might enhance the MDM2 self-ubiquitination.

RASSF1A contributes to p53 activation in response to DNA damage

Recent studies have shown that an early step in the accumulation of p53 in response to DNA damage is an associated increase in MDM2 self-ubiquitination, which is probably facilitated by disruption of MDM2-DAXX-HAUSP interactions (Stommel and Wahl, 2004; Tang *et al*, 2006).

Figure 7 RASSF1A disrupts MDM2-DAXX-HAUSP interactions and is required for MDM2 self-ubiquitination upon DNA damage. **(A, B)** U2OS cells were treated with MG132 for 6 h and then with adriamycin (1 μ M) for the indicated times. MDM2-DAXX-HAUSP interactions **(A)** were analysed by immunoprecipitation (IP) with IgG (lane 1) and MDM2 (lane 2–8), followed by immunoblotting for ubiquitinated MDM2 **(B)**. Molecular weights are in kDa. **(C, D)** U2OS cells transfected with GFP siRNA or RASSF1A siRNA **(C)**, or *Rassf1a*^{+/+}, *Rassf1a*^{-/-} and *Rassf1a*^{-/-} MEFs complemented with pcDNA-RASSF1A **(D)**, were treated with adriamycin (1 μ M) for 30 min. Mdm2-Daxx-Hausp interactions were analysed by immunoprecipitation (IP) with the indicated antibodies (left) followed by immunoblotting for ubiquitinated Mdm2 (right). Molecular weights are in kDa.

Consistent with this early involvement of MDM2 self-ubiquitination in DNA damage response, we found that treatment of several cell lines with the DNA-damaging agent adriamycin reduced levels of MDM2 at early time points (Figure 8 and data not shown). The effect of RASSF1A on MDM2 destabilization led us to investigate whether DNA damage might regulate the interactions between RASSF1A, MDM2, DAXX, and HAUSP. Treatment of U2OS cells with adriamycin abolished MDM2-DAXX-HAUSP interactions after 30 min and was accompanied by enhanced MDM2 self-ubiquitination (Figure 7A and B). Interestingly, both RASSF1A-MDM2 and RASSF1A-DAXX interactions appeared to be slightly increased at 60 min after adriamycin treatment. By contrast, the MDM2-DAXX-HAUSP complex remained intact after adriamycin treatment of both RASSF1A-depleted U2OS cells (Figure 7C, left) and *Rassf1a*^{-/-} MEFs (Figure 7D, left). In agreement with this, adriamycin-induced MDM2 ubiquitination was significantly reduced in both RASSF1A-deficient U2OS cells (Figure 7C, right) and *Rassf1a*^{-/-} MEFs (Figure 7D, right). Taken together, these data suggest that

RASSF1A is involved in disrupting the MDM2-DAXX-HAUSP complex and promoting MDM2 self-ubiquitination upon DNA damage.

In response to DNA damage, activated p53 serves the important function of arresting the cell cycle in the G₁ or G₂ phase, a process in which the p53 target, p21, has a major function (Bunz *et al*, 1998). Given that RASSF1A mediates MDM2 destabilization and p53 stabilization, we examined whether RASSF1A might contribute to p53-dependent control of cell growth in response to DNA damage. Ectopic expression of RASSF1A in adriamycin-treated MCF7 cells, which do not express RASSF1A, induced G₁ arrest and promoted accumulation of both p53 and p21 compared with controls, suggesting that RASSF1A contributes to p53 activation at an early stage in response to DNA damage (Figure 8A). Moreover, the transient reduction in Mdm2 abundance at early time points after adriamycin treatment was not apparent in *Rassf1a*^{-/-} MEFs (Figure 8B). By contrast, restoration of RASSF1A expression in *Rassf1a*^{-/-} MEFs, however, recovered the increases in p53 and p21 levels, and

Figure 8 RASSF1A contributes to the efficient activation of p53 at early time points after DNA damage. (A) MCF7 cells transfected with pcDNA-CD4 and HA-RASSF1A (or with the corresponding empty vectors) were treated with adriamycin (1 μ M) for the indicated times. Cell lysates were analysed by immunoblotting for the indicated proteins. (B) *Rassf1a*^{+/+}, *Rassf1a*^{-/-} and *Rassf1a*^{-/-} MEFs complemented with pcDNA-RASSF1A were treated with adriamycin (1 μ M) for the indicated times, followed by immunoblot analysis of cell lysates. (C) *Rassf1a*^{+/+}, *Rassf1a*^{-/-} and *Rassf1a*^{-/-} MEFs complemented with pcDNA-RASSF1A were treated with adriamycin (1 μ M) for 12 h and with BrdU for 30 min prior to harvest. Cell-cycle profiles and fractions of S-phase (BrdU-positive) cells were analysed by flow cytometry. Data are expressed as means $\pm$ s.e.m. of values from three independent experiments. (D) A model depicting the postulated RASSF1A-mediated regulation of MDM2 and p53 stability following DNA damage through disruption of MDM2-DAXX-HAUSP interactions.

the early decrease in Mdm2 stability induced by adriamycin (Figure 8B). *Rassf1a*^{+/+} MEFs displayed reduced numbers of BrdU-labelled cells upon adriamycin treatment, indicating the cell-cycle arrest at G₁-S. By contrast, the numbers of BrdU-labelled cells were not significantly decreased in *Rassf1a*^{-/-} MEFs upon adriamycin treatment (Figure 8C). Restoration of RASSF1A expression in *Rassf1a*^{-/-} MEFs, however, restored the cell-cycle arrest in response to adriamycin. We also found that induction of RASSF1A expression in stably transfected U2OS cells markedly potentiated both γ -irradiation (IR)-induced p53 and p21 accumulation and the IR-induced decrease in MDM2 levels (Supplementary Figure S9). Collectively, these data suggest that RASSF1A contributes to the efficient activation of p53 by promoting MDM2 self-ubiquitination in cell-cycle checkpoint control in response to DNA damage.

Discussion

The results described in this study demonstrate an important function for the tumour suppressor RASSF1A in controlling the MDM2-p53 pathway. RASSF1A regulated G₁-S cell-cycle progression in a p53-dependent manner and had a function in regulating the MDM2-p53 pathway by promoting MDM2

self-ubiquitination and preventing p53 degradation at an early stage in response to DNA damage. Importantly, RASSF1A is associated with both MDM2 and DAXX in the nucleus, thereby disrupting MDM2-DAXX-HAUSP interactions and enhancing the intrinsic ubiquitin ligase activity of MDM2 (Figure 8D).

When cells are under certain types of stress, MDM2 is prevented from inhibiting p53 function, thus ensuring that p53 can execute an appropriate stress response (Vogelstein *et al*, 2000). Here, we show that the tumour suppressor RASSF1A is associated with MDM2 and promoted MDM2 destabilization. There is considerable evidence that MDM2 trans- and self-ubiquitinating activities can be differentially regulated through MDM2 interactions with other proteins. For example, MDM2 self-ubiquitination is regulated by MDM2-selective deubiquitination by the ubiquitin hydrolase, HAUSP (Li *et al*, 2004; Meulmeester *et al*, 2005). Intriguingly, the balance between self- and p53-ubiquitinating activities of MDM2 can be regulated by the combined actions of DAXX and HAUSP (Tang *et al*, 2006). Both DAXX and HAUSP stabilize MDM2 and increase MDM2 ubiquitin ligase activity towards p53, and thereby trigger p53 destabilization. Here, we demonstrate that RASSF1A bound to MDM2 and DAXX

and disrupted this complex (Figure 6A), resulting in increased MDM2 self-ubiquitination and degradation, and p53 stabilization. By contrast, both siRNA-mediated depletion of RASSF1A and genetic ablation of *Rassf1a* prevented dissociation of MDM2 from the complex, even after adriamycin treatment (Figure 7C and D). Thus, it is likely that RASSF1A contributes to the dissociation of MDM2 from DAXX and HAUSP, and increases MDM2 self-ubiquitination, at an early time point after DNA damage.

How does RASSF1A prevent DAXX–HAUSP-mediated MDM2 stabilization and stabilize p53 after DNA damage? First, consistent with a previous report (Tang *et al*, 2006), our study showed that DAXX is a key component of the MDM2–DAXX–HAUSP complex. RASSF1A binds to both the MDM2 C-terminal region, which contains the RING finger domain, and the DAXX N-terminal region, which contains the MDM2- and HAUSP-interacting domain (Figures 4 and 5) (Tang *et al*, 2006). Thus, RASSF1A may competitively bind to the DAXX–MDM2–HAUSP complex and sequester DAXX from MDM2 and vice versa. Second, the regulation of DAXX–MDM2 dissociation by RASSF1A in response to DNA damage may be more complex than suggested by this simple competition model; other factors may be involved. It has been proposed that MDM2 dissociation from DAXX and HAUSP is partially dependent on ATM kinase (Tang *et al*, 2006). In addition, transient destabilization of MDM2 appears to depend on the activities of kinases such as ATM, ATR, or DNA-PK, which are activated upon DNA damage (Khosravi *et al*, 1999; Maya *et al*, 2001; Stommel and Wahl, 2004). However, because the phosphorylation of MDM2 at Ser395 by ATM kinase is not likely to be essential for MDM2 release from DAXX in response to DNA damage (Tang *et al*, 2006), it is tempting to speculate that RASSF1A may function as a cofactor for DNA-damage-induced kinases in the regulation of the MDM2–DAXX–HAUSP complex. Intriguingly, we found that the interaction of MDM2 with RASSF1A was slightly increased upon DNA damage, whereas DNA damage led to a decrease in DAXX–MDM2 interactions (Figure 7A). Although the significance is not clear in this context, it is noteworthy that RASSF1A contains a putative phosphorylation site for ATM kinase (Kim *et al*, 1999). In any case, the functional relationship between RASSF1A and the MDM2–DAXX–HAUSP complex and potential modulation of these interactions, by DNA-damage-induced kinases warrant further investigation. Recently, RASSF1A has been shown to disrupt the Raf1–MST2 kinase complex, thereby enhancing MST2–LATS1 interactions (Matallanas *et al*, 2007). RASSF1A-mediated LATS1 activation promotes p73-directed transcription of the proapoptotic gene *puma*. Because the functions of p53 overlap with those of the p53-related proteins, p63 and p73, it is tempting to speculate that RASSF1A-activated MST2–LATS1 kinase also regulates the p53–MDM2 pathway.

RASSF1C has been shown to associate with DAXX in the nucleus (Kitagawa *et al*, 2006). Upon DNA damage, RASSF1C dissociates from DAXX, translocates to cytoplasmic microtubules, and participates in the activation of SAPK/JNK. However, because RASSF1A remains localized to the nucleus (MS Song and D-S Lim, unpublished data), whereas RASSF1C translocates to the cytoplasm upon DNA damage, RASSF1A and RASSF1C might have distinct functions following genotoxic stress. RASSF1C associated with DAXX *in vivo*, but interestingly failed to disrupt the MDM2–DAXX complex

in vitro and *in vivo* (Figure 6D and Supplementary Figure S7A). Moreover, RASSF1C did not affect cell-cycle distribution or the stability of MDM2 or p53 (Supplementary Figures S1 and S2). It is not entirely clear why RASSF1C fails to dissociate the MDM2–DAXX complex even it is able to associate with DAXX. However, differences in the N-terminal regions of RASSF1C and RASSF1A led us to speculate that the ability of RASSF1A to interfere with MDM2–DAXX interactions might require both MDM2 binding to the N terminus of RASSF1A and DAXX binding to the C terminus of RASSF1A. Moreover, the interaction of the deubiquitination enzyme, HAUSP, with either DAXX or MDM2 was not affected by exogenous RASSF1C (Supplementary Figure S7B and C), suggesting that RASSF1C alone is insufficient to both completely disrupt the MDM2–DAXX–HAUSP complex and regulate MDM2 and p53 stability.

The tumour suppressor ARF binds to MDM2 and preferentially inhibits MDM2 p53-ubiquitinating activity (Zhang *et al*, 1998; Sherr and Weber, 2000). The ARF-binding region of MDM2 is distinct from the RASSF1A-binding region, but the E1A-regulated transcription factor, E4F1, physically interacts with ARF and RASSF1A (Rizos *et al*, 2003; Fenton *et al*, 2004). Thus, it will be of interest to investigate the effects of RASSF1A on the binding of ARF to MDM2. Because it has been proposed that E4F1 is an atypical p53 ubiquitin ligase that regulates p53 effector functions independently of proteolysis (Le Cam *et al*, 2006), the effect of RASSF1A on E4F1-mediated p53 activation also warrants further investigation. The RING finger protein, MDMX, inhibits p53 transcriptional activity by directly interacting with p53 (Shvarts *et al*, 1996; Laurie *et al*, 2006). MDMX also binds to MDM2, but whether the main effect of this binding is to stabilize MDM2, and thus indirectly inhibit p53, or whether the interaction is more important for MDMX activity by MDM2, remains to be determined. Notably, RASSF1A binds directly to the RING domain of MDM2 but not to that of MDMX (MS Song and D-S Lim, unpublished data). Future studies will be required to address the possible role of RASSF1A in regulating MDMX–MDM2 interactions.

In conclusion, our study demonstrates an important function for RASSF1A in the negative regulation of MDM2. Because MDM2 destabilization is required for a proper p53 response at early time points after DNA damage, the tumour suppressor, RASSF1A, may have an important function in the early stages of the cellular response to genotoxic stress.

Materials and methods

Cell culture

Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. HCT116 (p53^{+/+} or p53^{-/-}) cells were kindly provided by B Vogelstein; *Mdm2*^{+/+} p53^{-/-} and *Mdm2*^{-/-} p53^{-/-} MEFs were the kind gifts of G Lozano; *Rassf1a* wild-type and null MEFs were kindly provided by G Pfeifer.

Plasmids and recombinant proteins

RASSF1A, HA-RASSF1A, Flag-RASSF1A, HA-p53, Flag-MDM2, HA-MDM2, HA-DAXX, GST-RASSF1A, GST-RASSF1C, GST-MDM2 and GST-MDM2(ΔRING) were constructed by subcloning the corresponding cDNAs into untagged pcDNA3.1 or pcDNA3.1 (Invitrogen) containing the DNA sequence for an N-terminal HA or Flag tag, or into the pGEX vector. The plasmid pGEX-ubiquitin was kindly provided by AM Weissman; His₆-ubiquitin was a gift from D

Lim; Flag-DAXX was kindly provided by E Choi; Myc-HAUSP was a gift from K Pak.

Antibodies

Guinea-pig polyclonal antibodies to RASSF1A were raised against a GST fusion protein containing an N-terminal fragment of human RASSF1A (residues 1–119). Specific antibodies were affinity purified using the RASSF1A antigen. Also used antibodies were against: HA (Roche); Flag and β -actin (Sigma); ubiquitin and MDM2 (SMP14) (BD Pharmingen); cyclin A, p53 (DO-1), p21, MDM2 (SMP14), GST, HDAC1, β -tubulin, and DAXX (Santa Cruz Biotechnology); Myc (Cell Signaling); p53 (Ab-6) and p53 (Ab-1) (Calbiochem); HAUSP and MDMX (Bethyl).

RNAi

The RASSF1A siRNA sequence was 5'-GACCUCUGUGGCGACUUC-3'. The DNA sequence for this siRNA was inserted into the plasmid pSUPER (Oligoengine) and introduced into cells by transfection using the Effectene reagent (Qiagen).

Immunoblot analysis, immunoprecipitation, *in vitro* binding assays, and immunofluorescence

Immunoblot and immunoprecipitation analyses were performed as described previously (Song *et al*, 2004). For *in vitro* binding assays, *in vitro*-translated 35 S-labelled products were generated using the TNT-Coupled System (Promega). The products were incubated with anti-Flag or anti-HA antibodies in binding buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 0.5% (w/v) Triton X-100, phosphatase inhibitors, and protease inhibitors) for 2 h at 4°C. The beads were washed four times with binding buffer, and bound 35 S-labelled products were detected by SDS-PAGE and autoradiography. For immunofluorescence, U2OS cells were permeabilized with 0.2% (w/v) Triton X-100 for 10 min, fixed with paraformaldehyde, and blocked with 10% (v/v) normal goat serum, followed by consecutive exposure to primary and secondary antibodies with intervening washes. Slides were mounted with a medium containing 4,6-diamidino-2-phenylindole (DAPI) and imaged using a Carl Zeiss LSM510 META microscope (C-Apochromat $\times$ 40/1.2W) equipped with a charge-coupled device and a PMT detector at the side port. The green and red channels used 488 and 543 nm argon laser lines for excitation. Data were processed with LSM 510 META System software.

Ubiquitination assays

To examine the effects of RASSF1A on ubiquitylation of p53 or MDM2 *in vivo*, cells were lysed by incubation for 10 min at 95°C

with two volumes of TBS (10 mM Tris-HCl (pH 7.5), 150 mM NaCl) containing 2% (w/v) SDS. After adding eight volumes of 1% (w/v) Triton X-100 in TBS, the lysates were sonicated for 2 min and then incubated with protein G-agarose beads. The beads were removed by centrifugation, and the lysates were immunoprecipitated with protein G-agarose-coupled anti-p53 (DO-1) or anti-MDM2 (SMP14) antibodies. The beads were first washed with 0.5 M LiCl in TBS and then twice with TBS, and boiled. Proteins were separated by SDS-PAGE and analysed by immunoblotting using an anti-ubiquitin antibody. Alternatively, cells were transfected with a vector for His₆-ubiquitin and His₆-proteins were purified from cell lysates using a Ni²⁺-NTA spin column (Qiagen) under denaturing conditions. For analysis of MDM2 self-ubiquitination *in vitro*, purified GST-MDM2 or GST-MDM2(Δ RING) (250 ng) was incubated for 90 min at 30°C with E1, Ubc5b (Boston Biochem), an ATP-regenerating system (7.5 mM creatine phosphate, 1 mM ATP, 1 mM MgCl₂, 0.1 mM EGTA, rabbit creatine phosphokinase type I (30 U/ml; Sigma)), and 32 P-labelled ubiquitin (2×10^4 c.p.m.) prepared with GST ubiquitin, as described previously (Fang *et al*, 2000). The extent of ubiquitination was then analysed by SDS-PAGE and autoradiography.

Cell-cycle analysis

Analysis of DNA content during cell-cycle progression was performed as described previously (Song *et al*, 2004). For analysis of BrdU incorporation, cells were labelled with 10 μ M BrdU (Sigma) for 1 h, fixed with 70% (v/v) ethanol, incubated with 2 M HCl for 20 min, washed with 0.1 M sodium borate (pH 8.5) and then with PBS containing 0.5% (w/v) BSA. Incorporation of BrdU was detected by immunostaining or flow cytometry using an anti-BrdU antibody (BD Pharmingen) and the percentage of BrdU-labelled nuclei was quantified.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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