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GENOTOXIC STRESS INDUCED POSTTRANSLATIONAL MODIFICATION OF TRANSCRIPTIONAL ADAPTOR PROTEIN ADA3

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Abstract: Posttranslational modification is one of the crucial mechanisms involved in initiating and propagating cellular defense response towards genotoxic insults. It is also a part of the fine tuning mechanism that allows the system to have precise control over protein stability and activity depending on the intensity of the stress signal. Human ADA3 has been implicated in the transcriptional regulation of DNA damage response genes by potentiating acetylation mediated stability and activity of p53. However, impact of posttranslational modification on hADA3 function has not been studied so far. Our current investigation shows that hADA3 is induced in response to DNA damage treatment with adriamycin in a p53 dependent manner. Further analysis revealed that prolonged adriamycin treatment imparts posttranslational modification and hence enhanced stability to hADA3 protein. Treatment with the kinase inhibitor caffeine prevented accumulation of slower migrating forms of hADA3 indicating a role of ATM/ATR kinases in DNA damage mediated modification of hADA3. In summary, our data shows that hADA3 undergoes posttranslational modification under genotoxic stress and suggests possible mechanism by which hADA3 may trigger activation of DNA damage response pathways.

Key Words: hADA3; p53; ATM/ATR; Genotoxic Stress; Posttranslational modifications.

Introduction

Maintenance of genomic stability is one of the key requirements for cell survival. DNA, being the most vulnerable component of the cell, is the main victim of various kinds of insults by both external and internal culprits. These include myriad of environmental genotoxic agents like alkylating compounds, polycyclic aromatic hydrocarbons, biphenyls heterocyclic amines, ultraviolet light, ionizing radiations, therapeutic drugs or cellular metabolic byproducts (Oberle and Blattner, 2010; Roos and Kaina, 2006). For healthy survival, all living organisms have evolved robust and efficient mechanisms of repair of a wide variety of DNA lesions. Typically, DNA damage elicits an orchestrated damage response that stalls the cell cycle and activates specific DNA repair mechanisms (Jackson and Bartek, 2009). If the damage is too severe to be repaired, the cells are eliminated by apoptosis. Failure to repair DNA lesion leads to genomic instability and contributes to pathogenesis like premature ageing and cancer (Hannah and Zhou, 2009). Elegant biochemical studies by several researchers revealed that various transcriptional and posttranscriptional mechanisms govern the execution of these damage responses. There exists a complex interplay between multiple protein modifications including phosphorylation, acetylation (Liu *et al.*, 1999), ubiquitination (Al-Hakim *et al.*, 2010), SUMOylation (Bartek and Hodny, 2010) and NEDDylation in relaying DNA damage signals leading to the onset of cell cycle arrest, DNA repair, apoptosis and senescence (Harris and Levine, 2005; Huen and Chen, 2008; Polo and Jackson, 2011). Phosphorylation is one of the commonly reported protein modifications that occurs in mammalian cells and serve as a switch to regulate protein-protein interactions (Huen and Chen, 2008). It is a rapid and reversible process required for the regulation of a number of proteins and sometimes a necessity for subsequent modifications (Bernardi and Pandolfi, 2007; Bode

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and Dong, 2004). A large-scale proteomic analysis has recently identified DNA damage induced phosphorylation of more than 700 proteins (Matsuoka *et al.*, 2007). The most intensively studied tumor suppressor gene p53 is a classical example of such regulation (Brooks and Gu, 2003). It is otherwise a short-lived transcription factor but when confronted with stress signals, gets stabilized by undergoing posttranslational modifications like phosphorylation (Banin *et al.*, 1998; Khanna *et al.*, 1998) and acetylation (Ashcroft and Vousden, 1999). While modifications like phosphorylation and acetylation stabilizes p53, acetylation alone has been shown to enhance the transcriptional activity by exposing the DNA binding domain (Avantaggiati *et al.*, 1997; Gu and Roeder, 1997; Sakaguchi *et al.*, 1998). In general, a complex and diverse array of covalent modification not only stabilize p53, but also influence the selection of specific p53 target genes resulting in induction of either growth arrest or apoptotic pathways(el-Deiry *et al.*, 1993; Lavin and Gueven, 2006; Meek, 2004; Miyashita and Reed, 1995).

Recently, it has been shown that the transcriptional coactivator hADA3 plays a key role in DNA damage induced acetylation of p53 leading to stabilization and augmentation of its transcriptional activity (Nag *et al.*, 2007, Sekaric *et al.*, 2007). Though hADA3 has been implicated to have important role in a number of diverse physiological processes including regulation of nuclear receptor functions (Zeng *et al.*, 2002), chromatin remodeling by virtue of its association with a number of HAT complexes like PCAF (Ogryzko *et al.*, 1998), p300 (Wang *et al.*, 2001), STAGA (Martinez *et al.*, 2001), GCN5 (Germaniuk-Kurowska *et al.*, 2007) and in senescence (Sekaric *et al.*, 2007), a lot remains to be explored. For instance, the status of hADA3 or related complexes under genotoxic stress still remains a mystery. Hence, to gain functional insights of hADA3, it is of key importance to decipher the mechanisms by which its levels and activity are regulated in response to genotoxic insult. Since posttranslational modification is an integral part of DNA damage response and no information is available regarding such modifications of hADA3, therefore the purpose of our current study was to examine the effect of DNA damage on the posttranslational modification and stability of hADA3. Using a simple approach of exposing mammalian cells to genotoxic agent like adriamycin, we showed a p53 dependent stabilization of hADA3. Our study also revealed that phosphorylation by ATM/ATR kinases is likely to be the key step in the subsequent posttranslational modification and stabilization of hADA3. These findings therefore highlight a previously unappreciated function of hADA3 in provoking DNA damage response to maintain genomic integrity.

Materials and Methods

Chemicals, Reagents and Antibodies – Adriamycin, caffeine, and NP-40 were purchased from Sigma (St. Louis, MO). Protease inhibitor cocktail was obtained from Roche Applied Science (Mannheim, Germany). Polyclonal antibody against hADA3 was a kind gift from Dr. Vimla Band (University of Nebraska Medical Center, Nebraska, USA), anti-p53 was bought from Santa Cruz (Santa Cruz, CA) and β-actin antibody was from Abcam Inc. (Cambridge, MA).

Cell culture – HeLa and MCF7 cell lines were generously gifted by Dr. Vimla Band (University of Nebraska Medical Center, Nebraska, USA). U2OS cell line was kindly provided by Dr. Pradip Raychaudhuri (University of Illinois at Chicago, Chicago, USA). HEK293T cell line was generously provided by Dr. Sagar Sen Gupta (National Institute of Immunology, New Delhi, India). Cell lines were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 100U/mL of penicillin and 100mg/ml streptomycin (Invitrogen) in a humidified incubator with 5% CO_2 atmosphere at 37° C.

Adriamycin and Caffeine treatment – Exponentially growing cells were plated at a confluency of 1.5 X 10^6 in a 60 mm culture dish and allowed to adhere to the plate overnight. Next morning, adriamycin (ADR) was added at a final concentration of 0.25µg/ml. For caffeine treatment, 2mM caffeine was added to cells 1h prior to ADR treatment.

Cell Lysis and Western blotting – Cells were harvested and lysed by incubating in buffer containing 50mM Tris.Cl (pH 7.5), 400mM NaCl,

0.2% NP40, 10% glycerol, 5mM EDTA and protease inhibitors. Equivalent amounts of whole cell lysates were resolved by SDS-PAGE, transferred to PVDF membrane and then subjected to immunoblot analysis with appropriate primary and secondary antibodies. The signals were detected by enhanced chemiluminescence (ECL) technique.

Results

Accumulation of hADA3 after ADR Treatment

Human ADA3 has long been associated with p53 in terms of its interaction, stability and function (Kumar *et al.*, 2002; Nag *et al.*, 2007; Shamanin *et al.*, 2008; Wang *et al.*, 2001). Recent reports (Kumar *et al.*, 2002; Nag *et al.*, 2007) have demonstrated that hADA3 interacts with endogenous p53 and enhances its transactivation function by acetylating and stabilizing it upon DNA damage. These findings signify the role of hADA3 in damaged DNA response and prompted us to study the status of hADA3 following DNA damage. To examine the involvement of hADA3 in genotoxic stress response, we treated HeLa and HEK293T cells with ADR for varying time periods followed by Western blot analysis with anti-hADA3 antibody. As anticipated, cells exhibited a normal response towards the DNA damage by upregulating the level of p53 upon the ADR treatment (Fig. 1). Interestingly, a dramatic increase in the level of hADA3 was also seen within few hours of cells sustaining DNA damage. Further, we observed a DNA damage induced appearance of hADA3 doublets in HEK293T cells (Fig. 1.B) which are typical of either phosphorylated or acetylated forms of protein. This raises the possibility that stabilization of hADA3 upon DNA damage may be mediated through posttranslational modifications. These results also suggest that hADA3 is capable of sensing the genomic insult and therefore may be involved in evoking the DNA damage response in these cell lines.

p53 Dependent Upregulation of hADA3 upon DNA Damage

The tumor suppressor protein p53 is a critical component of the cellular stress management system. It gets activated upon DNA damage and transcribes a plethora of genes that initiate DNA repair, growth arrest, senescence, and apoptosis (Lavin and Gueven, 2006). As an extension of the previous experiment, our next goal was to investigate whether p53 has any role in the DNA damage mediated upregulation of hADA3. For this, we exposed the cervical cancer cell line C33A which harbors a mutant p53 to ADR for various time intervals. Immunoblot analysis with hADA3 antibody failed to show an evident induction in the level of hADA3 protein after the ADR treatment (Fig. 2). Consistent with earlier findings, genotoxic stress had no effect on p53 protein level of C33A cells (Kessis *et al.*, 1993; Scheffner *et al.*, 1991). This shows that functional p53 is an essential requirement for genotoxic stress induced upregulation of hADA3. Since p53 is a known interacting partner of hADA3 (Nag *et al.*, 2007; Wang *et al.*, 2001), it is conceivable that DNA damage may induce formation of more stable hADA3-p53 complex. As a result, major proteolytic cleavage sites on hADA3 are masked making it less susceptible for degradation. Thus, our study reveals that hADA3 is induced in response to DNA damage in a p53-dependent manner.

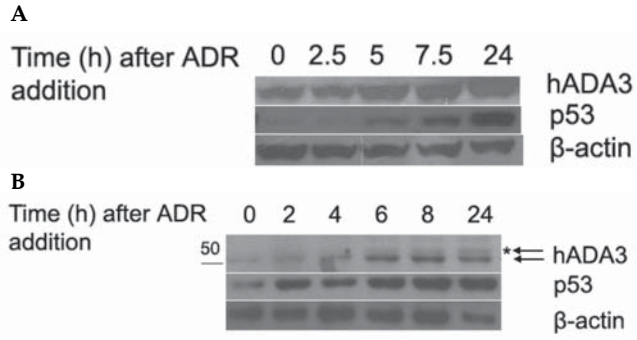

Figure 1: **Effect of adriamycin treatment on the levels of cellular hADA3. To induce DNA damage, (A) HeLa cells and (B) HEK293T cells were treated with 0.25μg/ml of ADR for the indicated time points prior to cell lysis. Total cell lysates (30μg) were run on SDS-PAGE gel followed by immunoblotting with antibodies against anti-hADA3 (asterisk * indicates modified form), anti-p53 and anti-β-actin (as loading control)**

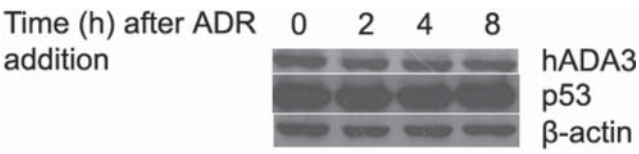

Figure 2: **Effect of DNA damage on the levels of hADA3 in cells with mutant p53. Following treatment with ADR (0.25μg/ml), C33A cells with mutant p53 were lysed. Cell lysates (30μg) were subjected to SDS-PAGE followed by Western blotting with anti-hADA3, anti-p53 and anti-β-actin antibodies**

ADR Treatment Induces Posttranslational Modification of hADA3

The principle that governs the complex transcriptional regulatory network can be attributed to the array of posttranslational modifications of sensing molecules during normal or stressed conditions. To explore whether ADR treatment provokes posttranslational modifications of hADA3, U20S cells with wild type p53 were exposed to ADR for varying time intervals. Whole cell lysates were immunoblotted with hADA3 specific antibody. Results obtained after probing the major part of the blot with anti-hADA3 antibody, showed a gradual appearance of ladder consisting of slower migrating species of hADA3 (upto ~98 kDa as shown in Fig. 3) with prolonged DNA damage treatment. Manifestation of this shift in electrophoretic mobility of hADA3 raises several possibilities. It could be due to the presence of multiple covalent posttranslational modifications on hADA3. Another possibility could be DNA damage induced dimerization of hADA3 since the apparent mobility of the major band corresponds to the molecular weight of approximately two molecules of hADA3. Therefore, the data demonstrated that DNA damage triggers a posttranslational modification cascade of hADA3 resulting in accumulation of differentially modified forms of hADA3.

Caffeine Attenuates ADR Mediated Posttranslational Modification of hADA3

DNA damage response is a fine tuned intricate network of signaling molecules that orchestrate multiple pathways to repair the lesion. The PI3K related protein kinase family comprising ATM, ATR, ATX/SMG-1, mTOR/FRAP and DNA-PK are at the top of the web of this pathway by sensing the damage and transmitting it to the downstream targets (Branzei and Foiani, 2008; Durocher and Jackson, 2001; Shiloh, 2001). Posttranslational modification of key regulators by protein phosphorylation has been the most extensively studied potential functional switch mechanism. To gain insight into the mechanism of posttranslational modification of hADA3, our next focus was to evaluate the possible role of the key sensor kinases like ATM/ATR in the generation of high molecular weight forms of hADA3. For this, HEK293T cells were incubated with caffeine, an inhibitor of ATM/ATR, followed by addition of ADR. Western blot analysis of the total cell lysates showed a clear reduction in the overall intensity of the slower migrating bands detected by anti-hADA3 antibody as shown in Fig. 4. The result shows that the hADA3 induction after DNA damage was blocked by the ATM/ATR specific inhibitor, caffeine. In other words, our data suggests the likelihood of ATM/ATR mediated phosphorylation as one of the potential DNA damage induced posttranslational modification of hADA3.

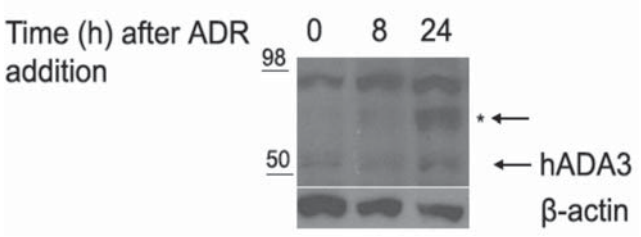

Figure 3: **DNA damage promotes posttranslational modification of hAda3. U2OS cells were treated with 0.25µg/ml of ADR. Cells were harvested after the indicated time of treatment and the whole cell lysates were resolved on SDS-PAGE. Western blot analysis was carried out using antibodies against hADA3 (asterisk * indicates modified form), p. 53 and β-actin**

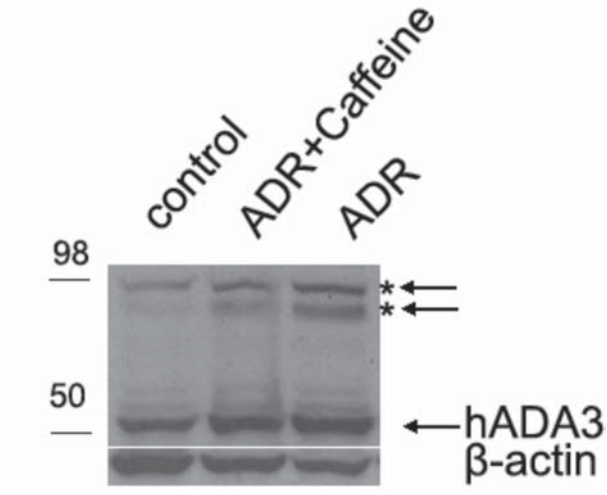

Figure 4: **Caffeine treatment abrogates the adriamycin induced accumulation of slower migrating forms of hADA3. Asynchronously growing HEK293T cells were treated with ADR (0.25µg/ml) in the absence or presence of 2mM caffeine for 1h. Whole cell lysates (30µg) were analyzed by Western blotting with antibodies (asterisk * indicates modified form) against hADA3 and β-actin**

Discussion

Unraveling the molecular basis of the pathways responsible for maintaining genomic integrity has attracted substantial interest of many investigators. Sensing, signaling and repair of DNA damage involves a plethora of proteins whose function at DNA damage sites is modulated by a myriad of posttranslational modification including phosphorylation, acetylation, SUMOylation, methylation and ubiquitination which are highly dynamic and reversible in nature (Babic *et al.*, 2004; Bawa-Khalfe and Yeh, 2010; Ivanov *et al.*, 2007). A growing list of proteins is being identified to be posttranslationally modified by several different enzymes and play important roles in response to genotoxic insult. Among all, the tumor suppressor protein p53 has been shown to be phosphorylated, acetylated, SUMOylated, methylated and ubiquitinated with the ultimate goal to stabilize and activate its role as a transcription factor for upregulating expression of series of proteins involved in cell cycle control (Appella and Anderson, 2000; Bode and Dong, 2004; Xu, 2003) and apoptosis. Tumor suppressor p53 is primarily known to be the foremost among the proteins induced after DNA damage and the mechanisms of its induction in response to DNA damage is well established (Canman *et al.*, 1998; Lakin and Jackson, 1999). However, the effect of DNA damaging agents on its coactivators still remains elusive. Human ADA3 is one such p53 coactivator that has been shown to play significant role in stabilization and functional activation of p53 (Nag *et al.*, 2007; Wang *et al.*, 2001). In order to gain full appreciation of hADA3 biology in DNA damage response, we performed simple experiments to explore the possibility of stress induced posttranslational modifications of hADA3. The data presented in this report demonstrate that hADA3, a core component of histone acetyl transferase (HAT) complex is induced following DNA damage by ADR. Interestingly, ADR mediated induction of hADA3 seems to be dependent on normal cellular status of p53 as its level remained unchanged in C33A cell line which harbors a mutant p53. This result can be explained by the fact that hADA3 is one of the interacting partners of p53 (Nag *et al.*, 2007; Wang *et al.*, 2001). Role of hADA3 in enhancement of acetylation, stability and function of p53 is well documented. Moreover, interaction of p300 with hADA3 has been implicated in facilitating acetylation of p53 possibly by formation of stable p300-p53 complexes. Based on these findings, we hypothesize that hADA3 and p53 play equally important role in stabilizing each other. Therefore, certain mutants of hADA3 may even exert dominant negative effect against endogenous p53. Similarly, mutant p53 may render hADA3 non-functional as well. This underscores the possible status of hADA3 and its significance in cancer patients carrying mutant p53.

Protein posttranslational modification has emerged as an important strategy for dynamically regulating activity of several key sensory molecules responding to a variety of cellular stresses (Wang *et al.*, 2008). To a great extent, cellular response to DNA damaging agents leads to activation of molecules participating in DNA damage sensing and execution of effector responses. The key sensor kinases like ATM/ATR trigger a range of differential posttranslational modification events that have been implicated in protein stabilization and enhancement of transcriptional transactivation (Shiloh, 2003). This process is responsible for relaying its response in an appropriate and proportionate manner according to the nature of the damage (McGowan and Russell, 2004). Our data provides evidence in support of posttranslational modification of hADA3 triggered by DNA damage. Since the high molecular weight bands of hADA3 were detected under denaturing condition of SDS-PAGE, the increment in the size of the protein could be due to introduction of multiple covalent modifications like SUMOylation, Ubiquitination or NEDDylation. Moreover, even though hADA3 is not a membrane associated protein, possibilities of hADA3 glycosylation cannot be totally ruled out. In our study, incidences of non-specific recognition by hADA3 antibody cannot be ignored and thus this report does not provide absolute evidence for a particular posttranslational modification of hADA3. Therefore, the results need to be verified by specific immunoprecipitation experiments with better controls and further experiments will be required to explore and address specific queries in detail. Nevertheless, our findings are definitely

suggestive of the stress induced existence of modified forms of hADA3. These posttranslational modifications provide extremely diverse and dynamic functional regulation to proteins ranging from changes in stability, localization to alterations in molecular interactions leading to modulation in its activity and function (Seo and Lee, 2004). A number of studies have suggested that hADA3 forms multiple, distinct coactivator complexes but the mechanism of their function is not well understood. We speculate that existence of differential posttranslational modifications on hADA3 under specific physiological conditions. This can serve as one of the key mechanisms by which hADA3 can interact with multiple transcriptional activators, coactivators, HATs, histone deacetylases (HDACs) and other members of the transcriptional machinery (Rowan *et al.*, 2000). Therefore, such modifications will further add up another layer of diversity to the function of hADA3. By this mechanism, the temporal and spatial expression pattern of hADA3 can also be modulated in response to various stimuli. While some modifications will stabilize the protein, others might make it more susceptible to degradation. Therefore, posttranslational modification pattern of hADA3 can serve as a molecular code that will dictate its dynamics of protein-protein interaction, subcellular localization, stability and stress response. Hence, disruption of this process can impair the potential tumor suppressor functions of hADA3.

The ATM and ATR kinases are master regulators of the DNA damage initiated activation of cell cycle checkpoints (Lobrich and Jeggo, 2005). Their action is mediated by phosphorylation and activation of checkpoint proteins including CHK1-CHK2 and p53 (Banin *et al.*, 1998; Bartek *et al.*, 2001; Khanna *et al.*, 1998; Lavin and Kozlov, 2007; McGowan, 2002). Caffeine is a methylxanthine that has been used extensively as an inhibitor of ATM and ATR kinase activity (Rowley, 1992; Schlegel and Pardee, 1986)). In presence of caffeine, cells are expected to overcome S-phase and G_2/M checkpoint responses to DNA damage. In our study, exposure of cells to caffeine prior to DNA damage resulted in an attenuated accumulation of the slower migrating forms of hADA3. This observation directed our attention towards the possible role of ATM and ATR kinases in phosphorylation of hADA3 following DNA damage. Further, this suggests a potential role of hADA3 in the tight controlling of these checkpoints. Taken together, the existing findings led us to propose a model (Fig. 5) in which DNA damage mediated activation of ATM/ATR may promote phosphorylation of hADA3, which in turn may provide platform for further modifications. These modifications can alter its preference for association with other protein partners by altering its own localization or dimerization ability which may play role in regulating various biological processes including DNA repair, cell cycle arrest, apoptosis, senescence and chromatin remodeling.

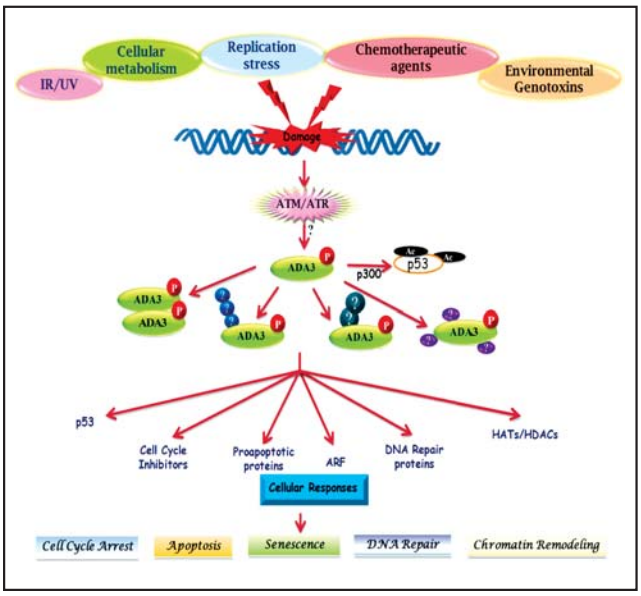

Figure 5: **A schematic representation of the hypothetical model for dynamics of DNA damage response induced posttranslational modification of hADA3. In response to a variety of DNA damage signals, activated ATM/ATR phosphorylates hADA3. Following phosphorylation, hADA3 undergoes subsequent posttranslational modifications, which enhances its binding affinity towards p53 and other molecules of the repair apparatus. Depending on the existing physiological conditions, different modifications as well as formation of diverse hADA3 complexes will be triggered. The composition and the local stoichiometry of the stable multiprotein complexes of hADA3 will drive the cellular response. We also propose that formation of such hADA3 complexes will help in triggering appropriate cellular self defense machinery. In this model, we have omitted many players in the ATM and p53 circuit in order to have a simplified representation of this intricate pathway**

Present study sheds light on the potential role of hADA3 containing complexes in the maintenance of genome integrity of the cell. The control of genome integrity is of pivotal importance for the cell and failure to do so inevitably results in the accumulation of mutations, genome rearrangements, cell death and cancer (Ou *et al.*, 2005). Therefore, our study will provide novel insights into mechanisms as to how hADA3 can function as a sensory molecule under stressful conditions and potentiate genomic stability. Hence, it will be interesting to unravel how multitude of hADA3 protein modifications combine to ensure efficient activation of cellular processes in response to genotoxic stress. The function of the hADA3 targets like p53 (Nag *et al.*, 2007; Wang *et al.*, 2001) and ER (Benecke *et al.*, 2002; Meng *et al.*, 2004) are well established in physiology and tumorigenesis. Moreover, the significance of hADA3 in cancer related studies is exemplified by its specific association with the high-risk (cancer-associated) variety of Human Papillomavirus oncoprotein E6 (Kumar *et al.*, 2002; Shamanin *et al.*, 2008; Zeng *et al.*, 2002). Studies aimed at elucidating the mechanism of hADA3's function are likely to provide a more detailed understanding of the basic cellular events contributing towards cancer as well as its protective role in safeguarding cells from undergoing oncogenic proliferation. Such work will also enrich our knowledge and provide valuable information for development of next generation diagnostics and novel therapeutic targets for treatments of cancer.

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Abbreviations

ADA3, alteration /deficiency in activation; PVDF, polyvinylidene difluoride; ATM, ataxia telangiectasia mutated; ATR, rad-3-related; ADR, adriamycin; HAT, histone acetyltransferases; GCN5, general control nonrepressed 5; PCAF, p300/CBP-associated factor; MCF7, human breast adenocarcinoma cell line; EDTA, ethylenediaminetetraacetic acid.

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