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ORIGINAL ARTICLE

Identification of novel interaction between Promyelocytic Leukemia protein and human Alteration/Deficiency in Activation 3 coactivator and its role in DNA damage response

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Abstract

Human Alteration/Deficiency in Activation 3 (hADA3) is an interesting transcriptional coactivator adaptor protein with predominant nuclear localization. Even though it has been shown to be an important mediator of diverse cellular processes including embryonic development, cell cycle progression and maintenance of genomic stability, the molecular mechanisms underlying its role in apoptosis and DNA damage response remain elusive. Our study for the first time revealed that hADA3 exhibits punctate nuclear pattern which interestingly colocalizes with Promyelocytic Leukemia Nuclear Bodies (PML-NBs). We also provide novel evidence for the physical interaction between them, which is further enhanced following DNA damage. Moreover, we demonstrate that cells expressing a hADA3 mutant which is unable to interact with PML, displayed impaired apoptosis indicating a clear role of hADA3 in PML-mediated apoptosis. These findings, therefore, highlight a previously unappreciated function of hADA3 and establish its novel functional link with PML in provoking DNA damage response.

Keywords PML · ADA3 · Coactivator protein · DNA damage · Tumor suppressor

Introduction

A key feature of cellular systems is maintenance of genomic stability, which is brought about by several coordinated events including DNA replication, recombination and repair, all of which require proper access to the chromatin. Chromatin remodeling is an incessant event carried on by a complex interplay among various proteins, which involve several activators and co-activators to mediate their function. One such coactivator is the evolutionarily conserved hADA3 (Alteration/Deficiency in Activation 3) protein which, along with ADA2 and GCN5 (general control non-repressed 5), acts as an essential bridge between components of basal transcription machinery and Histone Acetyl Transferases (HATs) (Horiuchi et al. 1995; Mirza et al. 2012; Wang et al. 2008). Several HAT complexes, including SAGA (SPT-ADA-GCN5 acetylase), STAGA (SPT3-TAF_{II}31-GCN5L acetylase), TFTC (TATA-binding protein-free TAF-containing complex), ATAC (Ada2A containing) complex have been found to contain hADA3 as a conserved component (Gamper et al. 2009; Hardy et al. 2002; Martinez et al. 1998). Importantly, hADA3 acts as a transcriptional coactivator of the tumor suppressor protein p53 and participates in p300/CBP-associated factor (PCAF) and p300/CBP-mediated p53 acetylation and stabilization (Nag et al. 2007; Wang et al. 2001). Additionally, hADA3 is responsible for transactivation of estrogen receptor-α (ERα) and Retinoic X Receptor-α (RXRα), which are known to be targeted by high-risk human papillomavirus (HPV) 16E6 in E6-driven pathogenesis (Li et al. 2010; Zeng et al. 2002). Recent work from our lab highlighted the role of SUMOylation in regulation of hADA3. HPV16E6 was shown to accelerate SUMOylation-mediated ubiquitination and hence destabilization of hADA3 in cervical cancer cells (Chand et al. 2014).

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The physiological relevance of hADA3 is affirmed by the observation that *Ada3*$^{-/-}$ mice die in utero, probably due to deregulated chromatin modifications (Mohibi et al. 2012). Additionally, conditional deletion of *mAda3* leads to delayed G1-S transition in *Ada3* null MEFs due to hypophosphorylation of pRb and elevated p27 levels, indicating its role in cell cycle progression (Mohibi et al. 2012). Moreover, MEFs derived from *Ada3*$^{-/-}$embryos exhibit spontaneous chromosomal abnormalities, which get exaggerated upon DNA damage (Mirza et al. 2012). In addition, loss of hADA3 has also been reported to trigger an increase in the levels of DDR (DNA damage response) proteins, such as γH2AX (H2A histone family member X), pATM (phospho-Ataxia Telangiectasia Mutated), p53BP1 (p53 Binding Protein 1), and pRAD51 and also a delayed disappearance of DNA damage foci for several DDR critical proteins after ionizing radiation, suggesting the importance of hADA3 in DDR (Mirza et al. 2012). In response to genotoxic assault, a cell either undergoes cell cycle arrest allowing time to repair the damaged DNA or more dramatically, undergoes apoptosis. Pertaining to the role of hADA3 in programmed cell death, N-terminal half of hADA3 has been shown to prohibit p53-mediated apoptosis (Wang et al. 2001) and a recent gene knockdown screen has identified hADA3 as a regulator of human granzyme-mediated apoptosis (Brasacchio et al. 2014). Apart from these few studies, however, the role of hADA3 in apoptosis has not received much attention.

During most of our hADA3 overexpression studies, we often noticed a punctate appearance of nuclear hADA3 resembling the typical pattern of PML-NB (PML-nuclear body). PML (Promyelocytic Leukemia) protein is a putative tumor suppressor that is found to be deregulated in various hematological malignancies, especially APL (acute PML), and solid tumors like breast carcinomas (Gurrieri et al. 2004; Salomoni et al. 2008). The role of PML in apoptosis is well established and extensively studied. In normal cells, PML colocalizes with several proteins as distinct macromolecular structures, called PML-nuclear body (PML-NB), which are suggested to be involved in maintenance of genomic integrity (Bernardi and Pandolfi 2007; Salomoni and Pandolfi 2002; Wang et al. 1998). *PML*$^{-/-}$ mice are found to be resistant to various pro-apoptotic stimuli, including lethal UV dosage, DNA-damaging agents and CD95/Fas (Wang et al. 1998). The p53 family of tumor suppressors plays an important role in DDR as well as apoptosis in concert with PML. Under various cellular or oncogenic stress, PML-NBs have been found to contain p53 along with multiple factors that regulate its activity, including p300, thereby promoting its acetylation and stabilization (Guo et al. 2000; Nag et al. 2007; Pearson et al. 2000). PML was also shown to stabilize and amplify p53 activity by preventing its ubiquitination or sequestering Mdm2 to the nucleolus (Bernardi et al. 2004; Kurki et al. 2003). Furthermore, PML associates in vivo with the DNA damage response protein TopBP1 in response to ionizing radiation, which in turn also co-accumulated with Rad50, Brca1, ATM, Rad9, and BLM (Xu et al. 2003). PML-NBs have also been displayed to provide a platform to a plethora of proteins such as Homeodomain-Interacting Protein Kinase-2 (HIPK2) and Sirtuin-1 (SIRT1), which play a major role in DNA damage response. In fact, depletion of PML was shown to abrogate HIPK2-mediated stabilization of p53 in case of severe genomic damage (Conrad et al. 2016). On the other hand, Daxx, a death domain-associated protein, is believed to exert pro- or anti-apoptotic effect through PML-NBs (Meinecke et al. 2007; Torii et al. 1999; Zhong et al. 2000). Yet, another study in primary mouse fibroblasts has shown that UV light-induced apoptosis follows a pathway that is p53 independent but dependent on JNK/c-Jun and the active, phosphorylated c-Jun physically interacts with and colocalizes into PML-NBs (Salomoni et al. 2005). Moreover, PML-NBs can also regulate apoptosis by associating with FLASH (FLICE-associated huge protein), a caspase 8 activator at DISC (death-inducing signaling complex), and TRADD, an adaptor protein responsible for recruiting caspase 8 to DISC (Krieghoff et al. 2007; Milovic-Holm et al. 2007; Morgan et al. 2002). Thus, PML plays a vital role in various apoptotic pathways depending on the apoptotic stimulus or cellular context.

In this study, we have identified PML as a novel interacting partner of hADA3 and this interaction has been found to upsurge during cellular stress. In-depth analysis also revealed the interacting domains involved and mutational study encompassing these domains highlighted the importance of this association in mediating apoptosis upon DNA damage. Overall, this study provides a new aspect to the well-known event of DNA damage response and programmed cell death, illuminating the novel hADA3-PML axis and its significance in preserving the genomic integrity.

Materials and methods

Cell culture, transfection and western blotting

HEK293T, U2OS, MCF7 and HCT116 cell lines were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL of penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA) in a humidified incubator with 5% CO_2 atmosphere at 37 °C.

For overexpression studies, HEK293T cells were transfected using calcium phosphate (Nag et al. 2001), while MCF7, HCT116 and U2OS were transfected using FuGENE HD (Promega, Madison, WI) transfection reagent and Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA), respectively, as per manufacturer's protocol. The medium was changed 16 h after transfection and the cells were lysed 42 h

post-transfection in lysis buffer containing 50 mM Tris–HCl, 400 mM NaCl, 0.2% Nonidet P-40, 10% glycerol and protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Equal amount of whole cell lysates were processed for SDS-PAGE and subjected to immunoblotting with appropriate antibodies.

Antibodies and plasmid constructs

Anti-Flag antibody was purchased from Sigma (St. Louis, MO), while antibodies against actin, PML, γH2AX, Daxx, p53, c-PARP, GAPDH and p300 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Myc antibody was obtained from Invitrogen (Carlsbad, CA). Plasmids expressing Myc-hADA3, Flag-hADA3 and its deletion mutants (1–144), (145–288), (289–432) were generated by inserting appropriate PCR fragments into *Hind*III and *Xho*I restriction sites in the pcDNA3.1 vector. Plasmid expressing HA-PML (a generous gift from Carl Maki, Rush University, USA) was used to generate various PML constructs namely Myc-PML, Flag-PML WT and its deletion mutants (1–211), (212–422), (423–633) within the *Hind*III and *Xho*I restriction sites in the pcDNA3.1 vector. Similarly, DsRed-PML construct was generated using *Xho*I and *Bam*HI sites. All the constructs were verified by DNA sequencing.

Generation of stable cell lines

For generation of stable cell line, Phoenix Ampho cells were transfected with pSUPER.retro.puro vector system using calcium phosphate method (Nag et al. 2001). hADA3 and PML-specific RNAi sequences (sense: 5′-GGC CAG CCA UCA CAA UCA ATT-3′; anti-sense: 5′-AGA UGC AGC UGU AUC CAA G-3′) were used in shRNA construct (Chand et al. 2014). Cells were transduced with either shADA3 or shPML retroviral supernatants as described earlier (Chand et al. 2014). Cells were selected in media containing puromycin (0.5 µg/ml) for 10 days, followed by maintenance in the same media. Single cell clones were grown and knockdown was verified by assessing the expression of endogenous hADA3 and PML by Western blotting.

Confocal microscopy

Cells transfected with either EGFP-hADA3 alone or in combination with DsRed-PML were fixed in 4% paraformaldehyde for 20 min at RT, washed thrice with PBS and stained with 1 µg/ml DAPI for 5 min at RT. Cells were washed again and mounted in Prolong® Gold, Anti-fade reagent (Molecular Probes, Eugene, OR) and captured using *Leica* SP-5 confocal laser-scanning microscope. The two-dimensional co-localization scatterplot was created using the *Leica* LAS-AF software.

Immunofluorescence

Cells were grown and fixed as mentioned earlier. Fixed cells were permeabilized in PBS-T (PBS containing 0.1% Triton X-100) for 20 min at RT, washed thrice with PBS and blocked using 5% FBS for 4 h at RT. Cells were immunolabeled with antibodies against PML, Flag, Myc, Daxx and γH2AX (1:200) overnight at 4 °C followed by three washes in PBS. Cells were then incubated with appropriate Alexa-conjugated secondary antibody for 2 h at RT followed by three washes in PBS-T. Nuclei were stained with DAPI before viewing under fluorescence microscope (Nikon-Eclipse-Ti-S, Tokyo, Japan) or Leica SP-5 confocal laser-scanning microscope.

DNA damage treatment

Transfected cells were subjected to various genotoxic stresses for 16 h by adding adriamycin (0.5 µg/ml), etoposide (3 µg/ml), actinomycin D (0.5 µg/ml), hydroxyurea (2 mM), separately. For UV treatment, cells were washed with PBS and then exposed to UV (10 J/m^2 or 40 J/m^2 using UV-cross-linker (UVC-5000, Hoefer, USA) and further cultured in the original conditioned medium.

Co-immunoprecipitation assay

Cell extracts were prepared in lysis buffer containing 150 mM NaCl, 1% NP-40, 5 mM EDTA, 50 mM Tris–HCl (pH 7.5), 2 mM PMSF (phenylmethanesulfonyl fluoride), 2 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail. Pre-cleared lysates were subjected to immunoprecipitation followed by thorough washing with binding buffer. Pulldown was accomplished using Protein-A/G beads (Jaiswal et al. 2015). The bound immunocomplexes were then eluted with Laemmli buffer and resolved by SDS-PAGE followed by immunodetection with appropriate antibodies.

Annexin-V staining

MCF7 cells stably expressing pSUPER.retro.puro control vector, shADA3 and shPML were grown on glass cover slips to achieve 50% confluency. Cells were then treated with 10 J/m^2 UV and allowed to grow for 24 h and then stained with annexin-V and propidium iodide as directed by the manufacturer (Cell Signalling, Danvers, MA). Coverslips were then mounted on slides using Prolong® Gold anti-fade reagent and the images were captured using fluorescence microscope.

Determination of Sub-G1 population by FACS analysis

HCT116 cells stably expressing pSUPER.retro.puro control vector were transfected with Flag-hADA3 in the presence or absence of Flag-PML. Additionally, HCT116 cells stably expressing shADA3 or shPML were transfected with Flag-PML and Flag-hADA3, respectively. These cells were subjected to selection in G418 (Sigma, St. Louis, MO) containing media for 10 days followed by treatment with UV (40 J/m^2). After 24 h of UV treatment, cells were harvested with 0.4% EDTA containing PBS and fixed in ice cold 85% ethanol for 2 h. Cells were then stained with propidium iodide (50 µg/ml) (Sigma, St. Louis, MO) containing 50 µg/ml RNaseA (Himedia, India) for 30 min at RT. Cell cycle progression was analyzed and sub-G1 phase cells were identified using FACSCalibur (BD biosciences, San Jose, CA) with at least 10,000 events for each sample and analyzed using CellQuest software.

Cell proliferation assay

MCF7 cells stably expressing pSUPER.retro.puro control vector, shADA3 and shPML were seeded at 50% confluency. The cells were treated with 5 and 10 J/m^2 UV and allowed to grow for 7 days. Media was changed every alternate day and the obtained colonies were stained with crystal violet (0.1% crystal violet in 10% ethanol) and counted manually.

Statistical analysis

Statistical analysis was performed with GraphPad Prism Software. Comparisons between two groups were determined by Student's *t* test. The *p* values are represented as *$\leq$0.05; **$\leq$0.01; ***$\leq$0.001. The values $\leq$0.05 were considered statistically significant.

Results

hADA3 colocalizes and interacts with PML-NBs

In our studies involving overexpression of EGFP-hADA3, we have consistently observed transfected cells to either express hADA3 homogeneously or in the punctate form. The latter appearance was usually found in the nuclear region which resembled the localization pattern observed for PML-NBs (Bernardi and Pandolfi 2007; Lallemand-Breitenbach and de The 2010). These observations intrigued us to check whether hADA3 and PML might colocalize and interact with each other. To address this question, we first co-expressed EGFP-hADA3 and DsRed-PML fusion proteins in U2OS cells and followed it up with confocal microscopy. The results shown in Fig. 1a demonstrated the appearance of more prominent fluorescence signals of hADA3 and PML in the nucleus. Also as expected, PML was found to form punctated PML-NBs in the nucleus with hADA3 also giving similar speckled pattern (Fig. 1a). Analysis through Z-stacking of the nucleus confirmed that the observed co-localization was not an artifact of microscopy and that the stochastically distributed speckled hADA3 colocalizes with PML-NBs in the axial plane as well. Moreover, maximum overlap between red (DsRed-PML) and green (EGFP-hADA3) pixels at diagonal indicated significant co-localization between the two proteins (Pearson's correlation coefficient, r = 0.72), Fig. 1b. The speckled hADA3 were found to colocalize with PML-NBs, in nearly 35% of successfully co-transfected cells (Fig. 1c). We also carried out the same in HEK293T cells and obtained similar punctate pattern of nuclear co-localization for EGFP-hADA3 and DsRed-PML (Fig. 1d). We further investigated the functional significance of this spatial co-localization by performing co-immunoprecipiation assay (Co-IP). Lysates prepared from HEK293T cells transfected with Myc-hADA3 alone or co-transfected with Flag-PML and Myc-hADA3 were subjected to immunoprecipitation with anti-Flag monoclonal antibody and analyzed by western blotting with anti-Myc antibody. Western data showed that PML was able to pull down hADA3 while, in control lane where the lysate contained only overexpressed Myc-hADA3, no band was observed (Fig. 1e). Similarly, hADA3 was also able to interact and pulldown PML when Co-IP assay was performed using Myc-PML and Flag-hADA3 constructs (Fig. 1f). Thus, these results complement the co-localization data, indicating that hADA3 is able to interact with PML and PML-NBs accommodate hADA3.

hADA3 speckle formation is dependent on PML

Since PML and hADA3 were found to interact with each other, next, we wanted to check if hADA3 and PML have any effect on each other's nuclear speckle formation. For this, HCT116-shADA3 and HCT116-shPML cells were transfected with DsRed-PML and EGFP-hADA3, respectively. HCT116 cells stably expressing pSUPER.retro.puro were taken as control. Western blot analysis revealed efficient knockdown of hADA3 and PML protein in the HCT116 knockdown stable cell lines, respectively (Fig. 2a). Analysis by fluorescence microscopy showed a drastic decrease in the hADA3 speckle formation in PML-depleted cells while no such effect was observed on PML-NB formation in hADA3 knockdown cells (Fig. 2b–e). Thus, the observations suggest that while hADA3 speckle formation is driven by PML, the formation of PML-NBs is independent of hADA3.

Though we were unable to visualize any perturbance in PML-NBs in hADA3-deficient cells, we suspect that

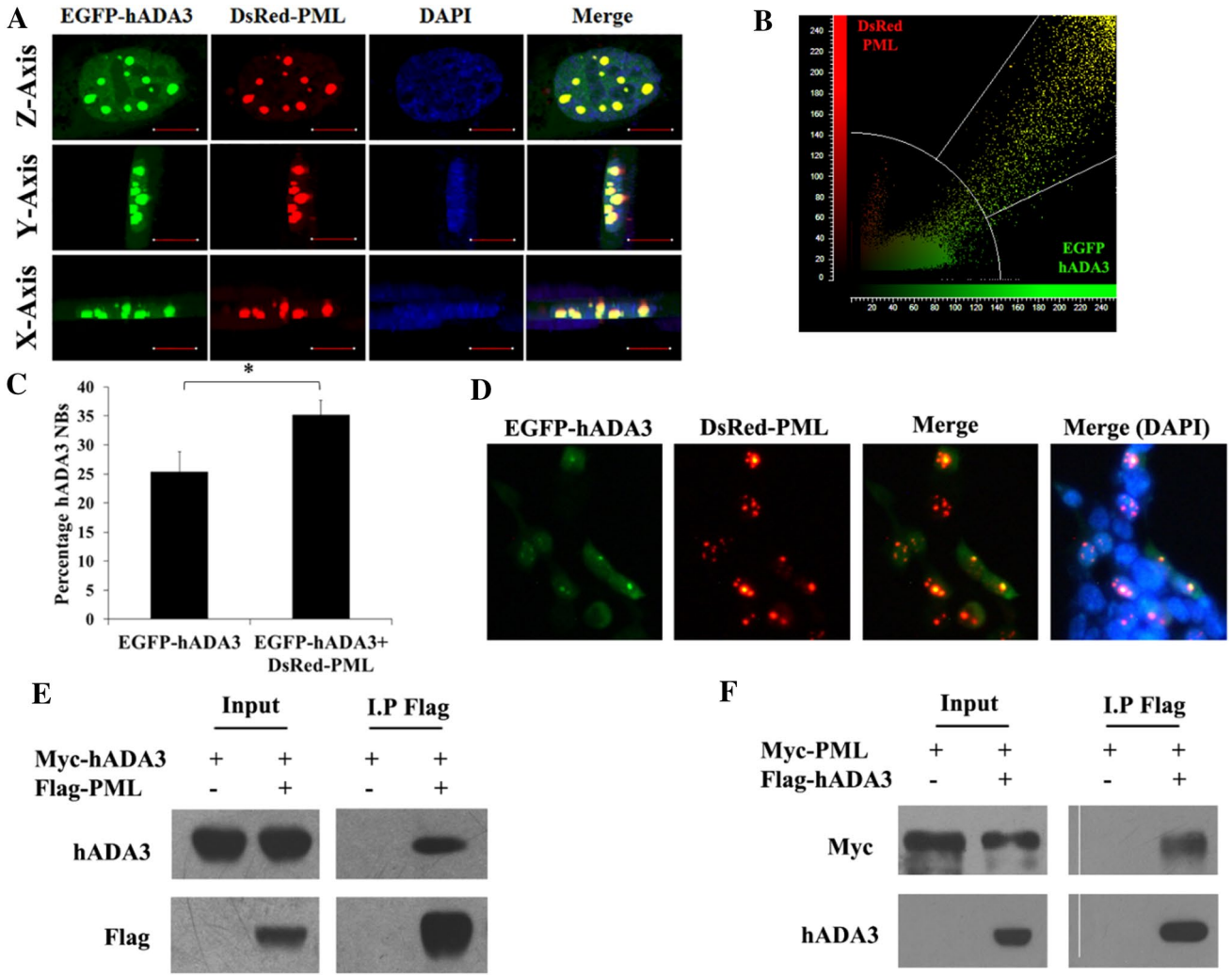


Fig. 1 hADA3 colocalizes and interacts with PML. **a** hADA3 and PML colocalize with each other. U2OS cells were transiently transfected with EGFP-hADA3 (Green) and DsRed-PML (Red) followed by confocal microscopy analyses 40 h after transfection. Scale bar is 10 µm. **b** Two-dimensional scatterplot for DsRed-PML and EGFP-hADA3 co-localization. **c** Quantification of hADA3 speckle formation in nucleus of U2OS cells in the presence and absence of PML. Results are given as standard error of mean ($*P \leq 0.05$). **d** Co-localization of EGFP-hADA3 and DsRed-PML in HEK293T cells. HEK293T were transiently transfected with EGFP-hADA3 (Green) and DsRed-PML (Red) followed by fluorescence microscopy 40 h post-transfection. Nuclei were counter-stained using DAPI. **e**, **f** In vivo interaction between hADA3 and PML. **e** Co-immunoprecipitation of hADA3 with PML. HEK293T cells were transiently transfected with Myc-hADA3 alone or co-transfected with Flag-PML and Myc-hADA3. The lysates prepared from these cells were subjected to immunoprecipitation with anti-Flag monoclonal antibody and analyzed by western blotting with anti-Myc antibody. **f** Co-immunoprecipitation of PML with hADA3. Lysates prepared from HEK293T cells transfected with Myc-PML alone or co-transfected with Myc-PML and Flag-hADA3 constructs were subjected to immunoprecipitation with anti-Flag monoclonal antibody and western blot analysis with anti-Myc antibody

hADA3 may be required for carrying out downstream coactivator functions. The coactivator functions of hADA3 are known to involve other proteins such as p300, a histone acetyltransferase, which is also reported to interact with PML and has been implicated in acetylation-mediated stabilization of p53 in PML-NBs upon genotoxic stress. The latter function has also been shown to be mediated by hADA3 (Nag et al. 2007; Wang et al. 2001). Thus, we investigated whether p300 recruitment and the resulting p53 retention in the complex at PML-NBs requires hADA3. To this end, we carried out co-immunoprecipitation of endogenous PML in control or shADA3 stably expressing MCF7 cell lines followed by western blot analysis. We observed that in the absence of hADA3, while PML is not able to interact with p300, its interaction with p53 is minimally affected (Fig. 2f).

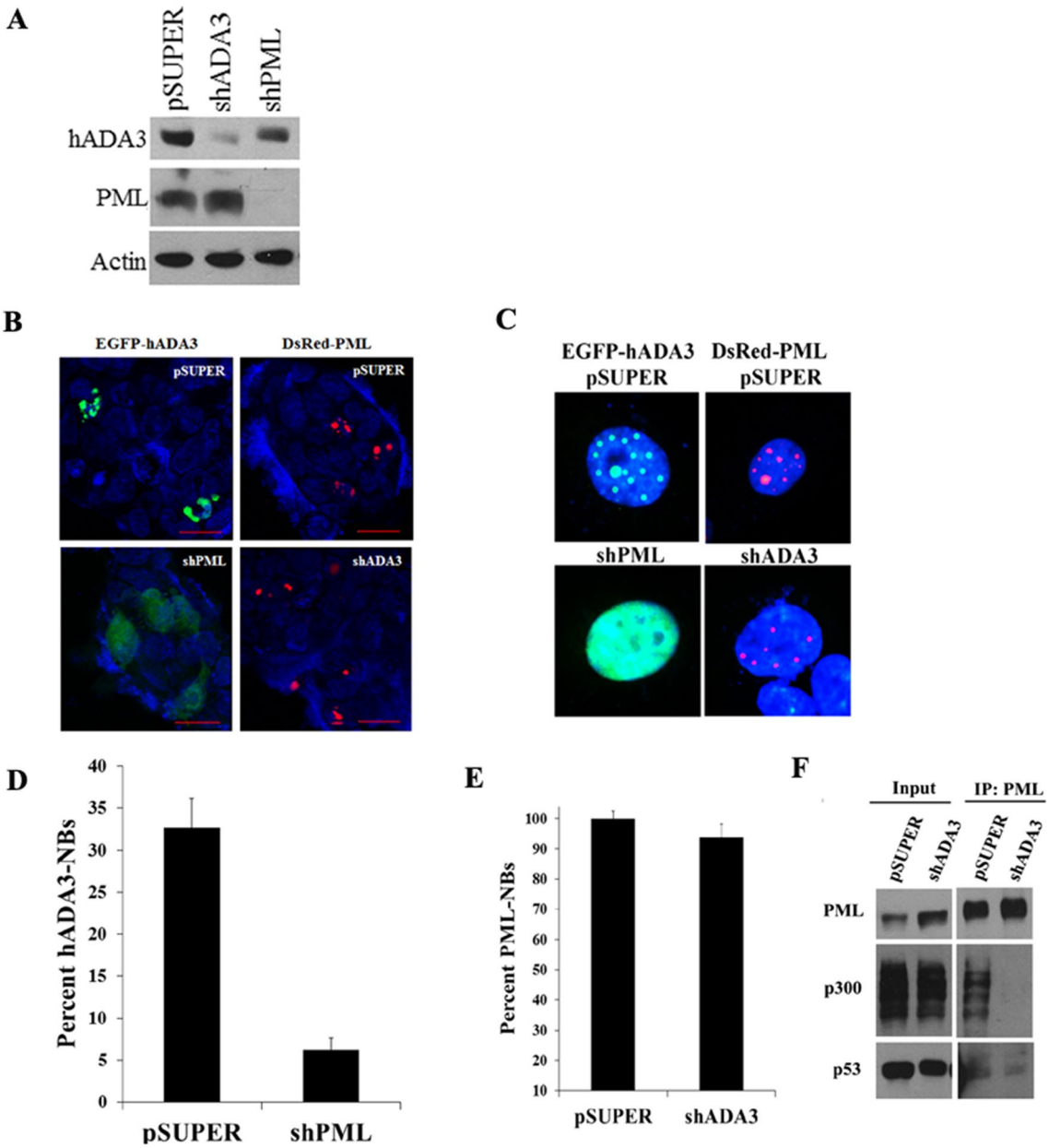


Fig. 2 hADA3 speckle formation is dependent on PML. **a** Western blot showing silencing control for hADA3 and PML. **b** Downregulation of PML negatively affects hADA3 speckle formation. HCT116 cells stably expressing control vector, shPML or shADA3 were grown on cover glass and transfected with either EGFP-hADA3 or DsRed-PML followed by fixing the cells with 4% paraformaldehyde and staining the nucleus with DAPI after 40 h of transfection. Images were captured under confocal microscope. Scale bar is 20 µm. **c** Magnified image of a single nucleus showing clear localization of hADA3 and PML-nuclear bodies in HCT116 cells stably expressing control vector, shPML or shADA3. The nucleus was stained with DAPI. **d** Bar graph depicting the percentage of hADA3-NBs in the presence and absence of PML in HCT116 cells. Results are given as standard error of mean (***$P \leq 0.001$). **e** Graphical representation of PML-NBs in the presence and absence of hADA3 in HCT116 cells. [Results are given as standard error of mean (NS—non significant)]. **f** hADA3 knockdown diminishes PML association with p300 but not with p53. MCF7 cells expressing control vector or s*hADA3* were subjected to immunoprecipitation with anti-PML antibody followed by western blotting with anti-PML, anti-p300 and anti-p53 antibody. Figure is the representative of at least three independent experiments

Mapping of interaction interfaces on hADA3 and PML

To characterize the interaction in detail and identify the domains responsible for mediating protein–protein interaction between hADA3 and PML, a series of deletion mutants of hADA3 namely [N-terminal (residues 1–144), Medial (residues 145–288) and C-terminal (residues 289–432) expressing constructs of Flag-hADA3] and PML were generated. Each of these truncated constructs

were co-transfected individually with wild-type Myc-PML in HEK293T cells and the lysates prepared 40 h post-transfection were subjected to co-immunoprecipitation assay. Briefly, anti-Flag monoclonal antibody was used to pull down the mutant hADA3 forms and anti-Myc antibody was used to detect PML. Our results show that both the N-terminus and C-terminus containing hADA3 mutant proteins interact with PML (Fig. 3a). While a strong interaction of PML was seen with hADA3 N-terminus, a significantly weaker one was observed with C-terminus of hADA3 protein. We also noticed an almost negligible involvement of medial domain of hADA3 in mediating the interaction with PML as shown in Fig. 3a. Next, we mapped the domains of PML that interact with hADA3 by employing a similar

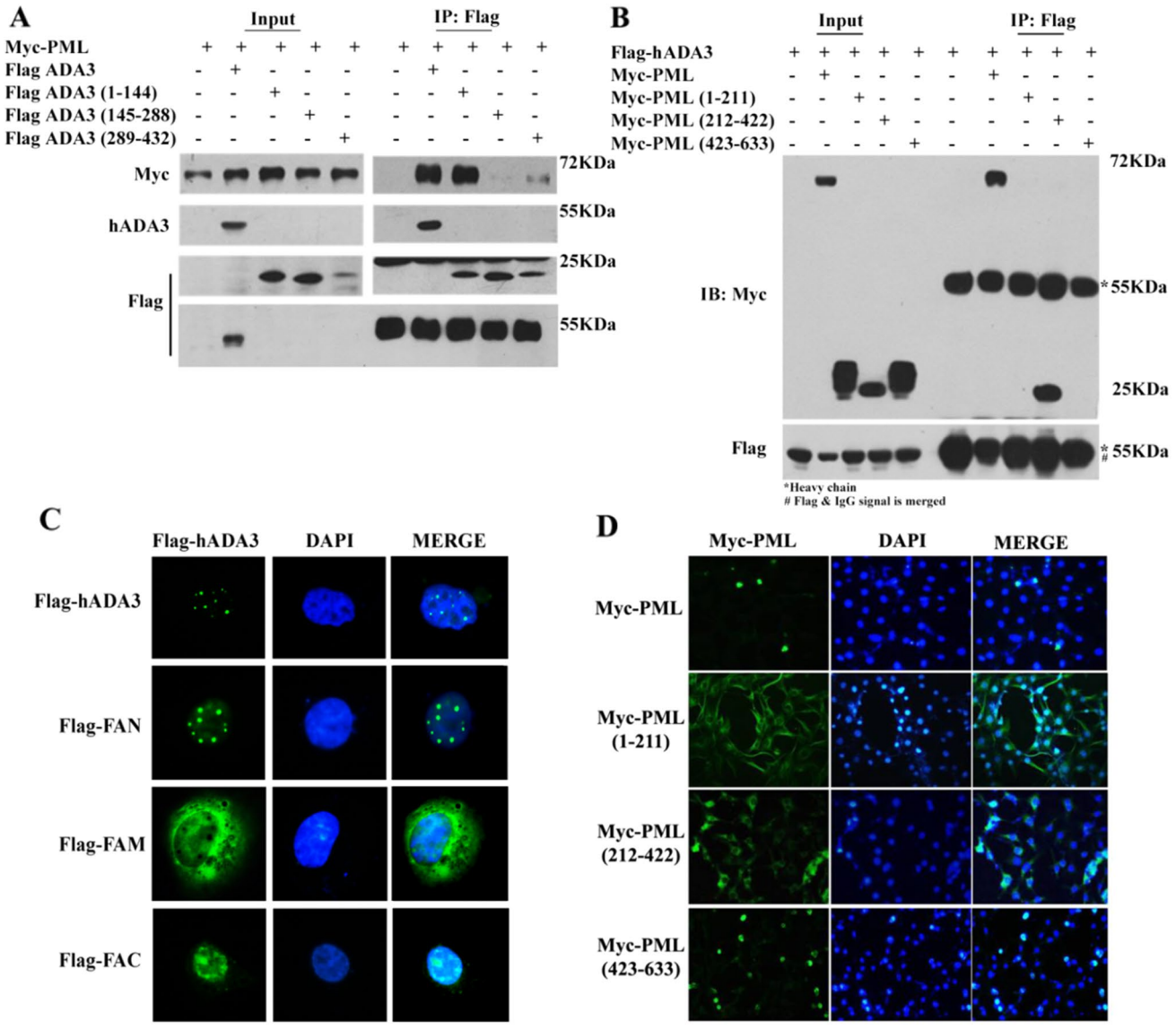


Fig. 3 hADA3 utilizes its N- and C-terminus while PML employs its middle domain for protein–protein interaction. **a** N- and C-terminus of hADA3 are involved in interaction with PML. HEK293T cells were transfected with either PML-WT alone or along with hADA3-WT or its deletion mutants. Lysates prepared were subjected to co-immunoprecipitation with anti-Flag antibody followed by western blot analysis with indicated antibodies. **b** A central fragment of PML (amino acids 212–422) interacts with hADA3. Lysates prepared from HEK293T cells transfected with hADA3-WT alone or co-transfected with PML-WT or its deletion mutants were immunoprecipitated using anti-Flag antibody. Afterwards, western blot analysis was performed using the indicated antibodies. **c** hADA3 and its mutants display distinct localization pattern. U2OS cells transfected with Flag hADA3-WT or its deletion mutants were immunostained followed by immunofluorescence detection. **d** PML and its mutants display a discrete accumulation pattern. U2OS cells transfected with MycPML-WT or its deletion mutants were subjected to immunostaining followed by immunofluorescence detection. Nuclei were stained with DAPI

strategy. The results show that the coiled coil region encompassing PML medial domain (amino acids 212–422) is involved in the interaction with hADA3 (Fig. 3b). However, the N-terminal proline-rich region or the C-terminal region containing serine–proline did not co-precipitate with hADA3. We also checked the localization pattern of these hADA3 mutants by immunofluorescence in U2OS cells. Interestingly, we found the N-terminal mutant (FAN) to mimic the WT foci pattern in the nucleus, whereas the C-terminus mutant (FAC) showed an almost diffused expression in the nucleus. On the contrary, the medial domain mutant (FAM) was present predominantly in the cytoplasm (Fig. 3c). Additionally, distinct nuclear localization, similar to the pattern exhibited by WT-PML, was obtained for Myc-PML (423–633) mutant whereas Myc-PML (1–211) mutant demonstrated prominent cytoplasmic localization (Fig. 3d). Interestingly, the Myc-PML (212–422) mutant was found to exhibit both nuclear and cytoplasmic localization, corroborating with the interaction study. Hence, the above results led us to conclude that hADA3 utilizes bipartite motif for interacting (via its N- and C-terminus domains) with medial domain of PML.

Genotoxic assault augments hADA3-PML interaction to restrict cellular proliferation

To investigate the functional relevance of PML-hADA3 association under genotoxic conditions, HEK293T cells were transfected with EGFP-hADA3 and DsRed-PML followed by exposure to two different doses of the DNA-damaging agents such as UV (causes cross-links and DNA adducts formation), etoposide (topoisomerase inhibitor and causes DSBs), hydroxyurea (ribonucleotide reductase inhibitor), adriamycin (DNA intercalator) and actinomycin D (DNA intercalator). Fluorescence microscopy revealed a significant increase in the number of cells exhibiting hADA3-PML

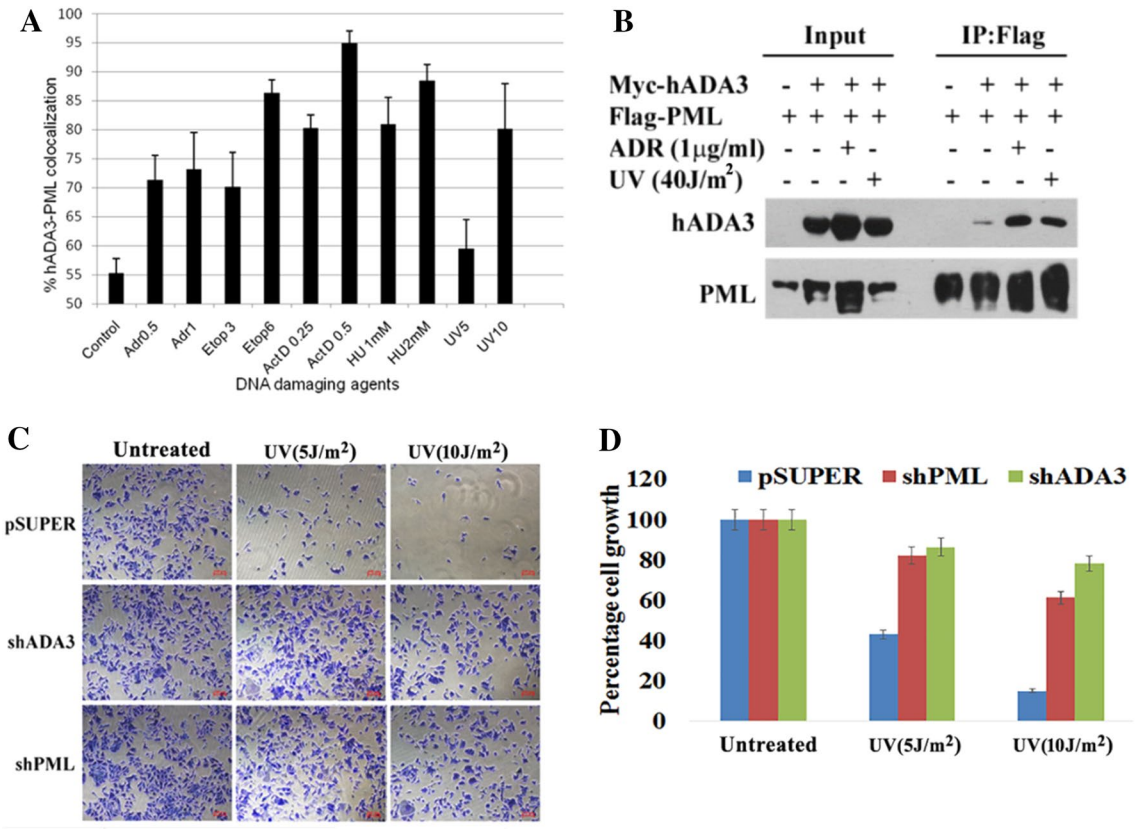


Fig. 4 Significance of hADA3-PML interaction upon DNA damage. **a** DNA damage enhances hADA3-PML co-localization. HEK293T cells were transfected with EGFP-hADA3 (Green) and DsRed-PML (Red) followed by exposure to various genotoxic agents: UV, adriamycin, actinomycin D, etoposide and hydroxyurea at the indicated doses. The percentage of cells showing overlap of hADA3 and PML were calculated and represented graphically. **b** Genotoxic stress enhances hADA3-PML association. HEK293T cells were transiently transfected with Flag-PML alone or together with Myc-hADA3. These cells were left untreated or treated with ADR or UV as indicated in the figure. Lysates were prepared and subjected to immunoprecipitation using Flag antibody followed by immunoblotting with anti-hADA3 and anti-Flag antibody. **c** ADA3 or PML knockdown increases cell viability. MCF7 cells (50% confluent) stably expressing pSUPER.retro.puro control vector, shADA3 and shPML were treated with 5 and 10 J/m^2 UV and allowed to grow for 7 days. Cells were then fixed and stained with crystal violet stain. **d** The cells were microscopically counted and graphically plotted

co-localization in treated sets as compared to control suggesting a high degree of co-localization of hADA3 in PML-NBs upon exposure to DNA damage (Fig. 4a, Supplementary Fig. 1). With the increasing doses a concomitant elevation in co-localization was also observed, except adriamycin which displayed a minimal change. We also observed around 55% co-localization of hADA3 speckles with PML-NBs in control cells thereby indicating the possibility of involvement of hADA3-PML complex in various other biological functions apart from DNA damage response. Further characterization of enhanced PML and hADA3 association under genotoxic stress was accomplished by co-immunoprecipitation assay wherein cells transfected with Myc-hADA3 and Flag-PML exhibited a positive interaction. Exposure to Adriamycin and UV stress culminated in increased hADA3-PML interaction (54.4% and 35.2%, respectively) in comparison to control cells (8.6%) (Fig. 4b). Furthermore, cell growth was assessed in MCF7 cells stably expressing pSUPER.retro.puro control vector, shADA3 and shPML treated with 5 J/m^2 and 10 J/m^2 UV. The knockdown cells exhibited an enhanced growth rate compared to control cells upon DNA damage (Fig. 4c, d). Clearly, the above findings suggest a functional importance of hADA3-PML interaction in DNA damage sensing and response.

Ablation of PML-hADA3 axis fails to induce apoptosis in response to DNA damage

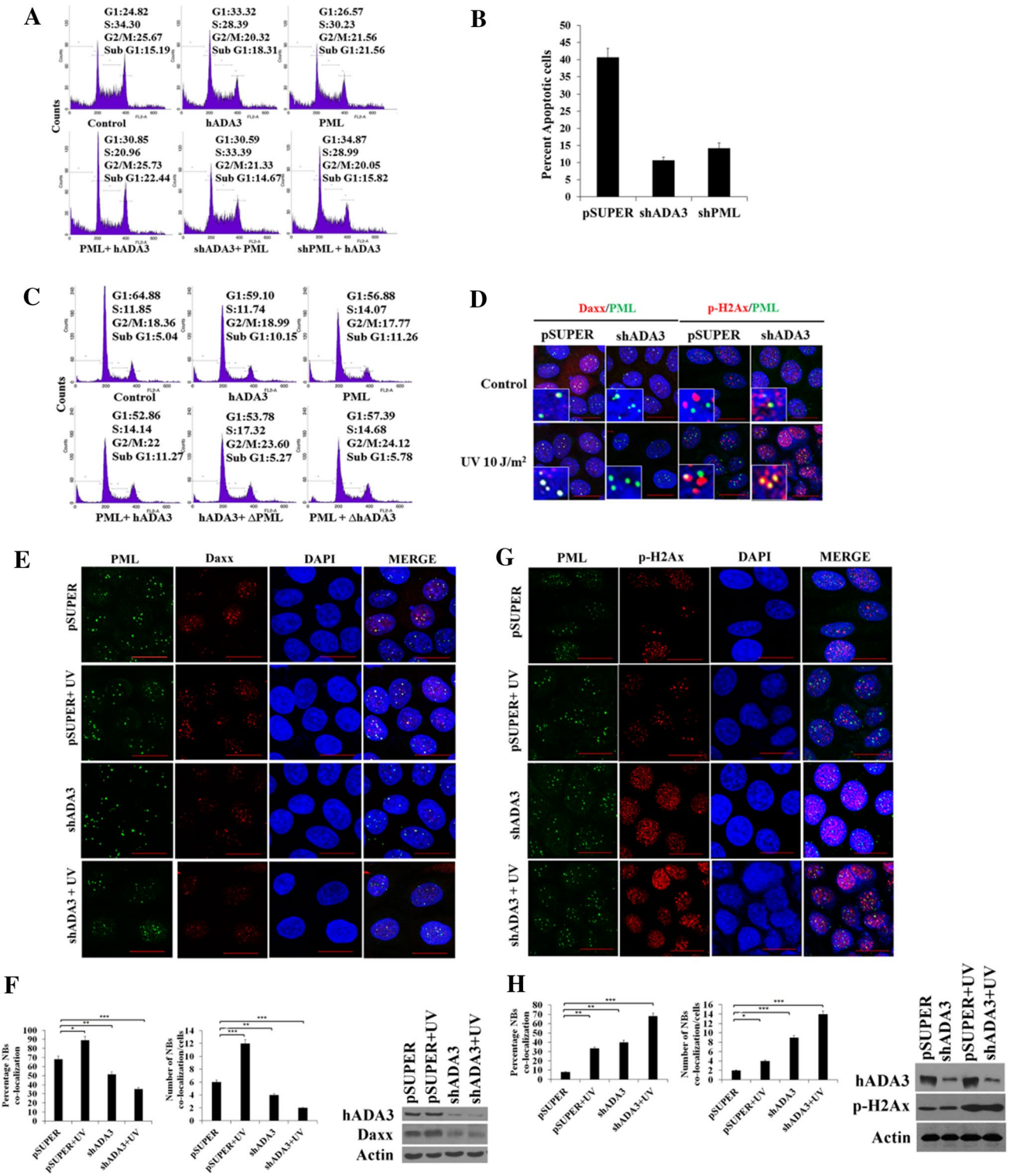
An initial response to cellular stress is cell cycle arrest and MEFs lacking hADA3 have previously been shown to undergo delay in G1-S progression (Mohibi et al. 2012). Therefore, we reasoned that hADA3 together with PML may have a role in bringing about cell cycle arrest upon DNA damage. To study this, HCT116 cells stably expressing control vector were transfected individually with control vector, Flag-hADA3, Flag-PML or combination of Flag-tagged hADA3 and Myc-PML. Additionally, stable lines of HCT116-shADA3 and HCT116-shPML cells expressing Myc-PML and Flag-hADA3, respectively, were allowed to grow for 24 h following UV exposure. Interestingly, the FACS analysis results showed an accumulation in the G1 phase cells ectopically expressing hADA3 alone or both hADA3 and PML as compared to control cells, after UV treatment (Fig. 5a).

Control cells overexpressing PML also exhibited minor increase in G1 population upon UV treatment. Strikingly, overexpression of hADA3 in PML-depleted cells yielded a slightly higher percentage of G1 population in comparison to cells lacking hADA3 and overexpressing PML indicating that hADA3 may be involved in mediating G1 arrest independent of PML (Fig. 5a).

Several reports have established the importance of hADA3 in DNA damage response (Mirza et al. 2012). Independent studies on PML also highlight its role in DNA damage sensing and subsequent response (Dellaire and Bazett-Jones 2004). However, the contribution of the hADA3-PML axis in DNA damage repair has not been explored till date. This intrigued us to investigate the potential function of this complex in cellular response to genotoxic stress. To further corroborate our findings, we prepared MCF7 cells stably expressing pSUPER.retro.puro control vector, shADA3 or shPML and performed annexin-V staining to gauge the extent of apoptosis. Our results demonstrated that cells containing knockdown of hADA3 or PML exhibited a drop in annexin-V signals, thereby implying decreased apoptosis in these cells (Fig. 5b). Moreover, we aimed to further clarify if ectopic expression of hADA3 in PML-depleted cells and vice versa could sensitize cells to UV-induced apoptosis. As shown in Fig. 5a, overexpression of hADA3 or PML led to ~ 1.2- to 1.4-fold increase in sub-G1 population after UV damage (apoptotic cell population), compared to the control set. Likewise, cells depleted of hADA3 and expressing PML or vice versa exhibited levels of sub-G1 population comparable to that of control group. These findings thus strongly hinted towards the hADA3-positive role of hADA3-PML collaboration in mediating apoptosis (Fig. 5a).

We also performed similar FACS analysis in HCT116 cells expressing either full length hADA3 and PML proteins or Flag-hADA3 WT with Myc-PML (212–422, hADA3 non-interacting mutant, represented as ΔPML) or Myc-PML WT with Flag-hADA3 (145–288, represented as ΔhADA3) mutant. The results revealed ~ twofold suppression in the Sub-G1 population of cells expressing either hADA3 or PML non-interacting mutants. This observation further substantiated our previous assumption of hADA3 and PML interaction being a key component in regulating apoptosis (shown in Fig. 5c).

Given that Daxx is a key player in the coordination of PML-mediated apoptosis (Torii et al. 1999; Zhong et al. 2000), we were, therefore, driven to examine the effect of hADA3 on the co-localization of endogenous PML and Daxx proteins upon DNA damage. Confocal microscopic analysis revealed a considerable enhancement of 22% in the co-accumulation of PML with Daxx in cells expressing pSUPER construct upon UV treatment. However, this association was severely compromised upon hADA3 depletion, diminishing from 50% (untreated) to 37% in UV-treated cells, suggesting the importance of hADA3 in PML-mediated apoptosis (Fig. 5d–f). Finally we explored the relevance of hADA3 and PML interaction in mediating DNA repair processes. To this end, we immunostained the same set of cells for PML and γH2AX and checked for their co-localization following DNA damage. Interestingly, upon UV damage, γH2AX co-localization with PML was substantially enhanced from 40 to 70% in hADA3-depleted cells (Fig. 5d, g, h). However,



γH2AX-PML co-localization was found to escalate from 7 to 35% in pSUPER-expressing MCF7 cells as well. Taken together, these data support the concept that the functional complex between hADA3 and PML has crucial roles in DNA damage repair and apoptotic processes.

Discussion

Human ADA3 serves as a key adaptor protein and is present in various HAT complexes including SAGA, STAGA and ATAC. It also recruits p300 and PCAF to facilitate

◂**Fig. 5** Ablation of PML-hADA3 axis causes defective cellular response to DNA damage. **a** hADA3 and PML may be involved in DNA damage sensing and G1 arrest upon genotoxic stress. HCT116 cells stably expressing pSUPER.retro.puro control vector were transfected with either Flag-hADA3 or Flag-PML or both. Additionally, shADA3 or shPML stable cell lines of HCT116 cells were transfected with Flag-PML and Flag-hADA3, respectively. Transfected cells were treated with UV (40 J/m^2) and 24 h later harvested with 0.4% EDTA containing PBS and fixed in 85% ethanol. Afterwards, propidium iodide (50 µg/ml) staining was performed and cell cycle distribution and the sub G1 phase cell identification was done using FACSCalibur (Becton–Dickinson, USA) with at least 10,000 events for each sample. Analysis was performed using CellQuest software. **b** Knockdown of hADA3 or PML lead to decreased apoptosis. MCF7 cells (50% confluent) stably expressing pSUPER.retro.puro control vector, shADA3 and shPML were treated with 10 J/m^2 UV and allowed to grow for 24 h. Subsequently, cells were stained with annexin-V and propidium iodide as directed by the manufacturer protocol followed by fluorescence microscopy and Annexin-positive cells were counted and graph was plotted. Results are given as standard error of mean (***P ≤ 0.001). **c** Non-interacting mutants of PML and hADA3 bring about suppression of sub-G1 cellular population. FACS analysis of HCT116 cells stably expressing either control vector, Flag-hADA3, Myc-PML alone or together or Flag-hADA3 with ΔPML (423–633) and Myc-PML with ΔhADA3 (145–288) non-interacting deletion mutants. **d**–**h** hADA3 is required for enhancing Daxx and PML co-localization. **d** MCF7 cells stably expressing control (pSUPER) or shADA3 constructs were left untreated or subjected to 10 J/m^2 UV dosage followed by immunofluorescence detection of PML co-localization with Daxx and γH2AX (merged). Figure is the representative of at least three independent experiments. Scale bar is 20 µm. Representative immunofluorescence images showing individual localization of PML and Daxx (**e**) and **g** γH2AX. **f** Graphical depiction of the percentage and number of PML-nuclear bodies colocalizing with Daxx. Western blot analysis showing expression of Daxx in pSUPER and shADA3 MCF7 cells in the presence and absence of UV treatment. Similar quantification and western blot analysis was performed for γH2AX as well (**h**)

acetylation-mediated stabilization and activation of p53 upon genomic stress (Gamper et al. 2009; Hardy et al. 2002; Martinez et al. 1998; Nag et al. 2007; Wang et al. 2008). Tumor suppressor protein PML, on the other hand, is found to characteristically exist as nuclear bodies in the nucleus which gives a punctate appearance (Lallemand-Breitenbach and de The 2010). Additionally, PML can colocalize with p14ARF to recruit p53 and HDM2 for proteasomal degradation (Kashuba et al. 2003). In a similar fashion, p14ARF also stimulates hADA3 to cause p53 acetylation-dependent senescence (Sekaric et al. 2007). Lastly, one of the common characteristics shared by PML-interacting proteins is that they all undergo SUMOylation, which has been recently found to hold true for hADA3 as well (Bernardi and Pandolfi 2007; Chand et al. 2014).

In this study, we have identified PML as a novel interacting partner of hADA3 (Fig. 1). Further delineation of protein–protein interaction sites on these two proteins revealed that hADA3 employs its N- and C-terminal to interact with the medial domain of PML (Fig. 3a, b). PML-NBs have been known to associate with transcription sites for both activated and repressed genes (Eskiw et al. 2004; Shiels et al. 2001; Tsukamoto et al. 2000). Considering the role of hADA3 as transcriptional coactivator and our observation that hADA3 localizes with only a subset of PML-NBs, it could be possible that hADA3 preferentially colocalizes with PML associated with the activated genes. Additionally, we observed that hADA3 speckle formation is dependent on PML but not the vice versa alluding to the likelihood that hADA3 is not required for maintaining PML-NB structural integrity. However, the option for PML-mediated recruitment of hADA3 at transcriptionally active sites (Fig. 2a–c) still exists. One of the common interacting partners of both hADA3 and PML is p300, a HAT (Nag et al. 2007; Pearson et al. 2000; Shima et al. 2008; Wang et al. 2001). A subset of PML-NBs has previously been shown to colocalize with p300 at sites of active transcription. Also, in response to genotoxic or oncogenic stress, PML-NBs can induce senescence by mediating acetylation-dependent stabilization of p53 by recruiting various post-translational modifiers including p300 (Bernardi et al. 2008; Boisvert et al. 2001; Guo et al. 2000). Previous reports demonstrate that hADA3 mutant, defective in acetylation by p300, fails to stabilize p53 (Sekaric et al. 2007). Here, we found that in absence of hADA3, PML is not able to interact with p300, however, its interaction with p53 remains unaffected (Fig. 2d). This suggests that p300-mediated p53 acetylation in PML-NBs might involve hADA3, notwithstanding, the hypothesis warrants further investigations.

PML-NBs are dynamic structures that are known to undergo change in number, size and structure in response to cellular stress such as DNA damage, heat shock and exposure to heavy metals (Bernardi and Pandolfi 2007; Dellaire and Bazett-Jones 2004). Interestingly, our interaction studies also revealed that interaction of PML and hADA3 was significantly enhanced upon genotoxic stress implicating the role of this interaction in DNA damage sensing and response (Fig. 4a–d). Apart from this elevated co-localization, a significant co-existence in the control cells implies the role of hADA3-PML interaction in various other cellular processes, which demands further investigation. Various proteins involved in DNA damage repair (DDR) and checkpoint signaling have previously been shown to associate with PML-NB upon genotoxic stress (Dellaire and Bazett-Jones 2004). Notably, p53 is recruited to PML-NBs upon treatment with γ-irradiation, UV or arsenic trioxide for post-translational modifications like phosphorylation and acetylation (Guo et al. 2000). Additionally, PML-NBs, even in the absence of DNA damage, can serve as a reserve depot for DDR proteins such as, MRE11-RAD50-NBS1 complex, CHK2, ATR, RPA and BLM (Dellaire and Bazett-Jones 2004). Interestingly, hADA3$^{-/-}$ MEFs have been found to exhibit genomic instability as evident from chromosomal breaks, fragmentation, deletion and translocation which gets further aggravated

upon DNA damage (Mirza et al. 2012). These data indicate that hADA3 and PML may function together or synergistically in DDR pathway, however, it necessitates further study.

Excessive DNA damage that compromises genomic integrity beyond repair can lead to apoptosis or type I programmed cell death (PCD), an active process that involves controlled elimination of unwanted cells from the body of multicellular organism without provoking inflammation. PML is known to pleiotropically regulate function of several pro-apoptotic factors such as p53 and death domain-associated protein 6 (also known as Daxx) (Li et al. 2000; Torii et al. 1999; Zhong et al. 2000). Homeodomain-interacting protein kinase-2 (HIPK2) which is known to phosphorylate p53 also mediates the same effect on PML (D'Orazi et al. 2002; Hofmann et al. 2002). Interestingly, upon UV stress, HIPK2-dependent phosphorylation of p53 in PML-NBs followed by its CBP-dependent acetylation is involved in p53 transactivation (Shima et al. 2008). Considering p300/CBP interacts with hADA3, it is conceivable that hADA3 may be involved in this process. Daxx is a multifunctional subcellular protein with ambiguous role in apoptosis (Tang et al. 2015). In agreement with the previous report (Li et al. 2000), nuclear Daxx was found to associate with PML-NBs wherein it corresponds to increase in pro-apoptotic function (Fig. 5d–f). FACS analysis further unfolded the cell cycle regulatory affair of hADA3-PML, where an increment in sub-G1 population overexpressing hADA3 and PML was observed (Fig. 5a). Furthermore, cells depleted of hADA3 and expressing PML or vice versa exhibited levels of sub-G1 population comparable to that of control group. Similar effect was observed in cells expressing WT-hADA3 and PML deletion mutant or vice versa. However, overexpressing hADA3 or PML with the non-interacting mutants of PML or hADA3 did not cause any enhancement in the sub-G1 population which might be due to the sequestering of the activity of wild-type proteins. Hence, even though the wild-type proteins are expressed, they still cannot perform their normal function (Fig. 5c). This indicated that, functionally, hADA3 and PML interaction might be involved in mediating apoptotic process. This speculation was confirmed by our observation that hADA3-depleted cells show decrease in co-localization of Daxx with PML upon genotoxic stress, indicating decrease in apoptosis. At the same time, these cells exhibited increased co-localization of γH2AX with PML which is in accordance with a previous report wherein γH2AX is required for maintenance of MRN complex in irradiation-induced foci (IRIF) (Paull et al. 2000). Recently, it has also been reported that hADA3$^{-/-}$ MEFs display delay in the disappearance of γH2AX and other DNA damage response protein containing foci post-IR treatment (Mirza et al. 2012). An interesting observation from this investigation is the build-up of γH2AX-PML foci in the control cells, which amplified from a mere 7% in the pSUPER-expressing untreated cells to 40% in the hADA3-depleted cells (Fig. 5d, g, h). This basal increase can be attributed to the knockdown of hADA3, which in turn emphasizes its role in DNA damage response. Considering the already established role of PML-NBs in DDR, we speculate that hADA3 and PML may act together in the repair-sensing process as depletion of hADA3 may not allow PML to mediate its proper effect in DNA damage response (Dellaire and Bazett-Jones 2004).

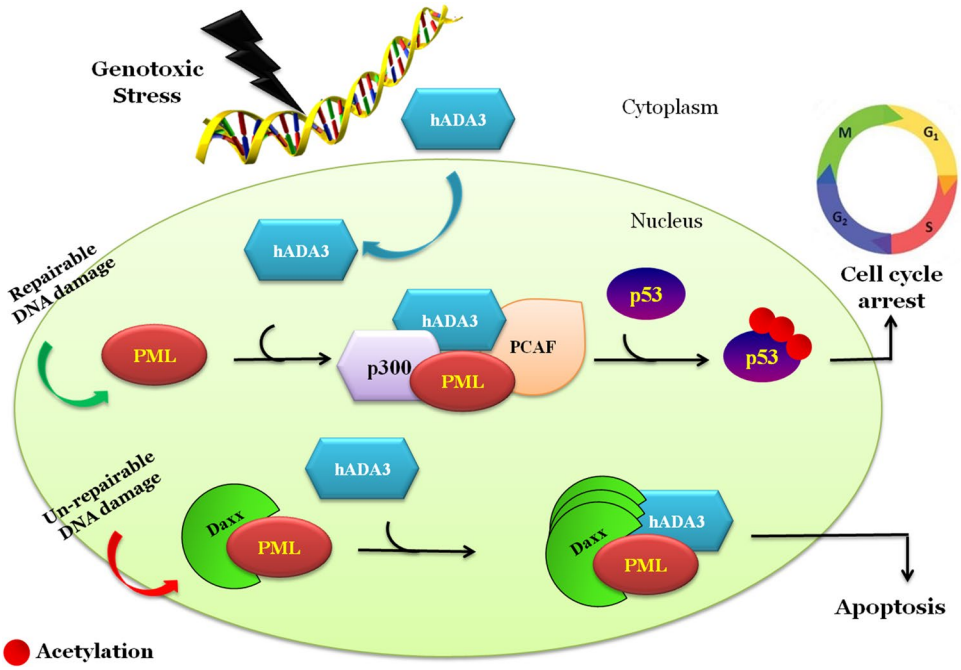


Fig. 6 Schematic model summarizing the mechanisms and functions of PML-hADA3 under varying conditions of genotoxic stress. As part of cellular response to varying DNA-damaging stimuli, hADA3 translocates to the nucleus and undergoes enhanced association with the PML-NBs. This complex then either induces cell cycle arrest via p53 acetylation through recruitment of different acetylating modulators, like PCAF, p300, or stimulates apoptosis by heightened engagement with Daxx

Taken together, we have identified PML as a novel hADA3-interacting protein and our report for the first time demonstrates that PML plays an important role in hADA3-induced DNA damage response. Based on our findings, as well as published literature, we propose a model (Fig. 6). Genotoxic stress induces hADA3 translocation in the nucleus where it associates with PML along with p300 and PCAF, and this complex causes p53 acetylation and stabilization to trigger cell cycle arrest. Severe DNA damage, on the other hand, prompts the hADA3-PML complex to dynamically recruit Daxx protein for mediating cellular apoptosis. As shown in our report (Fig. 5f) the Daxx levels severely decline upon hADA3 knockdown thus hampering the PML-mediated DNA damage response. Thus, hADA3 forms an essential component of the PML-induced genotoxic stress response.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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