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PML NBs (ND10) and Daxx: from nuclear structure to protein function

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1. ABSTRACT

Proteins that combine PML NBs (ND10) can be divided into two groups: "transient" (that accumulate at PML NBs upon over-expression, interferon-induced up-regulation, block of proteosomal degradation, environmental stress or viral infection) and "constitutive" that co-localize with PML in the majority of cultured cells. One of the few "constitutive" components of PML NBs is the death domain-associated protein Daxx. While PML NBs are the most obvious depositories of Daxx, there are multiple alternative localization of this protein in the nucleus and cytoplasm, suggesting differential functionality of Daxx at different cellular compartments and stages of the cell cycle. The purpose of this review is to analyze Daxx spatiotemporal behavior within and outside of PML NBs and to discuss functions attributed to these localizations. We suggest that Daxx can participate in numerous cellular functions as a mediator of protein interactions, thus acting as a fine tuning instrument in highly orchestrated cellular processes; we also envision PML NBs accumulation of Daxx as an "out of action" storage depot.

2. INTRODUCTION

Numerous ambivalent issues are associated with the biology of the death-domain associated protein Daxx. Specific functions of Daxx in apoptosis and transcription regulation are not clear; intracellular localization of Daxx is a subject of debate; and validation of (50+) Daxx interactions is controversial. As an example, Daxx was originally discovered as an interacting partner with the transmembrane death receptor Fas in yeast screen (1), but later was shown incapable of binding to this protein (2). Daxx is indispensable for embryo development, at least in mouse: partial or complete Daxx knockout leads to extensive apoptosis and embryo lethality at E 9.5 – 10.5 (3); (4). One of the few aspects of Daxx biology that become repeatedly confirmed is its accumulation at PML nuclear bodies (PML NBs, also known as Nuclear Domain 10 (ND10), Kremer bodies or PML oncogenic domains (PODs)) via interaction with sumoylated (modified by small ubiquitin like modifier SUMO) protein PML. Originally attracting the attention of the scientific community as a potential player in acute promyelocytic leukemia pathogenesis, during almost twenty years of study

these nuclear structures were sequentially suggested to participate in almost all known nuclear function, ranging from transcription to DNA repair (see this issue). The two most common (and partly overlapping) models for PML NBs function are described as "catalytic surfaces" where domains are envisioned as nuclear scaffolds that create ideal conditions for biochemical reactions and as "nuclear depots" where proteins are inactivated in storage compartments. While exact function (s) of these nuclear domains remain the subject of extensive discussions with controversial conclusions, the mechanism of PML NBs assembly has become more clarified in recent years with the identification of the promyelocytic leukaemia protein PML as a keystone component of PML NBs formation and sumoylation as the most likely mechanism of additional protein attraction.

The current review does not attempt to cover issues of Daxx participation in apoptosis and interaction with sumoylated substrates. These issues were recently covered in reviews by Salomoni and Kelifi (5) and Shih and co-authors (6), correspondingly; neither will this review discuss and provide complete analysis of the growing list of potential Daxx interactions, which would be appropriate for an entirely separate review. Instead, this review will focus on Daxx localization at PML NBs as the central landmark to discuss several spatiotemporal aspects of Daxx functionality and localization.

3. DAXX IN THE NUCLEUS: PML BODIES AND EXTRA-LOCATIONS

3.1. Daxx in PML NBs

3.1.1. Mechanism of deposition: interaction with sumoylated PML

Mouse Daxx was originally cloned as a binding partner with the transmembrane death receptor Fas (1); this interaction presumably happens in the cytoplasm, specifically at the cytoplasmic face of the plasma membrane; thus, the primary predicted localization of Daxx was cytoplasmic. Surprisingly, the first paper describing cloning of human Daxx as a protein binding to a steroidogenic promoter also had identified Daxx as mostly a nuclear protein consistent with mapping of potential nuclear localization signals within the Daxx polypeptide (7). Soon Daxx was shown to localize in the nucleus at PML NBs (8); (9); (2) and identified as an interacting partner with PML (9); (10); (11). Based on protein distribution in $PML^{-/-}$ cells, this interaction was sufficient for Daxx deposition at PML NBs (9). Interestingly, another PML NBs protein, Sp100, that was previously shown to be not essential for PML NBs formation (9), can form domains in $PML^{-/-}$ cells upon over-expression and recruit Daxx and other PML NBs components into these structures (Staege and Will, this issue). This observation suggests that some PML NBs associated proteins can re-form PML NBs upon over-expression; to what extend those structures can behave as endogenous PML NBs in the regard of controlled recruitment and release of proteins is an open question.

Sumoylation of PML was proposed to be necessary for interaction with Daxx and sequential attraction of Daxx from nucleoplasm to PML NBs (9, 10). An elegant study by Shih and co-workers has recently identified the last ten amino acids of the carboxyl terminus of Daxx as a SUMO-recognition motif (12). This study shed a new light on the question of Daxx attraction to PML NBs, explaining 1) impossibility of PML deltaSUMO mutants to rebuild PML NBs in $PML^{-/-}$ cells, specifically to recruit Daxx upon transient transfection (9); 2) disappearance of Daxx from PML NBs upon over-expression of sumoisopeptidase SENP-1 and after heat shock, when PML is de-sumoylated (13); 3) it also underpins the mechanism of PML NBs disassembly at the entrance of mitosis, at the prophase/prometaphase transition, when PML becomes de-sumoylated by cytoplasmic sumoisopeptidases and therefore cannot interact with Daxx. Moreover, identification of Daxx as a protein that interacts with sumoylated substrates can at least partly explain the constantly growing list of Daxx interaction partners, most of which can be sumoylated. Indeed, the majority of Daxx interaction studies are based on experiments using yeast two hybrid assay that pulled down almost exclusively the carboxyl terminus of Daxx, which includes the SUMO-recognition motif of the protein (reviewed in (6)).

3.1.2. Working place or storage compartment

PML NBs generally represent a heterogeneous population that is cell type and cell cycle dependent. Accordingly, the function (s) of PML NBs may vary between "catalytic surface" and "nuclear depot" models; thus, it is increasingly important to discuss functions of individual proteins associated with PML NBs within and outside of these domains. One of the most obvious functions of Daxx in the nucleus is transcription repression. Can Daxx accomplish this function while in PML NBs? One of many Daxx interacting proteins is HDAC2, a key transcriptional regulator in cells. Involvement of Daxx in transcription repression (discussed in part 5 of this review) is most likely mediated by attraction/stabilization of HDAC2 on specific promoters, as in the case of the c-met promoter (14). Daxx interacts with PML and HDAC2 (as well as with numerous other transcription factors, see Table 2) via the same carboxyl terminus. Thus, binding of Daxx to sumoylated PML (concomitant with accumulation of Daxx at PML NBs) presumably abrogates formation of Daxx-HDAC2 complexes (or other complexes involving the Daxx carboxyl terminus) resulting in the inactivation of Daxx-mediated transcription repression.

This model suggests that, in the case of Daxx, interaction with PML inactivates Daxx function and PML NBs serve as "nuclear depots" necessary for the maintenance of intranuclear homeostatic balance by recruitment/release of proteins to/from their place of action. It also emphasizes sumoylation as a regulator of this homeostasis. Indeed, several laboratories have shown that changes in the "nuclear depot" capacity of PML NBs can shift this balance. For instance, elevation of depot capacity by transient overexpression of PML reverses Daxx-mediated repression and increases its accumulation in PML NBs (10), (15), while de-sumoylation of PML upon heat shock leads to release of Daxx from PML NBs and

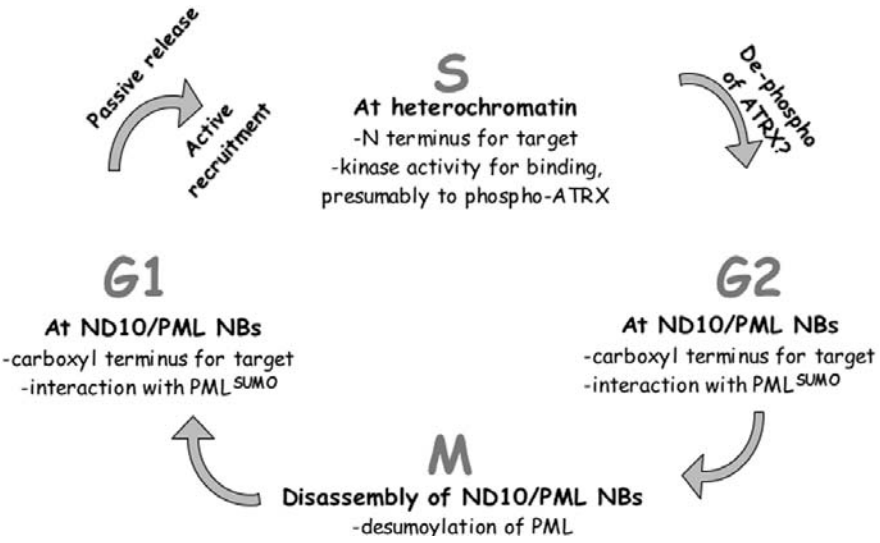

Figure 1. Cell cycle dependent intranuclear localization of Daxx. Daxx interacts with sumoylated PML that results in accumulation at PML NBs during G1 and G2 phases. At the end of S-phase, Daxx is deposited to the heterochromatin, presumably by interaction with phosphorylated ATRX. During mitosis, PML is de-sumoylated and PML NBs are disassembled.

increases transcription repression of Hsp25 (13). Interestingly, heat shock releases Daxx and Sp100 from PML NBs into the nucleoplasm due to rapid desumoylation of PML, while heavy metal exposure (Cd^{2+}) disperses Daxx and PML via a phosphorylation cascade with Sp100 retained at PML NBs (13). Thus, PML NBs –mediated "storage" of Daxx is: 1) Dynamic, in that environmental stress regulates PML NBs number and components; and 2) Regulated, in that the shift of sumoylation/phosphorylation can significantly change ratio of nucleoplasmic Daxx (functional) to PML NBs-associated Daxx (inactivated).

To address the issue of PML-dependent Daxx localization at PML NBs for the proper Daxx function, one can also compare phenotypes of Daxx knockout and PML knockout mouse models. While $Daxx^{-/-}$ embryos were lethal at E 9.5-10.5 (4, 16), $PML^{-/-}$ animals were indistinguishable at the gross phenotypic level from $PML^{+/-}$ and $PML^{+/+}$ (17). Since PML is essential for Daxx localization at PML NBs, one can conclude that this intra-nuclear deposition is not important for the proper Daxx function, at least at the organism level during embryogenesis.

3.2. Daxx at heterochromatin

Initial evidence for the potential association of Daxx with chromatin came from a PML-deficient model. In the absence of PML (in $PML^{-/-}$ cells) Daxx is not accumulated in PML NBs, but was found mostly associated with condensed heterochromatin (9); re-introduction of PML by transient overexpression or by cell fusion can recruit Daxx retroactively to PML NBs. Thus, heterochromatin was suggested as an alternative place of Daxx intranuclear localization and activity. Biochemical confirmation of chromatin association came from the work of Hollenbach and co-authors who, by size fractionation and co-immunoprecipitation experiments, had isolated Daxx and HDAC2 together with core histones and chromatin-associated protein Dek (18). The authors suggested that Daxx association with chromatin is critical for transcription repression function of this protein and is mediated via phosphorylation. Next, HIPK1 was identified as Daxx kinase that phosphorylates Daxx and moves Daxx to chromatin (19).

Daxx does not interact with DNA itself (20), but its association with chromatin-containing fractions can be disrupted by micrococcal nuclease treatment (4). What is (are) protein(s) that target Daxx to chromatin? Daxx forms a complex with ATRX, a putative member of the SNF2 family of ATP-dependent chromatin remodeling proteins that is mutated in several X-linked mental retardation disorders (21). Interestingly, ATRX interaction is mediated via the amino terminus of Daxx (22) and, presumably, is not regulated by sumoylation but by phosphorylation of ATRX (4). This interaction does not affect the ATPase chromatin remodeling activity of ATRX *in vitro* (22), but is critical to deposit ATRX at PML NBs in G1 and G2, and to accumulate Daxx at condensed heterochromatin at the end of S-phase (4) (see model on Figure 1). What could be the functional consequences of Daxx association with heterochromatin—specifically at this very short window during the cell cycle—is currently unknown. Increased occurrence of double nucleated $Daxx^{-/-}$ cells, however, may suggest a function of Daxx in proper progression of cell division (4). Daxx was found at XY bodies (transcriptionally repressed sex chromosomes) during meiotic prophase in spermatogenesis, specifically in pachytene spermatocytes (23), but functional significance of this accumulation is unknown. Considering the model presented in Figure 1, one can speculate that Daxx mediates accumulation/stabilization of some partners that interact with the carboxyl terminus of protein (which include HDACs, Dnmt1, DMAP1) at heterochromatin during the end of S-phase or at XY bodies during spermatogenesis, though identification of Daxx function at chromatin is a subject of further investigation.

3.3. Daxx at centromeres

Daxx interaction with centromere protein CENP-C and association with centromeres (24) was observed even earlier than its interaction with PML and association with PML NBs. The dynamic correlation between several proteins associated with PML NBs and centromeres was narrowed to G2 phase (8). In *S. pombe,* depletion of a Daxx-like motif-containing GATA factor Ams2 (that was isolated as multi-copy suppressors of cnp1-1, an *S. pombe* CENP-A mutant), results in a chromosome missegregation (25), though homology between Daxx and Ams2 is relatively low. Thus, as in case of chromatin association, the functional consequence of Daxx association with centromeres is unknown.

3.4. Daxx in the nucleolus

One of the most intriguing intranuclear deposition sites of Daxx are nucleoli. Daxx interacts with nucleolar microspherule protein MSP58 that leads to accumulation of both transiently expressed and endogenous Daxx in nucleoli (26). Interestingly, this interaction appears to inactivate Daxx mediated transcription repression; thus, nucleolar sequestration of Daxx (that was also observed for endogenous Daxx in some breast cancer cells (AMI and VMM, unpublished observation) can provide an alternative segregation mechanism that inactivates repressive functions of Daxx; alternatively, Daxx may inactivate PolI transcription.

4. DAXX IN THE CYTOPLASM: PRO AND CONTRA

4.1. Nuclear or cytoplasmic?

The sub-cellular localization of Daxx has been a controversy since it was discovered as a factor involved in Fas-induced apoptosis (1). While Daxx localization was not characterized from this screening, these findings suggest that Daxx would be found as a cytoplasmic protein near the cell membrane. A subsequent paradox would be developed when Daxx was discovered as a predominately nuclear protein. By biochemical separation of HeLa cells into cytosolic, nuclear and mitotic chromosome fractions, Daxx was demonstrated as a protein largely associated with nuclear isolated fractions (24). Very detailed cellular fractionation of NIH-3T3 mouse fibroblasts into nuclear, cytosolic, low-density microsome, high-density microsome and plasma membrane fractions (27), showed the majority of Daxx accumulating in the nuclear fraction and a small percentage appearing in low-density microsomes. Using immunofluorescent visualization of endogenous Daxx, authors observed predominately nuclear staining along with a very faint speckle-like cytoplasmic Daxx pattern in 3T3 fibroblasts which, presumably, may be an indication of two intracellular pools of Daxx that exist in cells. Similarly, subcellular fractionation of SH-SY5Y neuroblastoma cells showed Daxx predominately associated with nuclear fractions, with a small portion as part of the cytosol (28).

Immunofluorescent imaging described endogenous Daxx as nuclear with "punctuate staining pattern" (24), which was later identified by several groups as co-localizing with PML NBs (see above). Subsequent studies validated Daxx interaction with apoptosis signal-regulating kinase 1 (ASK1) and show co-localization and interaction of the two proteins in the cytoplasm, in correlation with induction of apoptosis, though the bulk of these experiments were based on transient over-expression (29). At the same time, interaction between endogenous Daxx and other nuclear proteins including chromatin remodeling proteins ATRX and HDAC1/2 (19, 21), nuclear sub-domain constituent PML (9), nuclear protein kinase HIPK1 (19), co-fractionation with chromatin-associated protein Dek and core histones (Hollenbach *et al.*, 2002) among others, suggests that Daxx is predominately a nuclear protein, at least when cells are not exposed to stress. In the absence of PML, the major Daxx housing domain in interphase, Daxx adopts a primarily chromatin-based localization in the nucleus (9). Thus, if Daxx resides in the cytoplasm at any period of time, it is most likely a result of specific relocation as part of signaling networks.

4.2. Localization upon stress (should I stay or should I go…)

Several reports describe detailed mechanisms of Daxx re-localization under various stress conditions (30); (31); (32); (33); (34). In many cases, this change in distribution of Daxx was shown to be critical for cell survival under stress. During glucose deprivation, Daxx is re-located from the nucleus to the cytoplasm (34); (35). Mutation of Trpytophan 621 and Serine 667 of human Daxx, moreover, was sufficient to block nuclear export in these stress conditions, which relied on stable adenoviral expression of Daxx in adenocarcinoma DU-145 cells. Chemical hypoxia-induced Daxx relocalization to the cytoplasm was eloquently shown by (31) using detailed confocal imaging analysis of endogenous Daxx in Chinese hamster ovary cell line PS120. Oxidative stress was also reported to influence the localization of Daxx to the cytoplasm in DU-145 cells, while over-expression of catalase inhibited nuclear export of Daxx and its glucose deprivation-induced cytotoxicity (34, 36). Upon ox-LDL treatment of THP-1 macrophages, faint and uniform cytoplasmic staining of Daxx, changed to a brighter, diffuse staining which appeared throughout the cell compartments (37) suggesting a change in Daxx localization. A contradictory report by Khelifi and co-authors, however, showed via biochemical separation that Daxx remains in the nucleus after exposure to hydrogen peroxide or UV treatment (38). In the majority of studies the primary means of determining stress induced Daxx localization was accomplished by immunofluorescence staining of transiently over-expressed protein (see Table 1); it can be beneficial to corroborate these data with endogenous protein study and alternative methodology, such as subcellular fractionation.

Daxx protein trafficking in response to stress stimuli may also be cell-line specific. Thus, Zhong and co-authors report cytoplasmic association of Daxx in mouse splenocytes; upon Con A stimulation, Daxx is imported to the nucleus where it interacts with PML (39). In the same study authors used another cell line, APL-derived NB4, where Daxx was observed as a strictly nuclear protein that remains associated with reconstituted PML NBs after

Table 1. Daxx translocation to cytoplasm upon stress

Daxx Translocation to Cytoplasm/Nucleus	Endogenous or Exogenous Daxx	Cellular Stress	Method of Study	Cell Line	Ref
Translocation to cytoplasm	Endogenous	Oxidative stress, myloid beta	Biochemical fractionation	SH-SY5Y	28
Remains in nucleus	Endogenous	UV, TNF-alpha	IF[1]	HeLa	66
Translocation to cytoplasm	Exogenous	Fas, zVAD-fmk	IF	293	67
Remains in nucleus	Exogenous	Cisplatin	IF	U2OS	56
Translocation to cytoplasm	Endogenous	Chemical hypoxia	IF/confocal	H9C2	30
Translocation to cytoplasm	Endogenous	Chemical hypoxia	IF/confocal	PS120	31
Translocation to cytoplasm	Exogenous	Oxidative stress, MPP^+[2]	IF	SH-SY5Y	32
Translocation to cytoplasm	Endogenous	MPP^+	IF	SH-SY5Y	33
Remains in nucleus	Endogenous	IFN-gamma, As_2O_3	IF	HeLa	68
Remains in nucleus	Endogenous	UV, Oxidative stress	IF, Biochemical fractionation	BJ fibroblasts	38
Remains in nucleus	Endogenous	Heat shock, Heavy metal	IF	HEp2	13
Translocation to cytoplasm	Exogenous	Oxidative stress, glucose deprivation	IF	DU-145	34
Translocation from cytoplasm to nucleus	Endogenous	ox-LDL[3]	IF	TH-1 macrophage	37
Remains in nucleus	Exogenous	Fas, TNF-alpha, As_2O_3	IF	HT1080	2
Translocation to nucleus	Endogenous	Con A, Retinoic acid	IF	Murine splenocytes	39

[1]IF = immunofluorescence, [2]MPP^+ = 1-methyl-4-phenylpyridinium, [3]ox-LDL = oxidized low density lipoprotein.

retinoic acid exposure. Exposure of different cell lines to oxidative stress (28); (38) or Fas stimulation (40); (2) also yields different results depending on the cell line that was studied.

Can re-localization of Daxx be explained by a general relocation of nuclear proteins into the cytoplasm upon stress conditions? To date, few studies have effectively incorporated these controls. If a general re-distribution of nuclear proteins is observed in these cases, it is possible that these phenomena are less attributable to Daxx function and more explainable as a general cellular stress response. While there is some tantalizing evidence to suggest stress-induced accumulation and function of Daxx in the cytoplasm, the majority of studies still do not conclusively give us a clear picture of Daxx behavior in this regard. Therefore, more extensive studies of endogenous protein trafficking in relation to cytoplasmic and nuclear localization are required to finalize stress-type and cell line-type dependent behavior of Daxx.

5. DAXX AND TRANSCRIPTION REGULATION

5.1. Interaction with transcription factors

A role of Daxx in transcriptional repression was first suggested by Hollenbach and co-authors who found that Daxx interacts with transcription factor Pax3 (20). This study has shown that Daxx can act as a transcriptional repressor when tethered to DNA through the GAL4 DBD and that co-expression of Daxx with Pax3 results in an 80% repression of Pax3 activity in a transient transfection assay. Evidence which has accumulated as a result of almost a decade of inquiry after this publication suggests that Daxx can regulate transcription through interaction with a growing number of transcription factors such as Ets1 (41), Pax5 (42), p53 and p53 family members p73 and p63 (43, 44), glucocorticoid receptor (45), androgen receptor (46), Smad4 (47), STAT3 (48), RelB (49), RelA (p65) (50) (see Table 2 for summary). Overall, Daxx represses transcription activity of most of these factors, though it also can behave as a transcription co-activator. Thus, Emelyanov *et al.* have found that Daxx can either repress or activate the transcriptional activity of Pax5 depending on the cell type and promoter context (42).

5.2. Chromatin modification

Besides affecting transcription by interaction with transcription factors, Daxx may regulate transcription by epigenetic mechanisms changing chromatin modifications on target promoters. Daxx was purified as a component of a multiprotein repression complex that includes HDAC1 and HDAC2, Dek and acetylated core histones (10, 18), and also interacts with DNMT1 and DMAP1 (16, 51). These interactions point towards a potential mechanism of Daxx-mediated repression as a result of histone deacetylation, chromatin remodeling or DNA methylation activity. Thus, a study describing interaction between Daxx and HDAC1 (10), demonstrated that treatment with a histone deacetylase inhibitor, trichostatin A (TSA), reverses the repressive effect of Daxx in a transient transfection assay. Recently, Morozov *et al.* (14) have demonstrated repression of the endogenous c-met promoter in $Daxx^{+/+}$ mouse cells compared to $Daxx^{-/-}$ cells, accompanied with increased HDAC2 binding and histone H4 deacetylation, but not with changes in c-met promoter DNA methylation. These data suggest that histone deacetylation, but probably not DNA methylation, is involved in Daxx-mediated repression at least in the case of the c-met promoter. In concordance with these observations, Daxx knockout embryos or cell lines do not have defects in global DNA methylation (16).

Daxx also modulates activity of histone acetylase CBP. Sumoylation of CBP recruits Daxx, which mediates inhibition of CBP transcriptional activity in transactivation assay through attraction of HDAC2 (52). Another study demonstrated that Daxx interaction with p65 (RelA, subunit of NF-kB) inhibits acetylation of this transcription factor by CBP that in turn, impairs NF-kB transcriptional activity (50). However, this effect was HDAC-independent since treatment with TSA failed to restore p65 acetylation (50). Conversely, Daxx enhances Pax5-mediated reporter activity by recruiting CBP (42), which implies that Daxx-CBP interaction can lead to different events depending on cellular and gene context.

Table 2. Daxx interaction with transcription factors

Transcription factor	Region of interaction	Method of interaction	Cell Line	Target gene/promoters	Functional consequence	Ref
Pax3	635-740	Co-IP[1]	COS1, NIH 3T3	PRS-9TK-CAT reporter	Daxx represses Pax3-driven transcription	20
Ets1	577-740	YTH[2] GST[3]	COS-1	Exogenous MMP1 or BCL2	Exogenous Daxx represses Ets1-driven transcription	41
Pax5	626-740	YTH; GST	Mouse B cells M12.4.1, A20, BL-2, HS-Sultan, 293T, HeLa	Exogenous multimerized Pax5 binding site from CD19 promoter, E1b promoter, TK	Daxx activates or represses transcriptional activity of Pax5 depending on the cell type and promoter	42
p53	434-493, 625-740	YTH; GST; Co-IP	Saos2, HT-29	N/D	Daxx interacts with tumorigenic mutant p53 175RH, 248RW, 273RH, 281DG, 143VA but not with wild-type 53	69
p53, p63, p73	644-740	YTH; GST; Co-IP	Saos2	G5p53-CAT, 17m5-TATA-CAT, p21WAF1-CAT reporter	Daxx inhibits p73 and p53 transactivation in a dose-dependent manner, potentially via deacetylation.	43
p53	434-496	YTH; Co-IP	HCT116, HCT116p53$^{-/-}$, Saos2, p53/MDM2 DKO MEF	Exogenous p21 and pAIP1	Daxx repress p21 and pAIP1 reporter construct in p53-dependent manner; Daxx protects cells from p53 mediated apoptosis	44
TSG101	1-492	Co-IP	293T	MMTV-LUC reporter	Enhancement of Daxx-mediated repression of GR-driven transcription	70
DMAP1	1-492	YTH; Co-IP	293T	MMTV-LUC reporter	Enhancement of Daxx-mediated repression of GR-driven transcription; Daxx protects DMAP1 from the proteasomal degradation	51
CBP	N/D[4]	Co-IP	BL-2, M12.4.1, 293T	Exogenous E1b and TK promoters	Daxx enhances Pax5-mediated reporter activity through the recruitment of CBP to Pax5-Daxx complex	42
CBP	N/D	YTH; Co-IP	HeLa, COS-1, 293, Daxx$^{+/+}$ and Daxx$^{-/-}$ mouse EC	P5xGal-E1B-LUC	Daxx mediates sumoylation-dependent inhibition of CBP transcriptional activity through attraction HDAC2	52
Dnmt1	N/D	YTH; Co-IP	293T	N/D	N/D	16, 51
HDAC1, HDAC2, HDAC3	N/D	Co-IP, GST	HeLa, HEK293	N/D	TSA reverses Daxx-mediated repression in a transient-transfection assay	10
HDAC2	573-740	Co-IP, co-fractionation	293T	N/D		18
GR	501-740	Co-IP, GST bettaGal assay	COS-1	MMTV-LUC reporter	Daxx represses GR-mediated transcriptional activity	45
AR	N/D	YTH; GST; Co-IP bettaGal assay, EMSA	COS-1, LNCaP	MMTV-LUC reporter, Endogenous human PSA promoter and rat probasin promoter	Daxx inhibits AR DNA binding and sumoylation-dependent repression	46
ATRX	N/D	Co-IP	HeLa	N/D	Daxx does not affect the remodeling activity of ATRX.	21
ATRX	1-160	Co-IP, co-fractionation, IF	HeLa, 293T	Exogenous luciferase reporter	ATRX-mediated transcriptional repression is reversed by Daxx	22
ATRX	1-625	Co-IP, IF	Daxx$^{+/+}$, Daxx$^{-/-}$ reconstituted with Daxx and mutants	N/D	ATRX attracts Daxx to heterochromatin at the end of S-phase, Daxx deposits ATRX to PML NBs in G1 and G1	4
Smad4	570-740	YTH; GST	COS-1, MDA-MB-468, Mv1Lu	3TP-Luc reporter w/TGF-beta-responsive elements from the PAI-1 and collagenase promoters; SBE4-Luc w/four copies of the Smad-binding CAGA	Daxx suppresses Smad4 transcriptional activity	47
HSF1	625-740	YTH; Co-IP	HeLa, Daxx$^{+/+}$, $^{+/-}$, $^{-/-}$	Exogenous and endogenous hsp70 promoter	Daxx enhances HIF1 activity	71
STAT3	1-240	Co-IP	HeLa, Hep3B	STAT3-Luc reporter, Endogenous SOCS3	Daxx inhibits IL-6/STAT3-mediated gene expression	48
Tcf4	N/D	YTH; Co-IP	HEK293T, Hct116, SW480	Exogenous reporter, Endogenous cyclin D1 and Hath-1	Daxx represses transcriptional activity of Tcf4	64
RelB	N/D	co-IP, ChIP	HT1080, MCF7	cIAP2, c-FLIP, Survivin	Daxx associates with RelB but does not prevent RelB from binding to target DNA; Daxx represses RelB-mediated transcription	49
RelA p65	N/D	Co-IP; GST	HeLa	3x kappa-B reporter	Daxx inhibits p65 acetylation by CBP and represses NF-kB transcriptional activity	50
Axin	1-197	YTH; Co-IP	293, H1299p53 null, H1299p53 reconstituted	p53-Luc reporter, PUMA, p21, Bax reporter	Activation of PUMA, but not p21 or BAX Axin or/and Daxx depletion decrease UV-induced cell death	54

co-IP[1]= co-immunoprecipitation; YTH[2]= yeast two-hybrid assay; GST[3] = GST pull-down assay; N/D[4] = not determined

5.3. Sumoylation as a control of Daxx interactions

It is obvious that Daxx can regulate transcription via multiple alternative means, but how is Daxx-mediated repression regulated? The most convincing answer comes from the work by Lin *et al.*, who demonstrated that Daxx binds to sumoylated proteins via its SUMO-interaction motif (SIM) ^{733}IIVLSDSD^{740} (12). Besides interaction with sumoylated PML, which targets Daxx to PML NBs (see above), Daxx interacts with several sumoylated transcription factors such as AR, Smad4, HDAC2 and GR, causing sumoylation-dependent transcriptional repression (6, 12, 46, 47). Thus, cell line/cell cycle specific sumoylation of Daxx-interacting proteins, including transcription factors, is one of the potential mechanisms that controls specificity of Daxx-mediated repression and can explain differential Daxx regulation of several target genes.

5.4. Intranuclear localization and transcription repression

Finally, the transcriptional repression function of Daxx is modulated by sub-nuclear compartmentalization through protein-protein interactions. Thus, the ability of Daxx to repress transcription is inhibited by its sequestration to PML-NBs. Coexpression of PML reverses the transcriptional repression of Daxx in a dose-dependant manner, which, in turn, correlates with the recruitment of Daxx to PML NBs (10). Sumoylation of PML is required for both recruitment of Daxx to PML-NBs and inhibition of Daxx-mediated repression. Stimuli that increase the level of sumoylated PML, such as As_2O_3 or interferon treatment, elevate Daxx recruitment to PML-NBs and release the constraints of Daxx-repressive transcriptional activity (12, 15). Vice versa, heat-shock induced desumoylation of PML and release of Daxx from PML NBs correlated with Hsp25 suppression (13).

The additional mechanism of relocalization of Daxx from PML-NBs, which can be mediated by its interaction with HIPK1 was proposed by Ecsedy *et al.* (19). Although the relocalization of Daxx out of PML NBs was phosphorylation independent and required only interaction with HIPK1, Daxx phosphorylation on Ser 669 by HIPK1 was important in modulating the ability of Daxx to function as a transcriptional repressor. Interestingly, Daxx-S669A mutant represses CRE-, E2F1- and Sp1responsive promoters in luciferase reporter assay to a greater degree than wild-type Daxx, whereas the c-met promoter shows no difference in repression between wild-type Daxx and Daxx-S669A. Therefore, phosphorylation at Ser 669 by HIPK1 diminishes the ability of Daxx to repress transcription, however, this modulation of Daxx activity is promoter-specific (19). HIPK2 modulates Daxx function releasing Daxx from PML NBs into the nucleoplasm (53) where Daxx, in cooperation with Axin, can stimulate HIPK2-mediated Ser^{46} phosphorylation and transcriptional activity of p53 (54). Again, Daxx and Axin display strong selectivity in regulation of p53-dependent genes. Among the reporter genes tested, only PUMA was activated and not p21 or Bax (54). As a potential negative feed back, the formation of tertiary Daxx-HAUSP-MDM2 complex reduces auto-ubiquitination and thus stabilizes MDM2, that activates p53 ubiquitination and degradation (55).

5.5. Target promoters

How is Daxx tethered to target promoters? Even though hDaxx was first isolated in one-hybrid assay as the steroidogenic promoter binding protein (7), it is unable to associate with DNA in the EMSA assay (20). Thus, Daxx is associated with target promoters most likely indirectly, either by interaction with DNA–binding transcriptional factors (see above) or with scaffolding chromatin-associated proteins such as ATRX.

Daxx specifically represses the p21 promoter, however, no significant effect was observed on several pro-apoptotic gene promoters, such as Bax, PIG3, and AIP1, whereas transactivation of the PUMA promoter was increased (56). Moreover, Daxx has been shown to differentially modulate transcription from different p53- and NF-kB-responsive promoters (49, 56). Potentially Daxx can selectively affect p53 and NF-kB transcription and through this be involved in regulation of the transcriptional balance between genes that induce cell-cycle arrest or apoptosis.

A major limitation of these studies is that they are based on artificial transcriptional assays and use over-expressed Daxx. To this end, a recent study (14) has shown that endogenous Daxx is a repressor of c-met transcription. Daxx mediates c-met repression through association with a specific region of the endogenous mouse c-met promoter concomitant with HDAC2 binding and decrease of transcription-associated modifications of chromatin, specifically, H4 acetylation. The wide variety of Daxx targets and the cell type dependent regulation of pro- and anti-apoptotic genes (49, 56), may, at least partly, provide a potential explanation for pro- and anti-apoptotic functions of Daxx.

6. DAXX FUNCTION IN PATHOGENESIS: CANCER PROGRESSION

A majority of Daxx studies have focused on regulation of apoptosis in relation to Fas and are effectively reviewed in Salomoni and Khelifi (5). Some evidence suggests a functional implication between Daxx and neurodegenerative diseases, including Alzheimer's disease (28, 57). However, the majority of this review will focus on the roles of Daxx in relation to PML in cancer progression.

One of the most clinically relevant aspects of PML biology is the frequent chromosomal translocation t (15;17) occurring in acute promyelocytic leukemia (APL) patients which involves the fusion of the PML gene with the retinoic acid receptor alpha gene (RAR-alpha). Expression of mutant PML/RAR-alpha protein results in altered localization—which is dissimilar to normal PML body localization—that attracts other nuclear body components. Treatment of cells expressing PML/RAR-alpha with retinoic acid (RA) is sufficient to catabolize this fusion protein resulting in reorganization of nuclear body associated proteins, including Daxx, in PML NBs (reviewed in (58). PML/RAR-alpha fusion protein is known to be a potent repressor of transcription which is

important in APL pathogenesis (59). The K160 sumoylation site of PML/RAR-alpha was shown to be critical for leukemic transformation *in vivo* and *ex vivo* as primary hematopoietic cell precursors with K160 mutations were less effective at proliferating (60). Moreover, mutation of this sumoylation site disrupted interaction with Daxx, which may in part explain the strong PML/RAR-alpha repressor activity on genes potentially critical for cellular differentiation in APL leukemogenesis (60). In a similar study, (61) showed that, in addition to PML/RAR-alpha, Daxx/tet/RAR-alpha can also inhibit cell differentiation and promote immortalization *ex vivo*. Taken together, these studies may imply that the functional importance of PML in PML/RAR-alpha -induced transformation is for homodimerization and to provide a SUMO-dependent domain important for attracting transcription repressors like Daxx.

Among other targets of Daxx-mediated transcriptional repression includes the c-met protooncogene, a tyrosine kinase receptor for the hepatocyte growth factor/scatter factor (HGF/SF) (14). Cells lacking a functional Daxx protein were shown to have elevated levels of c-Met protein, which correlated with an increased mobility and invasive index of cells. In addition, there was an inverse correlation between Daxx and c-Met in numerous cancer cell lines which was also recapitulated in primary metastatic breast cancer specimens (14). Thus, Daxx may be an important repressor of c-met during cancer progression and metastasis formation.

Elsewhere, Daxx expression was found to be upregulated in tumor stroma of primary prostate tumor tissues (62), the importance of which may be partially explained by the function of Daxx as a corepressor of the androgen receptor (AR) (63), a nuclear receptor involved in normal development and differentiation as well as the progression of prostate cancer. These findings were emphasized by (46) showing that Daxx can downregulate AR expression in colon cancer cells by interaction with the DNA binding domain of AR in a sumoylation-dependent manner. Importantly, mutation of SUMO-conjugated sites in AR resulted in loss of Daxx binding and a concomitant increase in AR expression. TCF-4 DNA binding activity and transcript expression was also found to be regulated in a Daxx-dependent manner in colon cancer cells (64). This study found Daxx protein reduced in a number of human colon adenocarcinoma specimens, further suggesting that regulation of Daxx expression may be critical in the development of colon cancer.

Daxx-dependent regulation of other nuclear receptors has also been confirmed for the glucocorticoid receptor (GR), which plays important roles in control of development and growth of the immune system by induction of apoptosis. By binding directly to GR, Daxx is capable of inhibiting activation of GR-target promotors and its repressive function can be alleviated by co-expression of PML, which sufficiently sequesters Daxx into PML nuclear bodies (45).

Recently, Daxx was shown to have a novel, unexpected role in sensitivity to the chemotherapeutic agent paclitaxel (65). Paclitaxel remains the most frequently used anti-cancer compound worldwide and large numbers of patients are resistant or become resistant to this drug during treatment. Breast cancer cells expressing high level of Daxx exhibited a robust response to paclitaxel, which resulted in cells exiting mitosis and forming micronuclei. Breast cancer cells with low Daxx expression or Daxx-depleted cells, however, respond with a prolonged mitotic block in prometaphase, allowing cells to complete cell division after drug removal or decay. This suggests that Daxx may play a novel and direct role in mitotic progression and potentially is a predictive marker for paclitaxel response in patients. Thus, as we learn more about Daxx biology, we will continue to unravel important functions of this protein in disease pathogenesis.

7. CONCLUSIONS AND PERSPECTIVES

We have attempted to summarize the differential aspects of Daxx biology, including spatiotemporal localization and functionality. The growing list of Daxx-interacting proteins and several aspects of Daxx behavior upon stress can be confusing and may be interpreted as evidence of potential functional controversy. Alternatively, one can envision Daxx as a multi-functional protein that may have diverse roles depending on cellular model, stage of cell cycle progression and/or stress application participating in numerous cellular functions as a mediator of protein-protein interactions. Unfortunately, only a fraction of Daxx-interacting proteins, to date, have been unequivocally identified as endogenous binding partners of Daxx and from this evidence, we should only begin to understand the importance of Daxx function in normal and pathological conditions. To this end, some Daxx interactions that were originally identified in artificial systems have not been validated using endogenous protein binding, despite studies showing differential outcomes of regulatory processes attributed to these interactions. Our current understanding of Daxx is of a mostly nuclear protein, which can very rarely enter and function in the cytoplasm. If Daxx indeed exists as a cytoplasmic species, however, much stronger endogenous and repeatable evidence should be gathered to prove specifically what cellular stresses are important for this relocalization.

The potential plasticity of Daxx biology (including possible functions in human pathology) can be better uncovered when we can address the following criteria:

1) What is the cell- and tissue-specific function of Daxx *in vivo (*with application of conditional knockout model)?
2) What is crystal structure of Daxx, especially in regard to SUMO-dependent binding?
3) What are the function (s) of Daxx at centromeres, in nuclei, at chromatin and, finally, in cytoplasm?

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