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Daxx is reciprocally regulated by Mdm2 and Hausp

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ABSTRACT

Daxx is a multifunctional protein, regulating a wide range of important functions including apoptosis and transcription. However, the way Daxx is regulated is poorly understood. In our previous studies, we have found that Daxx forms a complex with the E3 ubiquitin ligase Mdm2 and the de-ubiquitinase Hausp. In the present work, we show that Daxx is ubiquitinated by Mdm2 in both in vitro and in vivo systems and Mdm2 reduces Daxx expression upon over-expression. We further demonstrate that Hausp critically controls the cellular level of Daxx most likely by inducing Daxx de-ubiquitination. These results reveal Mdm2 and Hausp as important regulators for Daxx functions by controlling Daxx ubiquitination and stability.

Introduction

Daxx is a multifunctional protein, regulating a wide range of important cellular functions such as apoptosis, transcription control and embryonic development. Initially identified as a Fas receptor binding protein in a yeast two hybrid screening, Daxx was implicated in Fas-induced apoptosis [1]. Subsequent studies indicate that Daxx is involved in other scenarios of apoptosis. Under stress stimulations, such as TNF-alpha treatment and oxidative stress, Daxx is up-regulated and engaged in JNK mediated apoptosis by activating ASK [2–4]. The Daxx–JNK pathway represents an important anti-tumor program, disruption of which may contribute to tumorigenesis. In contrast to its pro-apoptotic role, it is reported that knockdown endogenous Daxx sensitizes cells to multiple stimuli-induced apoptosis, suggesting Daxx has an anti-apoptotic function in certain cellular context [5]. Daxx is primarily localized in the nucleus [6], however, it can translocate into the cytosol upon stress stimulation such as glucose deprivation [7].

Daxx possesses transcriptional regulation activity, which is probably due to its interaction with various transcription factors and epigenetic modifiers, including ATRX, HADCs et al. [8–12]. By recruiting ATRX and HADCs to the promoter region through interacting with a specific transcription factor, Daxx suppresses the transcription. One example of Daxx to inhibiting gene expression is Daxx-mediated viral IE gene repression [13,14]. To counteract Daxx's defense, human cytomegalovirus releases tegument protein pp71 to interact with Daxx by replacing ATRX and inducing Daxx degradation [13–16]. Recent studies suggest that cellular Daxx is also critical for Mdm2 stability and inhibiting the tumor suppressor p53's functions through diverse mechanisms [17,18]. Daxx knockout in mice results in embryonic lethality suggesting cellular Daxx is required for development [19].

The differential functions of Daxx may be related to its protein level and posttranslational modifications. Peptidyl-prolyl isomerase Pin1 inhibits Daxx-involved apoptosis by inducing Daxx phosphorylation on ser178 which mediated Daxx ubiquitination and degradation [20]. HIPK1 reduces Daxx transcription regulation activity via phosphorylation of Daxx on ser669 [21]. Despite that Daxx level is very important in executing its functions; its regulation is poorly understood [20]. Pp71-induced Daxx rapid degradation is ubiquitin independent but proteasome dependant [15]. In contrast, peptidyl-prolyl isomerase Pin1-induced Daxx degradation is ubiquitin dependent, but the responsible E3 is not known [20]. The ubiquitin–proteasome pathway is a major route targeting an ubiquitinated protein for degradation, yet the Daxx level has been found to be controlled by a Cul3-based E3 ubiquitin ligase mediated ubiquitination and degradation process [22]. Concerning the multiple pathways that Daxx is engaged in, it is highly likely that Daxx stability is also regulated by other E3 ligases. De-ubiquitination is a reverse process opposing proteasomal degradation and increasingly recognized in the control of protein stability. However, there is no current report as to whether or not a de-ubiquitinase is involved in Daxx level determination.

In a previous study, we have found that Daxx physically interacts with Mdm2 and the de-ubiquitinase Hausp, and that Daxx facilitates Hausp to de-ubiquitinate Mdm2 whereby stabilizing

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Mdm2 [17]. It has been reported that Mdm2 can induce ubiquitination and degradation of many of its interacting proteins, including p53, Foxo1 and E-cadherin [23,24]. The interaction between Mdm2 and Daxx suggests that Daxx may be a potential ubiquitination target of Mdm2. In the present study we found that Mdm2 ubiquitinated Daxx in both in vivo and in vitro systems, and that Mdm2 reduced cellular level of Daxx upon over-expression. Furthermore, Hausp de-ubiquitinated Daxx and regulated stability of endogenous Daxx.

Material and methods

Cells and plasmids. U2OS and 293T cells were grown in Dulbeco's modified Eagles medium (DEME) supplemented with 10% fetus bovine serum and 1% Pen-Strep (Gibco) at 37 °C in a humidified incubator supplied with 5% CO_2.

For expression in mammalian cells, Daxx, Mdm2 and Hausp were fused an NH2-terminal Flag tag in pRK5 vector. GST-Mdm2 fusion protein was made in pEGX-1ZT and expressed in *Escherichia coli*.

Purification of recombinant proteins. To purify proteins from mammalian cells, expression plasmids encoding Flag-agged Daxx or -Hausp were transfected into 293T cells, respectively. After 24 h, the cells were lysed in lysis buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 0.5% Triton X-100, 1 mM DTT, 1 mM EDTA, 10% glycerol) supplemented with 1× complete protease inhibitors. Cell lysates were immunoprecipitated with anti-Flag M2 beads. The non-specific proteins were removed through sequential washes with lysis buffers containing 150 mM NaCl, 0.5 M NaCl and 1 M NaCl. F-Daxx and F-Hausp were eluted using Flag peptide. For F-Mdm2 purification, the above procedure was followed except that the cells were treated with 20 μM MG132 for 4 h before lysis. To purify GST-Mdm2 from *E. coli*, the expression plasmid was transformed into strain BL21. Protein expression was induced by 1 mM IPTG for 1 h at room temperature. GST-Mdm2 was purified with glutathione-sepharose.

In vitro ubiquitination and de-ubiquitination assay. For ubiquitination assay, purified F-Daxx was mixed with F-Mdm2 or GST-Mdm2 together with E1, E2, his_6-Ub, Mg_2-ATP and 2 mM DTT as described in [17]. The reactions were stopped by adding 2× sample buffers followed by boiling for 5 min. The protein mixture was either directly resolved by SDS–PAGE or diluted 10 times with lysis buffer to reduce the concentration of SDS. F-Daxx was then purified with M2 beads and analyzed by Western blot.

For de-ubiquitination assay, Daxx was ubiquitinated by F-Mdm2 as above and then diluted five times with de-ubiquitination buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM DTT and 5 mM $MgCl_2$). Afterwards F-Hausp was added to the reaction. The samples were incubated at 37 °C for indicated time.

In vivo ubiquitination assay. U2OS cells were transfected with Ha-Ub and indicated plasmids, 24 h later treated with MG132 for 4 h. The cells were then lysed in 1% SDS. After boiling for 5 min, lysates were diluted 10 times with lysis buffer supplemented with 10 mM *N*-ethylmaleimide (NEM). F-Daxx was then purified with M2 beads and subject to Western analysis. Ubiquitinated Daxx was detected by an anti-Ha antibody.

siRNA and transfection. Plasmids or si-RNAs were transfected into U2OS cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. The target sequence for the Hausp siRNA is CCCAAATTATTCCGCGGCAAA. Both control siRNA and the Hausp siRNA were purchased from Qiagen.

Cycloheximide treatment. U2OS cells were treated with 50 μg/ml cycloheximide for indicated durations and then collected for Western analysis.

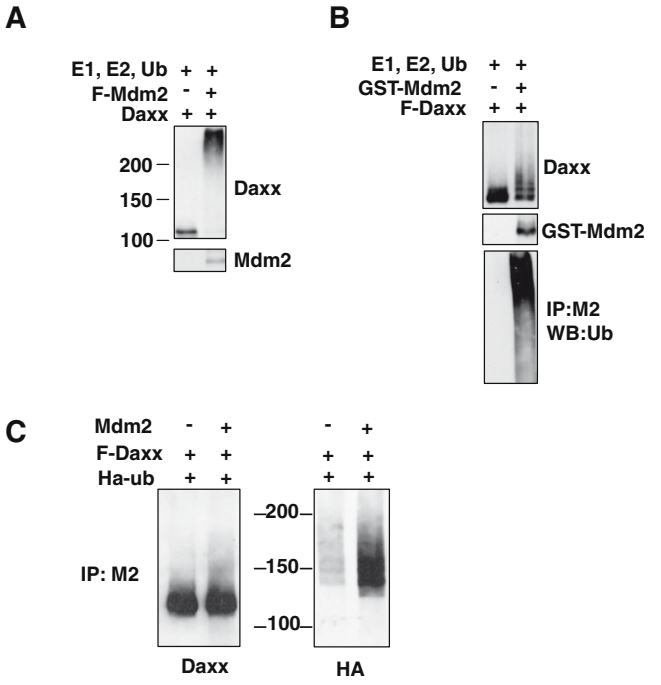

Fig. 1. Mdm2 induces ubiquitination of Daxx. (A, B) Mdm2 induces ubiquitination of Daxx in vitro. F-Daxx and F-Mdm2 were purified from 293T cells (A). Recombinant GST-Mdm2 was purified from bacteria (B). In vitro ubiquitination assay of Daxx was performed as described in Material and Methods. (C) Mdm2 induces ubiquitination of Daxx in vivo. F-Daxx and Ha-ub were co-transfected with or without Mdm2 into U2OS cells for 24 h. F-daxx was then pulled down by M2 beads and subject to Western analysis.

Results and discussion

Daxx is ubiquitinated by Mdm2 in vitro and in vivo

Daxx interacts with E3 ubiquitin ligase Mdm2 [17], hinting that Daxx may be subject to Mdm2-induced ubiquitination. To test this possibility, we performed an in vitro ubiquitination assay. Recombinant Mdm2 purified from 293T cells exhibited a strong activity towards Daxx, converting nearly all of the Daxx into high molecular weight species, likely ubiquitinated Daxx (Fig. 1A). To confirm this result, we purified the GST-Mdm2 from bacteria and performed the same assay. Compared with the Mdm2 purified from the mammalian expression system, the bacterially purified Mdm2 may exhibit weaker ubiquitination activity, but has the advantage of excluding other E3s which might be co-purified with Mdm2 as in the mammalian system. Like mammalian recombinant Mdm2, bacterial Mdm2 also induced the generation of slow migrating species of Daxx. The high molecular weight species were then determined to be ubiquitinated Daxx (Fig. 1B). These results indicated that Daxx can be ubiquitinated by Mdm2 in vitro.

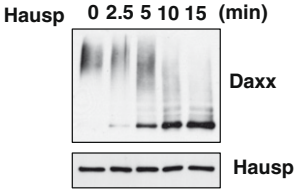

Fig. 2. Hausp de-ubiquitinates Daxx in vitro. Ubiquination of Daxx was first induced by F-Mdm2 by an in vitro ubiquitin assay. Hausp was then employed for the indicated periods of time at 37 °C.