Multiple neurotoxic stresses converge on MDMX proteolysis to cause neuronal apoptosis

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MDMX has been shown to modulate p53 in dividing cells after DNA damage. In this study, we investigated the role of MDMX in primary cultures of neurons undergoing cell death. We found that DNA damage, but also membrane-initiated apoptotic stresses (glutamate receptor; Amyloid β precursor) or survival factor deprivation downregulated MDMX protein levels. Forced downregulation of murine double minute X (MDMX) by shRNA induced apoptosis suggesting that MDMX is required for survival in neurons. Protease inhibitors prevented the loss of MDMX after neurotoxic treatments, indicating a regulation of protein stability. Some, but not all, neurotoxic stresses induced phosphorylation of MDMX at serine 367, further supporting regulation at the protein level. Interestingly, we found that depending on the stimulus either p53 or E2F1 was induced, but overexpression of MDMX inhibited the transcriptional activity of both proapoptotic factors, and maintained neuronal viability upon neurotoxic stresses. Taken together, our data show that MDMX is an antiapoptotic factor in neurons, whose degradation is induced by various stresses and allows activation of p53 and E2F-1 during neuronal apoptosis.

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Cell fate during development and pathogenesis depends on a complex equilibrium between pro- and antiapoptotic pathways in which p53 plays an important role.¹ Many cellular insults are capable of initiating a stream of events that activate and stabilize p53. This transcription factor stimulates the transcription of genes encoding proteins involved in cell cycle arrest or apoptosis, the outcome depending on the cells and the type and strength of the particular insult. The activity of p53 requires a tight control, achieved in part by a feedback loop involving the transcription of the negative regulator MDM2. Through its E3-ubiquitin ligase activity, MDM2 induces the ubiquitination and subsequent degradation of p53 by the proteasome.²

More recently, a MDM2 homolog called murine double minute X (MDMX) was identified.³ Similarly to MDM2, the function of MDMX is linked to the regulation of p53 activity, since the embryonic lethality of MDMX^{−/−} mice is fully rescued in MDMX/p53 double knockout animals.^{4,5} However, both the regulation and function of MDM2 and MDMX are clearly distinct. For example, transcription of the MDMX gene appears not to be regulated by p53. Furthermore, MDMX lacks a robust E3-ligase activity and, therefore, the ability to induce p53 degradation. However, MDMX is able to bind and inhibit p53-mediated transcription in several contexts.^{3,6,7} It has been suggested that MDMX can also stimulate p53 degradation by stabilizing MDM2, and in some cases MDMX overexpression would induce cell death by stabilizing p53.⁸

However, recent data obtained with different mouse models suggest that the main function of MDMX is to repress p53-mediated transcription without having a major function in the regulation of p53 levels.^{9,10} Some of these discrepancies may be due to the use of different cell lines, reflecting either tissue-specificity or transformation bias. MDMX is phosphorylated at multiple sites after cellular stresses, and that these modifications play a critical role in the stability and function of the protein upon DNA damage.^{11–13} Finally, given that MDMX has been reported to interact with other apoptosis modulating proteins such as E2F-1,¹⁴ ARF,¹⁵ 14-3-3,¹¹ HAUSP¹⁶ and the p53-related proteins p73,¹⁷ it is likely that complex mechanisms underlie MDMX regulation.

Most of the existing data on the roles and the regulation of p53, MDM2 and MDMX have been obtained from studies performed in cancer cells. However, it is also known that the various members of the p53 family (p53, p73, p63) play important roles in the nervous system development and in the control of neuronal survival. Indeed, p53 rapidly accumulates in response to various neuronal insults, including DNA damage, oxidative-stress, and cellular calcium overload.¹⁸ Interestingly, MDMX-null mice and MDM2/MDMX-null mice display neuronal defects and p53-dependent neuronal apoptosis.^{5,19} Thus, MDMX may play a critical role in regulating p53 pathway and neuronal fate. Furthermore, E2F-1, another MDMX partner, is also a proapoptotic factor that has been shown to play a key role in neuronal apoptosis.^{20,21} Both p53

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Abbreviations: AP, alkaline phosphatase; APP, amyloid β precursor protein; Bp, base pairs; CGN, cerebellar granule neurons; CIS, cisplatin; CMV, cytomegalovirus; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; GLU, glutamate; HK, high potassium; LK, low potassium; Luc, luciferase; NCS, neocarzinostatin; PAF, paraformaldehyde; PBS, phosphate-buffered saline; PEI, polyethyleneimine; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; shRNA, small hairpin RNA

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and E2F-1 proteins have been found upregulated in Alzheimer's disease,^{22,23} suggesting that MDMX may be involved in neurodegenerative diseases.

Therefore, we investigated the role and the regulation of MDMX in neuronal fate by using primary cultures of cerebellar granule and cortical neurons.

Results

Neuronal stress signals induce the loss of MDMX. The aim of our study was to decipher the role of MDMX in stress situations that lead to neuronal death via different signaling pathways. We wanted to compare the effects of stresses that involve DNA damage *versus* stresses that are more specific to neurons and that are not related to DNA damage. We utilized two DNA-damaging agents: Cisplatin (CIS) and Neocarzinostatin (NCS). CIS is an anticancer drug that

induces formation of intrastrand DNA adducts, primarily ascribed to its interaction with N7-sites of purine bases in DNA.²⁴ CIS is used in cancer therapies despite its neurological side effects.²⁵ Neocarzinostatin is also an anticancer drug that belongs to the enediyne antibiotic family and induces single- and double-strand DNA breaks, mimicking thereby the effect of γ -irradiation.²⁶ We employed two neuron-specific stresses: (i) low potassium concentration (LK), a well-described culture condition inducing a loss of electric activity, which can be considered as a starvation from neurotrophic signals²⁷; (ii) glutamate treatment (GLU), which induces an excitotoxic stress.

Figure 1a shows that the drugs tested induced a loss of undifferentiated and differentiated cerebellar granule neurons (CGN). At 100 nM of NCS, neuronal loss was similar to that induced by LK and CIS (20 μ M) and therefore these concentrations were chosen for the following experiments (Figure 1a). NCS, CIS, GLU and LK-induced nuclear

Figure 1 Neuronal stresses induce apoptosis of cerebellar granule neurons via different pathways. **(a)** Primary cultures of immature (undifferentiated) and mature (differentiated) cerebellar granule neurons were left untreated (CT) or treated for 48 h as indicated (LK: low potassium; CIS: Cisplatin; NCS: Neocarzinostatin; GLU: Glutamate) and survival was assayed by a MTT reduction test. Survival of untreated cells was set at 100%. **(b)** Nuclear morphology of neurons left untreated (CTRL) or treated 16 h as indicated. Nuclei were stained with Hoechst dye. Arrows point to fragmented and/or condensed nuclei. Scale (White bar) = 5 μ m. **(c)** Quantification of fragmented and condensed nuclei observed in panel **b**. Bars represent means $\pm$ S.D. Asterisks indicate statistically significant difference ($P < 0.05$) compared to control, as calculated by a one-way ANOVA test followed by a Newman-Keuls test over at least three independent experiments. **(d)** Neurons were left untreated (CTRL) or treated for 16 h with 100 nM NCS before fixation. Nuclei were stained with Hoechst (blue) and immunofluorescence was performed using the anti-p20 antibody (caspase-3 active fragment, green). **(e)** Western blot analysis of whole protein extracts from mature neurons treated for 2, 6 and 24 h as indicated. Immunoblotting was successively performed with the anti-p20 antibody (left panels: Active Casp3) and the anti-actin antibody (right panels) as a loading control. **(f)** Western blot analysis of serine 139 phosphorylation of histone H2AX 24 h after the indicated treatment. Note that proteins were acid-extracted and 10 μ g were run on a 12% SDS-PAGE. Proteins were then transferred on nitrocellulose subsequently stained with Ponceau S to control loading and transfer

fragmentation and shrinkage, two hallmarks of apoptosis (Figure 1b). Quantification indicated a substantial effect of NCS, CIS and LK on neuronal apoptosis, while GLU treatment only slightly affected nuclear morphology (Figure 1c). Immunofluorescence and Western blot analyses showed that NCS, CIS and LK treatments activated caspase-3 after 6 h and 24 h, while GLU did not (Figure 1d and e, and data not shown).

Several studies performed in cancer cells have shown a regulation of MDMX expression after DNA damage.^{11–13} So, we tested whether the expression of MDMX was regulated in neurons by neurotoxic stresses and whether this regulation was dependent on DNA damage-induced pathway. We first investigated the ability of neurotoxic stresses to induce DNA damage using as a marker the phosphorylation of histone H2AX.²⁸ CIS and NCS induced phosphorylation of histone H2AX, while LK had no effect (Figure 1f). GLU only modestly induced H2AX phosphorylation.

Subsequently, we examined whether MDMX expression was regulated by the neurotoxic stresses that either involve DNA damage (NCS, CIS) or that are independent of DNA damage (LK, GLU). First, we noted that MDMX protein levels remained unchanged during terminal maturation of CGN (Figure 2a) when compared to the neuronal marker MAP2.²⁹ In contrast, MDMX protein levels progressively decreased after different kinds of apoptotic stresses in mature neurons (Figure 2b for LK, NCS, GLU).

Western blot analysis of MDM2 protein levels showed that a DNA-damaging agent NCS increased MDM2 protein levels, while LK and GLU had no significant effect or a minimal effect (Figure 2c). These results show that MDMX and MDM2 expression are distinctly regulated upon treatment of neurons with neurotoxic agents.

Silencing of MDMX expression by shRNA favors neuronal apoptosis. As multiple neurotoxic stresses reduced MDMX expression, we assessed the consequences of MDMX silencing by shRNA expression vectors. The downregulation of MDMX by shRNA expression vectors was verified in the neuronal cells N2A (Figure 3h). Then, cerebellar granule neurons were cotransfected with a control or MDMX shRNA vector and a GFP expression vector. Neurons were further treated with LK or NCS for 24 h before Hoechst nuclear staining and immunofluorescence analysis to detect the activity of caspase-3. GFP-positive neurons were identified by fluorescence microscopy. On panels a–c (Figure 3), characteristic images of healthy neurons are shown while on panels d–f an apoptotic neuron characterized by its nuclear condensation, fragmentation and caspase-3 activity is shown (Figure 3). Apoptotic neurons in the GFP-positive population were counted and represented as percentages (Figure 3g). By this approach, we observed that the silencing of MDMX induced an increase in the number of apoptotic neurons, both in the absence of neurotoxic stress and after treatment with LK or NCS. This result indicates that MDMX is a survival factor for cerebellar granule neurons.

Caspase and proteasome inhibitors restore MDMX protein level after neurotoxic treatment. The loss of

Figure 2 MDMX is lost upon apoptotic treatment of granular neurons. (a) Cerebellar granule neurons were collected and cultivated *in vitro* for the indicated number of days. Whole cell extracts were analyzed by immunoblotting with the anti-MDMX antibodies (top panel), an anti-MAP2 antibody, and the anti-actin antibody as loading control (bottom panel). (b) Differentiated neurons were subjected to LK, NCS or GLU treatment for the indicated time and whole cell extracts were analyzed by Western blot as in panel a. (c) Primary cultures of cerebellar granule neurons were treated as indicated and whole cell extracts were analyzed by immunoblotting with a mixture of anti-MDM2 antibodies (top panel) and the anti-actin antibody as loading control (bottom panel)

MDMX following apoptotic treatments could arise from a transcriptional or a post-translational regulation. To address this issue, we assessed *Mdmx* mRNA levels. RT-PCR experiments showed no significant alteration of *Mdmx* mRNA levels after LK or NCS treatments (Figure 4a), suggesting rather a regulation at the protein level. Indeed, protease inhibitors, including Z-VAD FMK (a pan-caspase inhibitor), Z-DEVD (a caspase-3 inhibitor), MG132 (a proteasome inhibitor), prevented at least partly the downregulation of MDMX protein (Figure 4b and c). These data suggest that the loss of MDMX in apoptotic conditions is due to degradation of the MDMX protein.

Recent studies have shown that the degradation of the MDMX protein upon DNA damage is dependent on the phosphorylation of MDMX on several serines, including serine 367.^{11–13} Therefore, we examined the phosphorylation of MDMX at residue 367 after neurotoxic stresses. Interestingly, the phosphorylation of MDMX at residue 367 was induced by both NCS and GLU (Figure 4d), whereas LK had no significant effect, even after more than 10 h of treatment (data not

Figure 3 Silencing of MDMX by shRNA induces neuronal apoptosis. Cerebellar granule neurons were cotransfected with MDMX shRNA expression vector (shMDMX1, shMDMX2) and a GFP expression vector. Twenty-four hours after transfection, cells were treated for 24 h with NCS or LK. Cells were then fixed, active caspase-3 was detected by immunocytofluorescence and nuclei visualized by Hoechst staining. As control, a scrambled shRNA expression vector was used (CT). GFP positive neurons were observed by fluorescence microscopy and neurons with a condensed or fragmented nuclei and an active caspase-3 were counted as apoptotic neurons (d–f), in contrast to healthy neurons (a–c). (g) The transfection efficiency was 4% and 150 GFP-positive neurons were counted on each slide in at least 10 independent fields. Results are presented as means $\pm$ S.D. of three slides for a representative experiment out of three. (h) Control and MDMX shRNA expression vectors were transfected in the neuronal cells N2A for 24 h. After 72 h, RNA and proteins were extracted and subjected to RT-qPCR (top panel) or Western blot (bottom panel). *Mdmx* mRNA level were compared to *TBP* mRNA level for normalization. Blots were reprobed for actin as control for loading and transfer. Results are presented as means $\pm$ S.D. Statistically significant difference ($P < 0.05$) with control condition (scrambled shRNA) is indicated by an asterisk (*) as given by a one-way ANOVA followed by a Newman–Keuls test

shown). These results suggest that the degradation of MDMX protein in CGN after excitotoxic stress or DNA damage may be dependent on serine 367 phosphorylation. However, this

phosphorylation pathway does not seem to be necessary for MDMX degradation induced upon neurotrophic signal deprivation (LK).

Figure 4 Caspase and proteasome inhibitors restore MDMX protein level. **(a)** RT-qPCR performed with specific primers for *Mdmx* and *18s* on total RNA extracts from cerebellar granule neurons treated as indicated 3 days post-plating. Results correspond to the *Mdmx/18s* ratio for each condition and are presented as means $\pm$ S.D. with the ratio set at 1 for untreated cells. **(b and c)** After 3 days of culture, granule neurons were cotreated for 8 h with a protease inhibitor (MG132: 10 μ M; Z-VAD-FMK: 25 μ M; Z-DEVD-FMK: 50 μ M) and LK or NCS. Western blots were performed with the anti-MDMX antibody (top panel) and the anti-actin antibody to check equal loading (bottom panel). **(d)** Primary cultures of cerebellar granule neurons were co-treated with MG132 and LK, NCS, or GLU for 6 h at the indicated concentration. MDMX proteins were immunoprecipitated from whole cell extracts and separated by SDS-PAGE. MDMX and MDMX phosphorylation at serine 367 (P-MDMX) were detected by Western blotting with an anti-MDMX antibody and a S367 phospho-specific antibody, respectively

Neurotoxic stresses differentially regulate p53 and E2F-1 expression, but MDMX inhibits both p53 and E2F-1 activity in primary neurons. The transcription factors p53 and E2F-1 are both involved in neuronal apoptosis,^{18,20–23} and have both been described to interact with MDMX in cancer cells. Therefore, we examined the ability of the diverse stress signals to regulate p53 and E2F-1 and whether MDMX can inhibit their activity in neurons.

We first observed that p53 levels are increased upon treatment with NCS, CIS and GLU, but not significantly with LK (Figure 5a, upper panel). To investigate the function of MDMX in primary cultures of neurons, we examined its effect on p53 transcriptional activity using various p53 reporter genes. Figure 5b shows that overexpression of MDMX inhibited in a dose-dependent manner, the transcriptional activation of a reporter gene containing a p53 responsive element from the *Bax* promoter (Figure 5b, *Bax* min luc). A similar inhibitory effect was observed on reporter genes containing full-length promoters of p53 target genes (*Bax*, *Mdm2* and *p21* promoters) (Figure 5b, Supplementary Information 1).

In contrast to p53, E2F-1 protein and mRNA levels were increased upon LK treatment (Figure 6a and b). NCS, CIS and GLU did not significantly affect E2F-1 levels in neurons (Figure 6a). Concomitantly to E2F-1 upregulation, we

observed after LK treatment an increase of mRNA levels of E2F-1 target genes, such as *caspase-8* (Figure 6b) or *Apaf1* (data not shown). As LK upregulated E2F-1 expression, we tested the potential regulation of E2F-1 activity by MDMX using an E2F-1 luciferase reporter gene. Upon increasing doses of MDMX, transcriptional activity of E2F-1 was progressively reduced (Figure 6c, Supplementary Information 2).

These data indicate that apoptosis induced by certain stress signals correlates with the activation of p53 (NCS, CIS and GLU), while apoptosis induced by other treatments, such as LK, appears to be independent of p53 activation, but correlates with increased E2F-1 levels. However, both signaling pathways can be controlled by MDMX.

MDMX contributes to the survival of neurons. To confirm that a sustained expression of MDMX can block or at least reduce the apoptosis induced by neurotoxic treatments, we cotransfected a MDMX expression vector and a GFP expression vector into neurons undergoing apoptosis. We counted healthy and apoptotic neurons among transfected cells following proapoptotic LK or CIS treatments (Figure 7). While in control conditions, the number of apoptotic neurons was comparable to that measured by the MTT assay (about 60% in both LK and CIS treatments, see Figure 1a), it was significantly decreased in MDMX-transfected cells

Figure 5 Regulation of p53 by neurotoxic stresses and MDMX in neurons. (a) Western blot analysis of whole protein extracts from primary cultures of cerebellar granule neurons treated as indicated. Immunoblotting was successively performed with the anti-p53 antibody (top panel) and the anti-actin antibody (bottom panel) as a loading control. (b) Cerebellar granule neurons were co-transfected with 0.2 μ g of a p53-responsive luciferase reporter vector (Bax min luc or Bax luc), 0.2 μ g of a p53 expression vector or the corresponding empty vector (CT) and increasing doses of a MDMX expression vector (0.2–0.6 μ g) or the corresponding empty vector with the total amount of DNA kept constant at 1 μ g. Luciferase assay was performed 16 h after transfection. Results (means $\pm$ S.D.) of a representative experiment are shown with the luciferase activity observed in the presence of p53 and absence of MDMX being set at 100%

(Figure 7b). Notably, MDMX rescued neurons from death induced by LK treatment, which did not stabilize p53 (Figure 5a). Using a dominant-negative mutant of p53 (p53DD, Figure 7b), we showed that neuronal apoptosis triggered by LK treatment was independent of p53 activity. In contrast, overexpression of p53DD reduced NCS-induced cell death (Figure 7b) and a similar effect was observed on both CIS and GLU-induced cell death (data not shown). Altogether, these results strongly indicate that MDMX inhibits neuronal apoptosis both in p53-dependent and p53-independent manner.

MDMX regulates amyloid β precursor polypeptide (APP)-induced apoptosis. The above experimental results showed that MDMX plays an antiapoptotic role in neurons facing either DNA damage or an altered electrical activity (induced by LK or GLU).

We hypothesized that MDMX downregulation may also take part in other pathways that control neuronal death and which are linked to neurodegenerative diseases. To address this possibility, we used a cellular model of Alzheimer's disease in which the Amyloid β Precursor Protein (APP) is

activated by an antibody (APP-Ab) directed against the extracellular portion of the protein leading to neuronal apoptosis.³⁰ In cultures of cortical neurons, p53 protein levels were induced after stimulation of the APP pathway with APP-Ab (Figure 8a). In parallel, we observed a progressive loss of MDMX protein and an increase in MDM2 expression (Figure 8a). RT-PCR showed no concomitant reduction of *Mdmx* mRNA levels upon treatment of cortical neurons with APP-Ab (Figure 8b). In contrast, protease inhibitors suppressed the loss of MDMX protein induced by APP-Ab (Figure 8c). Thus, stimulation of the APP pathway in cortical neurons also resulted in degradation of the MDMX protein. We examined phosphorylation of MDMX and observed that phosphorylation of serine 367 was already induced 6 h after treatment with APP-Ab and lasted at least up to 24 h (Figure 8d). Interestingly, phosphorylation of histone H2AX indicative of DNA damage was not observed after the APP-Ab treatment, in contrast with NCS treatment.

Finally, we tested the ability of MDMX to inhibit neuronal death triggered by stimulation of the APP pathway. Overexpression of MDMX rescued cortical neurons from apoptosis induced by a mutated form of APP (APPSW³¹) described in Alzheimer patients or by treatment with the APP-Ab (Figure 8f). These data suggest that APP affects neuronal viability in part by downregulating MDMX, which acts as a survival factor in diverse types of neurons and against various stress signals, through inhibition of p53 and/or E2F-1 activities.

Discussion

Neuronal death is a cellular event that occurs in multiple physiological and pathological situations, ranging from developmental selection by neurotrophic factor depletion and DNA damage by γ -radiation or chemotherapy, to neurodegenerative diseases due to genetic mutations. In this study, we analyzed the contribution of MDMX to neuronal survival.

MDMX has a neuroprotective activity and its down-regulation is a node for multiple neurotoxic stresses. A previous study has shown that during embryonic development, lack of MDMX severely altered the nervous system, likely by affecting the survival of neuronal precursors.⁵ Here, we have shown that in terminally differentiated post-mitotic neurons, not only DNA damage, but also various other neurotoxic stresses led to a downregulation of MDMX (Figures 2 and 8). Therefore, the downregulation of MDMX protein levels appears to be a node where several neurotoxic stresses converge (Figure 9), suggesting that MDMX has a neuroprotective function. This neuroprotective role of MDMX was supported by the increase of apoptotic neurons when MDMX expression was silenced by shRNA (Figure 3), and was further confirmed by the ability of MDMX to both inhibit the activity of two proapoptotic transcription factors, p53 and E2F-1 (Figures 5 and 6) and to significantly reduce the level of apoptosis induced by DNA damage, LK, GLU or APP activation in primary cultures of neurons (Figures 7 and 8). We also showed that the reduction of MDMX protein level was not strictly dependent on a DNA damage-induced pathway, as several neuronal

Figure 6 Regulation of E2F-1 by neurotoxic stresses and MDMX. (a) Western blot analysis of whole protein extracts from primary cultures of cerebellar granule neurons treated as indicated. Immunoblotting was successively performed with the anti-E2F-1 antibody (top panel) and the anti-actin antibody (bottom panel). (b) Quantitative RT-PCR performed with *E2F-1*, *caspase-8* and *18s* specific primers on total RNA extracts from granule neurons treated as indicated. Results correspond to the *18s* ratio for each condition and are shown as means $\pm$ S.D. (c) Neurons were transfected with 0.2 μ g of an E2F-1-responsive luciferase reporter vector (E2F min luc), 0.2 μ g of an E2F-1 expression vector or the corresponding empty vector and increasing doses of an MDMX expression vector or the corresponding empty vector. Luciferase activities (means $\pm$ S.D.) of a representative experiment are shown

Figure 7 Overexpression of MDMX protects granular neurons in primary culture against neurotoxic stresses. (a) Neurons were transfected for 8 h with 0.2 μ g of GFP expression vector to identify transfected cells (green) before overnight treatment with NCS or vehicle (CT). Nuclei were stained with Hoechst dye (blue). (b) Granular neurons were co-transfected with a MDMX expression vector, or a p53DD expression vector or the corresponding empty vector (PBI) and a GFP expression vector before overnight treatment with LK or NCS. Intact nuclei were counted among GFP-positive neurons and set to 100% for untreated cells transfected with the empty expression vector (PBI). Results are presented as means $\pm$ S.D. Statistically significant difference ($P < 0.05$) with control condition (empty vector) is indicated by an asterisk (*) for LK and a sign (#) for CIS as given by a one-way ANOVA followed by a Newman-Keuls test

stresses (LK, GLU and APP-Ab) did not significantly induce H2AX phosphorylation, indicative of DNA damage (Figures 1f and 8e).

Interestingly, our results indicate that depending on the stress applied to neurons maintained in primary culture, the nuclear morphological changes and the signaling pathways (caspase-3) induced are different (Figure 1). Especially, we found that several stresses increased p53 protein levels (DNA damage, GLU, APP-Ab), while depletion of potassium (LK) did not affect significantly p53 protein level (Figures 5a and 8a). Inversely, E2F-1 expression was induced by potassium depletion (LK) but not by DNA damage (Figure 6a and b). The lack of E2F-1 activation by CIS or NCS contrasts with recent data obtained with cancer cells.³² E2F-1 does not seem to be a major mediator of DNA damage-induced apoptosis in

neurons. However, the induction of E2F-1 expression after LK is consistent with previous studies that described the role of E2F-1 in neuronal apoptosis.^{20,21} The selective role of p53 during apoptosis is confirmed by the observation that a dominant-negative mutant of p53, p53DD, did not prevent the neuronal cell death induced by LK, but could inhibit the

Figure 9 MDMX is involved in neuronal survival by controlling p53- and E2F1-dependent apoptosis. In a physiological situation, MDMX can control the activity of p53 and E2F-1 in neurons allowing their survival. After neurotoxic stimuli (DNA damage, Excitotoxicity, APP stimulation), MDMX becomes phosphorylated and degraded by multiple proteases. Removal of MDMX proteins allows activation of p53 and E2F-1 activities, leading to neuronal apoptosis

Figure 8 MDMX antagonizes activated APP-induced apoptosis of cortical neurons. (a) Primary cultures of mouse cortical neurons were left untreated (CT) or treated with the antibody directed against the extracellular portion of APP (APP-Ab: 250 μ g/ml) for the indicated time and extracts were analyzed by immunoblotting with the anti-p53 antibody, the anti-MDMX antibody, the MDM2 antibody and the antiactin antibody to check protein loading. (b) RT-qPCR performed with *MDMX* and *18s* specific primers on total RNA extracts from cortical neurons treated with the APP-Ab for the indicated time 3 days post-plating. Results correspond to the *MDMX/18s* ratio for each condition and are presented as means $\pm$ S.D. (c) Immunoblotting with the anti-MDMX antibody (top panel) and the anti-actin antibody (bottom panel) on protein extracts of neurons co-treated for 12 h with APP-Ab and the indicated protease inhibitor (MG132 or Z-VAD-FMK). (d) Cortical neurons were treated with APP-Ab in the presence of proteasome inhibitor (MG132) for the indicated time. MDMX proteins were immunoprecipitated from whole cell extracts and separated by SDS-PAGE. MDMX and MDMX phosphorylation at serine 367 were detected by Western blotting with an anti-MDMX antibody and a S367 phospho-specific antibody (P-MDMX) respectively. (e) Western blot analysis of serine 139 phosphorylation of histone H2AX (p-H2AX) 24 h after the indicated treatment. (f) Cortical neurons were transfected with 0.6 μ g of either MDMX-expressing or empty vector (PBI), 0.4 μ g of wild-type (WT) or Swedish mutated (SW) APP expression vector as indicated. A 0.2 μ g of GFP expression vector was added in all conditions to visualize transfected cells. When indicated, cortical neurons were treated by the APP-Ab or with an antiactin antibody used as mock control. At 24 h after transfection, neurons were stained with Hoechst and apoptotic-transfected cells were counted. Results are presented as means $\pm$ S.D. Asterisks indicate statistically significant differences ($P < 0.05$) as given by a one-way ANOVA followed by a Newman-Keuls test

apoptosis triggered by the DNA-damaging agent NCS (Figure 7b).

These results suggest the existence in neurons of at least two independent pro-apoptotic pathways, one leading to the activation of p53 (DNA damage, GLU, APP stimulation), while the other might use E2F-1 (LK) (Figure 9). We cannot exclude that other stresses might lead to activation of both pathways. For example, β -amyloid peptide treatment induces both p53 and E2F-1.³³ However, our study illustrates that for various neurotoxic stresses, either p53 or E2F-1 would be preferentially activated in untransformed neurons. Interestingly, the pro-apoptotic gene *Siva* is a target gene for both p53 and E2F-1, and can induce apoptosis in neurons upon overexpression.³⁴ *Siva* might therefore represent the common relay targeted by the two signaling pathways, p53 and E2F-1, and thus be responsible for the induction of apoptotic effectors, such as caspase-3 and the caspase-activated DNase.

Our observations indicate that MDMX is a survival factor for neurons, and, therefore, explain why several neurotoxic stresses converge to induce neuronal death via the degradation of MDMX. The survival function of MDMX is likely related to its ability to inhibit p53 and E2F-1 proapoptotic function.

Regulation of MDMX expression in neurons. Our investigations on the mechanisms involved in the loss of MDMX revealed that it occurred without significant decrease in mRNA levels, but was prevented by treatment with caspase or proteasome inhibitors, suggesting a regulation at the protein level by various mechanisms (Figure 4). Similar lack of effect on *Mdmx* mRNA levels has been found in mouse embryonic fibroblasts.³ The fact that multiple protease inhibitors blocked MDMX degradation raises the questions whether different types of proteases performed this process concomitantly or sequentially, and what are the mechanisms involved. Several studies reported that MDMX can be ubiquitinated by MDM2 and subsequently degraded through the proteasome after a toxic treatment in various cell lines.^{35,36} Interestingly, Gentiletti *et al.*³⁷ proposed that MDMX was processed by caspase-3 and subsequently degraded by the proteasome. Therefore, caspase-3 might be responsible in part for MDMX protein loss in some conditions, but not all as GLU and APP-A β treatments do not activate caspase-3 (Figure 1).³⁰ When we further investigated the molecular mechanism that might control MDMX protein levels, we found that some neurotoxic stresses enhanced MDM2 expression and induced MDMX phosphorylation at serine 367 (Figures 4 and 8). Serine 367 phosphorylation has been described to be essential for degradation of MDMX by MDM2 upon DNA damage.^{11–13} Therefore, it is likely that MDM2 and S367 phosphorylation contribute to the degradation of MDMX in neurons upon neurotoxic treatment. However, it appears that in neurons the mechanisms leading to MDMX degradation can also be different since we did not see any modification of MDMX phosphorylation after LK treatment. Interestingly, we also showed that MDMX phosphorylation did not correlate with induction of DNA damage, as GLU and APP-A β did not induce H2AX phosphorylation, suggesting that alternate mechanisms, which remain to be identified, might be involved.

Role of MDMX in neurodegenerative diseases. We have found that MDMX protein is lost after alteration of the APP signaling pathway (Figure 8a). The fact that overexpression of MDMX can, to at least in part, counteract the neuronal death induced by APP alteration (Figure 8f), suggests that the loss of MDMX can indeed play a role in this neuronal death. We were not able to demonstrate that downregulation of MDMX is indeed a triggering mechanism in the APP-mediated cell death, but it appeared to be required to some extent. Furthermore, the fact that MDMX silencing favors neuronal apoptosis (Figure 3) suggests that the downregulation of MDMX is part of the mechanisms by which APP alterations induce neuronal apoptosis. It is, however, possible that it is not the triggering mechanism, but involves an amplifying mechanism that pushes the survival/apoptosis balance toward neuronal cell death. Indeed, activation of caspases might favor the degradation of MDMX, and in that way further induce the p53-dependent apoptosis pathway.

These findings that alteration of APP function affects MDMX regulation as well as the fact that both E2F-1 and p53 seem to be involved in apoptosis associated with Alzheimer's disease^{22,23} suggest that MDMX expression may be altered during Alzheimer's disease. It is plausible that APP deregulation might, through the loss of MDMX, activate multiple proapoptotic pathways dependent on p53 and E2F-1.

p53 activation has been described in other neurodegenerative diseases. We observed its activation in ALS,³⁸ which affects both motoneurons and muscles. Further understanding of the complex mechanisms involved in the regulation and function of MDMX or MDM2 in neurons may therefore provide a potential basis for therapeutic intervention against Alzheimer's disease or other neurodegenerative pathology.

Materials and Methods

Primary culture of cerebellar granule neurons and cortical neurons. Cerebellar granule neurons were obtained from 7-day-old mice as described previously.³⁹ Cortical neurons were prepared from 16-day-old embryos. Briefly, after enzymatic and mechanical dissociation, cells were plated at a density of 500 cells/mm² on 0.1 mg/ml polymethine-precoated culture dishes and were grown at 37°C in a humidified atmosphere (5% CO₂/95% air). Plating culture medium contained DMEM (Dulbecco's modified Eagle's medium; Life Technology, Paisley, UK) supplemented with 10% heat-inactivated horse serum (Life Technology), 100 nM insulin, 50 mg/ml gentamycin. For the cerebellar granule neurons, 25 mM potassium is added to the culture. At 48 h after plating, cells were switched to defined medium containing DMEM supplemented with 10 nM insulin, 100 mg/ml human transferrin, 60 mM putrescine, 30 nM sodium selenite, 50 mg/ml gentamycin. For the cerebellar granule neurons, 25 mM potassium is also added to the culture (HK medium). Low-potassium (LK) medium was obtained by omitting KCl (final [K⁺] = 5 mM).

Colorimetric MTT assay. Cells were cultured in 96-well culture dishes (Costar, Corning, NY, USA). Cultures were rinsed with DMEM and incubated for 1 h at 37°C in freshly prepared culture medium containing 0.5 mg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma, Steinheim, Germany). Medium was then removed and dark blue crystals formed during reaction were dissolved by adding 100 μ l/well of 0.04 M HCl in isopropyl alcohol. Plates were stirred at room temperature and read on a Bio-Rad 680 micro-ELISA plate-reader, using a test wavelength of 490 nm and a non-specific wavelength of 650 nm for background absorbency. Results are given as percentage of survival, taking cultures grown in HK medium as 100%.

Hoechst staining and immunofluorescence. Cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and subsequently incubated with 1% Triton X-100 in PBS for 10 min, and 0.5% Triton X-100 plus 5% BSA in PBS for 30 min. After overnight incubation at 4°C with the caspase-3 antibody (R&D system, Minneapolis, MN, USA) diluted 1/1000, cells were incubated with Cy3 (carbocyanine 3)-coupled goat anti-rabbit IgG diluted 1/500 (Jackson ImmunoResearch, Suffolk, UK). Cells were then incubated for 30 min with the nuclear Hoechst dye 33342 (1 µg/ml). Fixed cells were viewed on a Nikon fluorescence microscope (420 nm).

Western blot. Cultured cells were lysed in 120 µl of Laemmli's buffer (125 mM Tris-HCl, pH 6.7, containing 3.3% SDS, 0.7 M 2-mercaptoethanol, 10% glycerol and 0.02% Bromophenol Blue). Extracts were sonicated for 20 s and boiled for 5 min before centrifugation at 20 000 × g for 5 min. Proteins were separated on a 10% SDS-polyacrylamide gel, and subsequently electro-transferred to PVDF membranes. Equal loading was verified with an antibody directed against actin (1/200; Gift from Dr Aunis). Immunoprobings were performed with anti-p53 (1/3, mix of pAb421 and pAb240), anti-MDMX (1/10, mix of 6B1A, 7C8, 3G12), anti-phospho-MDMX (S367, 1/10¹¹), anti-MDM2 (1/5, mix of 2A10 and SMP14) or with polyclonal anti-E2F-1 (1/500, Santa Cruz Biotechnology) antibodies. The membranes were subsequently probed with a secondary horseradish-peroxidase-conjugated antibody (anti-rabbit or-mouse according to the first antibody) diluted at 1/2000. Finally, membranes were developed by enhanced chemiluminescence detection.

Phospho-H2AX detection. Cells were scraped and spun down for 15 min at 0°C and 1000 × g. Cell pellets were washed in PBS, homogenized in 0.2 N H₂SO₄, and centrifuged at 13 000 × g. Histones were spun down from the supernatant by adding 0.25 volume of 100% (w/v) trichloroacetic acid. The pellets were suspended in 100% ethanol overnight and centrifuged again at 13 000 × g. The pellets were dissolved in Ultrapure water and evaluated for protein concentration (Uptima). Aliquots corresponding to 10 µg of proteins were boiled in SDS sample buffer and loaded on a 12% SDS-PAGE. Immunoblotting with anti-phospho-H2AX antibody (Upstate) diluted at 1/3000 was performed to reveal γ-H2AX. Ponceau S staining was used to check equivalent protein loading and transfer.

Immunoprecipitation. Cells were grown in 10-cm round plates. After treatments, cells were washed with PBS and proteins were extracted with a TEGN extraction buffer (Tris-HCl, pH 7.4, 50 mM, EDTA 1 mM, NaCl 200 mM, Glycerol 10%, NP40 0.5%) supplemented with protease inhibitors (including MG132) and phosphatase inhibitors cocktails (Sigma). After centrifugation, 3 mg of each extract was incubated with a mix of MDMX antibodies (6B1A, 7C8, 3G12, 100 µl each) at 4°C overnight. Then, 50 µl of 50% slurry G-sepharose beads were added for 1 h. Beads were then washed five times before sample buffer was added and the immunoprecipitated proteins were separated on a 10% SDS-polyacrylamide gel. Phosphorylation of MDMX at serine 367 was detected by Western blotting using a phospho-specific antibody.¹¹

Transfections. Cells were cultured in 12-well dishes and grown for 3 days prior to transfection experiments. Gene transfer was performed using Polyethyleneimine (PEI) as DNA carrier.⁴⁰ Neurons were transfected with 1.2 µg of DNA mixed to PEI (0.33 µl/µg of DNA) and spun for 5 min at 1500 r.p.m. After 30 min, neurons were switched to fresh medium (HK or LK) and assays were performed 20 h later. For luciferase assays, cells were lysed 24 h after transfection using the luciferase assay lysis reagent (Promega, Madison, WI, USA) and cell extracts were mixed with D-luciferin (Sigma). Luciferase activity was measured by a luminometer (Berthold systems, Bad Wildbad, Germany). Normalization of luciferase activity was performed by replacing the reporter gene of interest by a CMV luciferase reporter vector. For GFP cotransfection, 0.2 µg of GFP encoding vector was added to the MDMX vector or the MDMX shRNA vector.

Quantitative real-time RT-PCR (RT-qPCR). Cells were grown on 10-cm round plates. After treatment, they were harvested and total RNA was extracted using Qiagen RNeasy kits. Reverse transcription was performed with 1 µg RNA using Biorad iScript kit. qPCR was performed in iCycler thermal cycler (Biorad, Hercules, CA, USA) using SYBR Green dye (iQ SYBR green Supermix, Biorad). For each gene, a standard curve based on successive cDNA dilutions was performed and was used to calculate starting quantities. To ensure a thorough calculation, starting quantities of genes of interest were reported to those of a housekeeping gene (18 s or TBP) in the same plate. After each qPCR, specificity of the

amplification was controlled by a melting curve ranging from 55 to 95° whereby a single peak corresponding to the amplicon was present.

All primers were designed in regions flanking introns to exclude data alteration by possible DNA contamination.

MDMX: forward 5'-GGCTTTTCAGAACTATGAGCCAAAT-3'; reverse 5'-ATTGGCTCATAGTTCTGAAAGCC-3'.

E2F-1: forward 5'-CCAGCTCATTGCCAAGAAGT-3'; reverse 5'-GCTTACCAATCCCCACAA-3'.

Caspase-8: forward 5'-TGCTTGGACTACATCCCACAC-3'; reverse 5'-TGCAGTCTAGGAAGTTGACCA-3'.

18S: forward 5'-CGTCTGCCCTATCAACTTTTCG-3'; reverse 5'-TTCCTTGGATGTGGTAGCCG-3'.

TBP: forward 5'-CCAATGACTCCTATGACCCCTA-3'; reverse 5'-CAGCCAAGATTCACGGTAGAT-3'.

Peptides, antibodies and vectors. The inhibitors z-VAD-fmk and MG132 were purchased from Calbiochem (Darmstadt, Germany). Polyclonal anti-E2F-1 (C-20) antibodies were from Santa Cruz Biotechnology. Ser 137 phospho H2AX was used at a dilution 1/3000 (Upstate, NY, USA). Monoclonal anti-p53 antibodies (pAb240, pAb 421) were a kind gift from Dr C Prives (Columbia, NY). MAP2 antibody was used at a dilution of 1/200 (Santa Cruz Biotechnology). Horseradish peroxidase (HRP)-conjugated secondary antibodies, goat anti-rabbit IgG and sheep anti-mouse IgG, were purchased from Chemicon (Billerica, MA, USA). Cy3-conjugated donkey anti-mouse IgG was from Jackson ImmunoResearch. MDMX expression vectors were previously described.¹¹ MDMX shRNA vectors contain the sequence: GAATCTTGTTACATCAGCT (shMDMX1) or TGTCTTGAAGCCGT GTAGC (shMDMX2). E2F-1 expression vector and reporter gene were a gift of Dr Emmanuelle Trinh. p53 reporter genes were described previously.⁴⁰

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