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Master Thesis

探討 Daxx 辨識類泛素化修飾蛋白之模組

Identification of Daxx Motif in Recognizing Sumoylated Proteins

陳彥伃

Yen-Yu Chen

指導教授：施修明 博士

Advisor: Hsiu-Ming Shih, Ph.D.

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口試委員會審定書

探討 Daxx 辨識類泛素化修飾蛋白之模組

Identification of Daxx Motif in Recognizing Sumoylated Proteins

本論文係 陳彥伃君（學號: R06448001）在國立臺灣大學分子醫學研究所完成之碩（博）士學位論文，於民國 108 年 7 月 8 日承下列考試委員審查通過及口試及格，特此證明

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中文摘要

類泛素化是很重要的後轉譯修飾，並調節各樣的生物途徑。Daxx 蛋白在過去的文獻發現它可以辨認經類泛素化修飾後蛋白並且去抑制基因轉錄活性以及其在細胞中的分佈。Daxx 蛋白透過其 SUMO interacting motif (SIM)去辨認類泛素化修飾後的蛋白，但這樣不足以提供 Daxx 專一性的辨認。因此，在此論文中我們要探討除了 SIM 之外，是否存在其他區域或模組使 Daxx 可以專一性的辨認其交互作用的蛋白。已知 Daxx 蛋白傾向與 Lys^{159} 類泛素化 Smad4 蛋白有交互作用並且抑制由 Smad4 轉錄因子調控的基因轉錄，因此首先，我們在細胞外的實驗中發現 Smad4 會與 $Daxx_{625-740}$ 有交互作用但 $Daxx_{660-740}$ 則無，接著利用點突變實驗，我們發現在細胞外與細胞內的實驗都驗證當 Daxx 631 到 633 位置突變後會降低與 Lys^{159} 類泛素化 Smad4 蛋白的交互作用，並且失去抑制 Smad4 轉錄的活性。此外我們也探討 Daxx 631 到 633 位置對於 Daxx 與 Promyelocytic leukaemia protein (PML)的交互作用，在細胞內外實驗也發現到突變的 Daxx 與 PML 有較弱的交互作用，並且影響其進入 PML-NB (PML-nuclear body)，綜而言之，$Daxx_{631-633}$ 對於其能專一辨認作用的蛋白是重要的，在未來我們將更廣泛去看 Daxx 這段區域與其他類泛素化蛋白的交互作用是否有保留性。

關鍵詞: 後轉譯修飾、類泛素化修飾、Smad4、Daxx、蛋白交互作用

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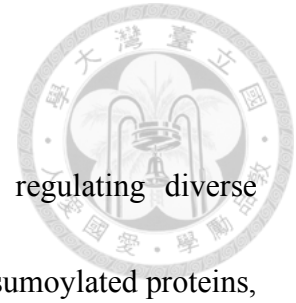

Abstract

Sumoylation is an essential post-translational modification regulating diverse cellular functions. Daxx has been reported to associate with several sumoylated proteins, which regulates the transcriptional activity and in certain cases affects their subnuclear compartmentalization. While Daxx can bind to specific sumoylated factors by its SUMO interacting motif (SIM), such binding cannot provide the specificity for substrate recognition. Thus, we hypothesized that additional region or motif within Daxx may contribute to its substrate recognition. In order to identify such substrate recognition motif within Daxx, we first selected its interacting substrate Smad4 for study. Our lab has shown that sumoylation of Smad4 Lys^{159} is critical for its interaction with Daxx, leading to Smad4 sumoylation-elicited transcriptional repression. By GST pull-down assay, I demonstrated that sumoylated Smad4 at Lys^{159} can be pulled down by $Daxx_{625-740}$ rather than by $Daxx_{660-740}$. Via site-directed mutagenesis, we found that $Daxx_{631-633}$ is critical for sumoylated Smad4 binding. Similarly, *in vivo* co-immunoprecipitation experiment presented that $Daxx_{631-633}$ mutant cannot interact with SUMO-modified Smad4. Additionally, reporter gene assays indicated that $Daxx_{631-633}$ mutant can mildly repress Smad4-mediated transcriptional activation. Furthermore, we also explored another Daxx interacting substrate, PML. GST pull-down and immunoprecipitation experiments

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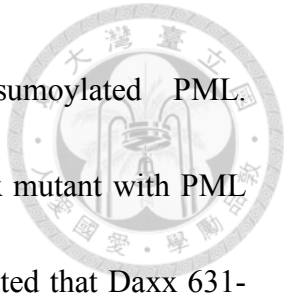

revealed that $Daxx_{631-633}$ mutant failed to interact with sumoylated PML. Immunofluorescence analysis indicated that the association of Daxx mutant with PML nuclear body was also reduced. Taken together, these results suggested that Daxx 631-633a.a. may play an important role in sumoylated Smad4 and PML recognition. In the future, we will examine whether $Daxx_{631-633}$ is a conserved substrate recognition motif for other substrates or only specific to Smad4 and PML.

Keywords: PTM, SUMOylation, Daxx, Smad4, protein-protein interaction

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Chapter I Introduction

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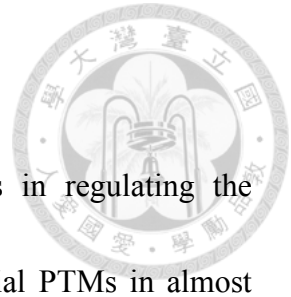

SUMOylation

Post-translational modifications (PTM) play important roles in regulating the cellular mechanisms of proteins. Sumoylation is one of the essential PTMs in almost eukaryotes participating in several mechanisms including genomic integrity, signaling transduction, transcriptional repression and viral infection. Sumoylation also has crosstalk with other PTMs such as phosphorylation [1], ubiquitination [2], methylation [3] and acetylation [4]. Moreover, sumoylation is also related to the development of disease and cancer. Several studies indicated that sumoylation is involved in DNA damage repair response since small SUMO ubiquitin-like protein (SUMO) modified substrates have been found upregulated in response to DNA damage [5]. Many tumor suppressors and oncogenes are sumoylated. The first link between cancer and sumoylation is from the discovery of promyelocytic leukaemia protein (PML) fused with retinoic acid receptor-α (RARα) in the acute promyelocytic leukemia (APL) patients [6]. With the increased number of sumoylated substrates, the regulation of the cellular sumoylation mechanism remains to be deciphered.

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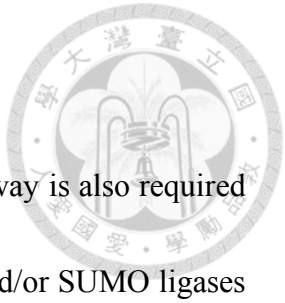

SUMO machinery

SUMO is structurally similar to ubiquitin and its catalytic pathway is also required SUMO-activating enzyme (E1), SUMO-conjugating enzyme (E2) and/or SUMO ligases (E3). A complete SUMO machinery is composed of SUMO maturation, conjugation and de-sumoylation [7]. Firstly, precursor SUMO is activated by sentrin-specific protease (SENP) to cleave C terminus and exposes its di-glycine residues for its conjugation with the lysine residue of substrates. Mature SUMO is then activated by SUMO-activating enzyme (SAE1/SAE2) by forming a thioester bond between the catalytic cysteine residue of SAE2 and C-terminal glycine residues of SUMO in ATP-dependent pathway [8]. Then, SUMO is transferred from SAE2 to SUMO E2 conjugating enzyme, Ubc9 (ubiquitin-conjugating 9), by forming a thioester bond. During conjugation step, Ubc9 recognizes SUMO consensus motif ψKXD/E (ψ, a large hydrophobic residue and X, any amino acid) of the substrates [9]. Of note, not all proteins with SUMO consensus motif ψKXD/E can be sumoylated. Then, SUMO protein is conjugated to protein substrate by forming an isopeptide bond between the C terminal glycine residue of SUMO and lysine of substrate. SUMO ligases including PIAS family of proteins, RAN Binding Protein 2 (RanBP2) and Polycomb protein (Pc2) enhance substrate sumoylation in certain situation [10]. As for

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de-sumoylation step, desumoylation enzymes (SENPs) de-conjugate the covalent linkage of SUMO to protein substrate which reverse the sumoylation.

SUMO interacting motif (SIM)

SUMO interacting motif (SIM) initially discovered by yeast two-hybrid screening, is a short sequence that allows protein binding to SUMO molecules [11, 12]. SIM is a hydrophobic core with a typically consensus sequence V/I-X-V/I-V/I. The SIM binds to SUMO at hydrophobic groove arranged by a β2 strand and a α1 helix [13]. Several proteins are found containing SIM, including SUMO substrates and sumoylation enzymes. One of SUMO substrates, Death-associated protein (Daxx) possesses a SIM at its C terminus I-I-V-L-S-D-S-D. Daxx SIM is important for its targeting to PML nuclear bodies (PODs), its own sumoylation and Daxx-dependent transcriptional regulation [14].

The role of Daxx

Daxx is first identified as an interaction partner of death receptor Fas by yeast two-hybrid screening [15]. Upon Daxx binds to Fas death domain, Jun N-terminal kinase (JNK) pathway is activated to induce apoptosis. Daxx is also found to interact with C terminal domain of TGF-β type II receptor which induces TGF-β apoptotic-signaling pathway [16].

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In the cytoplasm, Daxx acts as a pro-apoptotic protein. However, other study suggested that Daxx also has an anti-apoptotic role. Daxx knockout cells show the enhancement of apoptosis [17]. Besides the role of the signaling transducer, Daxx also acts as a transcriptional repressor in the nucleus. Several studies indicated that Daxx can suppress the transcriptional potential of several transcription factors, including glucocorticoid receptor (GR) [18], Smad4 [19], androgen receptor (AR) [18] and CREB-binding protein (CBP) [20] and p53 [21].

Moreover, further study indicated that the direct interaction between Daxx and several transcription factors is sumoylation-dependent. Daxx recognizes SUMO modification on these substrates by its C-terminal SIM and further represses transcriptional activity, including CREB-binding protein (CBP) [20], Smad4 [19] and androgen receptor (AR) [18]. Besides, Daxx is found to associate with other sumoylated proteins localizing in distinct subnuclear compartments. In PML-NBs, sumoylated PML is associated with Daxx, which efficiently sequestrates Daxx, leading to a loss of Daxx-mediated transcriptional repression [22]. Besides PML, Daxx is involved on interacting with centromeric component, including ATRX [23] and centromere protein-C (CENP-C) [24] during different phases of cell cycle, which implies that Daxx takes part in regulating other functions. However, not all sumoylated substrates can be recognized by Daxx.

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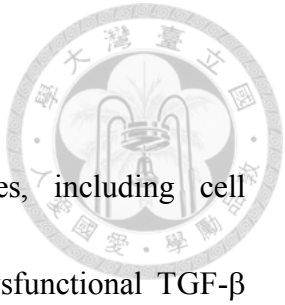

Overview of TGF-β signaling

TGF-β signaling is involved in diverse cellular processes, including cell differentiation, cell growth, cell migration and apoptosis [25]. Dysfunctional TGF-β signaling leads to many human diseases, such as cancer [26]. TGF-β receptors and SMAD protein family are important for mediating TGF-β signaling pathway. TGF-β receptor belongs to serine-threonine kinase receptor and can be divided into TGF-β type I and TGF-β type II. Upon TGF-β binds to TGF-β type II receptor, type I receptor is then recruited and phosphorylated. The type I receptor then phosphorylates regulated-Smad (R-Smads), which leads to decrease affinity of R-Smads for Smad anchor for receptor activation (SARA) and increase affinity for common Smads (Co-Smads). R-Smad and Co-Smad complex is then translocated into the nucleus and cooperated with co-activators and co-repressors to mediate the expression of its downstream genes. For example, upon the treatment of TGF-β, Smad3 forms complex with Smad4 and the complex is then translocated and accumulated in the nucleus. The complex binds to DNA with the help of other factors, such as FAST, and regulates Mix2 expression[27]. In addition to R-Smads and Co-Smads, inhibitory Smads (I-Smads) can act as an antagonist to inhibit TGF-β signaling pathway by competing with R-Smads for receptor interaction.

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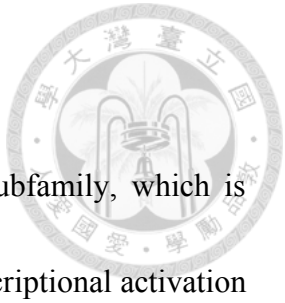

Smad4 as a co-Smad

Among Smads family, Smad4 is the only known co-Smad subfamily, which is essential for translocating R-Smads into nucleus and mediating transcriptional activation of TGF-β signaling. *Smad4*, as known as a tumor suppressor gene, has been found deleted or mutated in patients suffering pancreatic cancer [28]. Smad4 contains two conserved domains, MH1 domain and MH2 domain, which are separated by linker region and locate at N terminal and C terminal, respectively [29]. MH1 domain contributes to nuclear import activity and transcription regulation by binding to DNA. Its consensus-binding site on DNA is 8-bp GTCTAGAC, which is also known as Smad-binding element (SBE) [30]. As for MH2 domain, it regulates Smad oligomerization and interaction with other cytoplasmic factors. The proline-rich Smad activation domain (SAD) localizes at the linker region, which is responsible for full activation of Smads and important in stabilizing the interaction with other transcription factors.

The crystal structure of Smad3/Smad4 complex is heterotrimer composed of two Smad3 phosphorylated at serine 465 and serine 467 and one Smad4 [31]. Besides, the crystal structure of Smad4 fragment including the SAD and MH2 domain (S4AF) reveals that SAD enhances the structural core in order to present its hydrophobic proline-rich

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surface for interaction with other transcription factors. Thus, SAD provides transcriptional capability [32].

Recently, several reports demonstrated that post-translational modifications play essential role in regulating the activity of Smad4. When Smad4 threonine residue at 276 are mutated into alanine to abolish phosphorylation, its nuclear accumulation becomes less efficient with the TGF-β treatment and its transcriptional activity becomes low in the MDA-MB-468 cells [33]. Furthermore, ubiquitination on Smad4 at lysine 507 residue also regulates Smad4 function [34]. For example, oligo-ubiquitination positively regulates Smad4 function, whereas poly-ubiquitination occurs in unstable cancer mutants and leads to protein degradation. As for sumoylation, it has been found that there are two major Smad4 sumoylation sites, lysine 113 and lysine 159, respectively [19, 35, 36]. Mutation at lysine 159 affects its interaction with Daxx and abolishes its transcriptional activity [19]. Moreover, Smad4 interacts with Daxx in a SUMO-dependent manner.

PML and PML nuclear bodies

The promyelocytic leukaemia protein (PML) acts a tumor suppressor and forms unique sphere structure distinct from chromatin in the nucleus. PML is initially identified from the acute promyelocytic leukemia (APL) patients as the form of PML fused with

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retinoic acid receptor (RARα), resulting from reciprocal chromosome translocation [37]. There are seven PML isoforms generated by alternative splicing of C terminal exon. All PML isoforms consist of a zinc RING finger (R), two cysteine/histidine-rich B-Box domains (B1 and B2) and α-helical coiled-coil region (CC), which is also known as RBCC/TRIM motif [38]. The RBCC motif is essential for autoassembly and nucleation of nuclear bodies (NBs). As for C terminal part of PML, it contains serine and tyrosine residues which can be phosphorylated and is also important in regulating NBs formation [39]. The formation of NBs is activated not only by RBCC/TRIM motif multimerization but also by PML sumoylation. PML is sumoylated by SUMO-conjugating enzyme, UBC9, which catalyzes three major sumoylation sites, K65, K160 and K490 [40]. However, some groups found that sumoylation is not important for PML-NBs formation, since mutation of three sumoylation sites still forms PML-NBs [41, 42]. After PML-NBs formation, SUMO, several proteins and proteins containing SIM are recruited into inner core of the body. PML-NBs recruits several proteins into PODs, including nuclear protein Sp100 [43] and Daxx [22]. Previous study demonstrated that Daxx colocalizes with PML in POD and the recruitment of Daxx is PML sumoylation-dependent [44, 45]. The absence of SUMO-modified PML fails to recruit Daxx, which is associated with chromatin instead [22]. Moreover, the sequestration of Daxx by PML inhibits Daxx-

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mediated transcriptional repression, which leads to transcriptional activation of its downstream genes, such as GR [46]. PML-NBs acts as a hub to recruit many components for sequestrating their functions, mediating protein-protein interaction and facilitating post-translational modification. Moreover, PML-NBs also has diverse biological functions, including transcriptional regulation, DNA-damage response and apoptosis [47].

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Specific Aims

Previous studies indicated that Daxx acts as a SUMO reader via SIM and recognizes with several sumoylated transcription factors and subnulear factors, including Smad4, CBP and PML. Daxx represses transcriptional activity of these transcription factors and it could be recruited to PML-NBs by sumoylated PML. Since Daxx can only interact with specific sumoylated substrates, we tried to identify why Daxx possesses the substrate specificity. Thus, we hypothesize that Daxx contains a substrate recognition motif which cooperates with SIM to recognize substrates. Here, we utilize SUMO-modified Smad4 and PML to figure out their interplay with Daxx. We will elucidate the interaction between Daxx and sumoylated substrates by following specific aims:

1. To determine the substrate recognition motif on Daxx and its effect on binding to sumoylated Smad4 and PML
2. To characterize the transcriptional repression activity of the substrate recognition motif mutated Daxx
3. To analyze the recruitment of the substrate recognition motif mutated Daxx into PML-NBs

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Chapter II Materials and Methods

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Plasmid constructs and site-directed mutagenesis

The cDNA fragments of Daxx deletion mutants were subcloned into pGEX-4T-1 vector (Amersham Biosciences) to generate pGEX-5T-Daxx$_{625\text{-}740}$, pGEX-5T-Daxx$_{660\text{-}740}$ and pGEX-5T-Daxx$_{625\text{-}732}$. Twelve site-directed pGEX-5T-Daxx$_{625\text{-}740}$ mutants were generated by Pfu polymerase with the primer pairs indicted below. The Daxx$_{625\text{-}740}$ mutant included Daxx$_{SGP625\text{-}627AAA}$, Daxx$_{PCK628\text{-}630AAA}$, Daxx$_{KSR631\text{-}633AAA}$, Daxx$_{KEK634\text{-}636AAA}$, Daxx$_{KQT637\text{-}6639AAA}$ Daxx$_{GSG640\text{-}642AAA}$, Daxx $_{PLG643\text{-}645AAA}$, Daxx $_{NSY646\text{-}648AAA}$, Daxx $_{VER649\text{-}651AAA}$, Daxx $_{QRS652\text{-}654AAA}$, Daxx $_{VHE655\text{-}657AAA}$ and Daxx $_{KNG658\text{-}660AAA}$. The sequences of each construct were checked by DNA sequencing. pEGFP-Daxx was generated by subcloning full-length cDNA of Daxx into the pEGFP-C1 vector (Clontech). For constructing GST- Smad4-Linker-ΔMH1, cDNA fragment encoding the linker and MH2 domain was subcloned into the pGEX-4T-1 vector. The primer pairs used for generating Daxx$_{625\text{-}740}$ mutant were indicated in Table1.

GST Pull-down Assay

Ten microgram of bacterially purified GST fusion protein were incubated with 15μl GST resin in 300μl binding buffer (10mM HEPES pH7.5, 0.5mM EDTA, 0.1% NP-40, 50mM NaCl, 0.5mM DTT) for 1 hr at RT. The samples were then washed and blocked

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in buffer containing 5mg/ml of BSA for 1hr. Sumoylated-Smad4 were incubated with GST-fused protein in 300μl binding buffer for 1hr. The samples were then washed with high-salted washing buffer (binding buffer containing 100mM NaCl) four times and was added with 6μl 5X SDS loading dye for further analysis. Half of the sample were examined by Coomassie Blue staining to visualize GST fusion protein and the other half of the sample were analyzed by immunoblotting with anti-Smad4 antibody. As for the interaction of PML_{1-400} and GST fusion protein, the pull-down condition was the same and PML was analyzed by anti-PML antibody.

Cell Culture, Transient Transfection, and Luciferase Reporter Assay

Mv1Lu cell was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. For reporter assay, $2X10^5$ of cells were seeded on 24-well plate prior to transfection. HA-Daxx and HA-$Daxx_{KSR631-633AAA}$, together with SBE-4 reporter gene and TK-Renilla as an internal control were cotransfected by PolyJet transfection reagent (Signagen). The transfectants were starved for 12 hr followed by TGF-β treatment in DMEM supplemented with 0.2% fetal bovine serum for 12 hr. The cells were lysed for estimating protein expression and for assaying relative luciferase activity by Dual-Glo Luciferase Assay Kit (Promega).

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Cell lysis, Immunoprecipitation and Immunoblotting

5 x 10^5 cells of 293T cell were seeded on 10 cm dish the day before transfection. For Smad4-Daxx interaction, the expression vectors of mCherry-Sumo1, EGFP-Daxx or its mutants and pRK5-HA-Smad4 were transiently transfected into 293T cells. After 48 hr, the cells were lysed in lysis buffer containing 50 mM Tris (pH 7.8), 0.15 M NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 0.1% sodium deoxycholate, protease inhibitor mixture (Complete; Roche Applied Science) and 20mM *N*-ethylmaleimide (NEM). The lysates were mixed with anti-HA magnetic beads (ThermoFisher), incubated for 4-6 h on the gently rotary, and followed washed with the lysis buffer for 3 times. The beads-bound proteins were released in 2X SDS sample buffer and loaded onto SDS-PAGE, following by Western blot analysis with the indicated antibodies and ECL detection (Amersham). For PML-Daxx interaction, the expression vectors of mCherry-Sumo1, EGFP-Daxx or its mutants and HA-NLS-PML_{1-394} were transiently transfected into 293T cells. After 48 hr, the cells were lysed in lysis buffer and the cell lysate was then incubated with anti-HA magnetic beads for 4-6 hr. After washing step, the beads-bound proteins were released in 2X SDS sample buffer and loaded onto SDS-PAGE, following by Western blot analysis with the indicated antibodies and ECL detection. Anti-HA antibodies was from Covance and anti-GFP antibodies was from Abcam.

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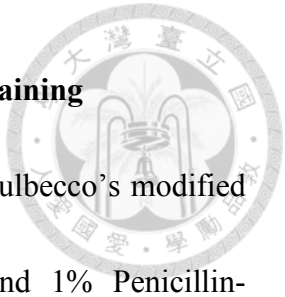

Cell Culture, Transient Transfection and Immunofluorescence staining

MDA-MB-468 cell and MCF7 cell (Daxx +/-) was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. $2X10^4$ of MDA-MB-468 cell and MCF7 cell were plated onto coverslips the day before transfection. MCF7 cell were transiently transfected with GFP-Daxx, GFP-$Daxx_{KSR631-633AAA}$ and GFP-$Daxx_{1-732}$. After 48 hours, the cells were then fixed in 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 1% Triton X-100, and then incubated with the anti-PML antibody overnight at 4°C. Following the incubation, the cells were washed three times for 3 min with phosphate-buffered saline containing 0.1% Triton at room temperature and the incubated with the Alexa Fluor 594 dye (anti-rabbit) for 2 hr at room temperature. After washing step, the nuclei were revealed by 4',6'-diamidino-2-phenylindole staining. The coverslips were inverted and mounted on slides. The images were visualized by confocal microscopy. The colocalization was quantified by Metamorph

Bacterial Expression and Purification of Daxx

pGEX-5T-$Daxx_{625-740}$ vectors were transformed into BL21 (DE3) *Escherichia coli* competent cells. The colonies were picked and incubated in 20ml LB broth. Then,

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bacterial culture was transferred to 400ml LB broth. Upon OD value reached to 0.6, expression was induced by treating the bacteria with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3hr at 37°C. The bacteria were harvested by centrifugation at 4000xg for 30 min at 4°C. The pallet was resuspended and lysed by High Pressure Cell Disruptor with lysis buffer (25mM Tris and 100mM NaCl). Next, the bacterial lysate was centrifuged and the supernatant was applied to a gravity-flow column (Bio-Rad) packed with Glutathione-Sepharose resin. The non-specific binding protein was washed with washing buffer containing 300mM NaCl. The GST-Daxx$_{625-740}$ was eluted by 10mM reduced glutathione. All the Daxx mutant was purified by the same method.

Bacterial Expression and Purification of Sumoylated Smad4

pGEX-5T-Smad4-ΔMH1 was co-transformed with pE1-E2-his-sumo1 in BL21 (DE3) *Escherichia coli* competent cells. After IPTG induction overnight at 16°C, bacteria were harvested by centrifugation at 4000g for 30 min at 4°C, resuspended with lysis buffer (25mM Tris and 100mM NaCl) and lysed by High Pressure Cell Disruptor. The cell debris was removed by centrifugation at 14,000g for 30 min at 4°C. The sumoylated Smad4 was applied to a gravity-flow column (Bio-Rad) packed with Glutathione-

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Sepharose resin. The resin was washed with washing buffer containing 300mM NaCl to avoid non-specific binding. The protein-bound resin was incubated with 50 unit of thrombin (GE Healthcare) overnight at 20°C. The supernatant was eluted from resin and then applied to column packed with Ni-NTA affinity resin for purification of sumoylated Smad4. The resin was washed with buffer containing 100mM NaCl buffer and followed by buffer containing 10% glycerol and increasing amount of Arginine. The Smad4-ΔMH1-his-sumo1 bound resin was eluted with 300mM imidazole. The protein was further purified by using Superdex 200 gel-filtration column (GE Healthcare).

Circular dichroism (CD) spectroscopy

The secondary structures were measured by circular dichroism (CD) spectroscopy, using Chirascan™-plus CD Spectrometer. For far-UV measurement, the spectra were obtained from 260 to 190 nm at 25°C in a 1mm pathlength cuvette at a bandwidth of 1 mm and 1s response time. Bacterial purified His-tagged $Daxx_{625\text{-}740}$ and His-tagged $Daxx_{K631\text{-}633A}$ in buffer containing 20 mM HEPES and 100 mM NaCl were diluted to 0.5 mg/mL prior to use. The experiment was conducted by BCF in Academic Sinica. CD data was analyzed by CDNN software to predict secondary structure.

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Chapter III Results

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Purification of sumoylated Smad4-ΔMH1 at K159

In the previous study, the linker region of Smad4 was sufficient for its interaction with the C terminal part of Daxx by the yeast-two hybrid. Thus, we purified SUMO modified $Smad4_{414-541}$ containing Linker region and MH2 domain. We co-transformed SUMO machinery plasmid (sumo E1, sumo E2 and his-tagged sumo1) with GST-Smad4-ΔMH1 to produce sumoylated Smad4 at K159 in E.*coli*. The SDS-PAGE staining indicated that after GST resin purification (Fig. 1B), thrombin digestion (Fig. 1C), Ni-NTA resin purification (Fig. 1D) and FPLC purification, there was a sumoylated Smad4 band at around 72kD (Fig. 1E). However, when Smad4-ΔMH1 K159R was purified, it didn't show a band. Then, we could use sumoylated Smad4-ΔMH1 at K159 to do further experiment.

Identification of the substrate recognition motif on Daxx for interaction with sumoylated Smad4

In yeast two-hybrid assay, the C-terminal domain of Daxx ($Daxx_{570-740}$) could interact with LexA-Smad4, rather than N-terminal fragment of Daxx. In order to examine the direct interaction between Daxx and sumoylated Smad4-ΔMH1, we utilized different truncated C terminal fragments of Daxx, $Daxx_{625-740}$, $Daxx_{660-740}$ and SIM-deletion mutant

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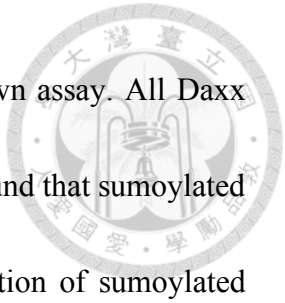

Daxx, $Daxx_{625\text{-}732}$, as a negative control to perform *in vitro* pull down assay. All Daxx mutants was fused with GST tag. In the GST-pull down assay, we found that sumoylated Smad4 could be pulled down by $Daxx_{625\text{-}740}$. However, the interaction of sumoylated Smad4 and $Daxx_{660\text{-}740}$ became as weak as $Daxx_{625\text{-}732}$ (Fig. 2). It implied that Daxx relied not only on its SIM to recognize sumoylated Smad4 but also other region between amino acid from 625 to 660. Here, we named the region as substrate recognition motif. Furthermore, in order to narrow down the region containing substrate recognition motif, we then did the $Daxx_{625\text{-}740}$ mutagenesis. Triplet of the amino acids from 625 to 660 were mutated into alanine so there were twelve Daxx mutants. In the GST-pull down assay, we observed that only when the three amino acid from 631 to 633, KSR were mutated, its interaction with sumoylated Smad4 became weak (Fig. 3A). As for other Daxx mutant, their interaction with sumoylated Smad4 were not affected (Fig. 3C). The relative quantification of band on western blot showed that the binding of $Daxx_{K631\text{-}633A}$ mutant with sumoylated smad4 had 80% reduction, compared to wild-type $Daxx_{625\text{-}740}$ (Fig.3B and C). Meanwhile, we also mutated KSR residues into alanine, respectively and found that single mutation still could interact with sumoylated Smad4 (Fig. 4). These results indicated that KSR, three amino acids from 631 to 633 might localized at substrate recognition motif and were important for sumoylated Smad4 recognition.

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Identification of the substrate recognition motif on Daxx for interaction with sumoylated PML

Daxx was known to be recruited to PML nuclear body (POD) in SUMO-dependent manner. Without sumoylated PML expression, Daxx could not target to POD. To know whether three amino residues, KSR for substrate recognition was conserved to other sumoylated substrates, next, we tested the interaction between Daxx and sumoylated PML by pull down assay. We purified $PML_{1\text{-}400}$ (K65R, K380R, K394R) and sumoylated at K160, the common lysine residue for sumoylation. In the GST pull down assay, sumoylated PML was pulled down by $Daxx_{625\text{-}740}$ rather than $Daxx_{K631\text{-}633A}$ and $Daxx_{625\text{-}732}$ (Fig. 5). These results indicated that KSR residues were also important for Daxx recognizing sumoylated PML.

Secondary structure comparison between $Daxx_{625\text{-}740}$ and $Daxx_{K631\text{-}633A}$ mutant

Mutagenesis on the amino acid sequence could alter the secondary structure of the protein, which would change the protein-protein interface and affect their interaction. To rule out the possibility that the mutagenesis on these three residues affected the secondary of Daxx, we did circular dichroism spectrum to estimate secondary structure and folding property of Daxx after its three amino acid residues were mutated. We purified wild-type

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and $Daxx_{625-740}$ mutant fused with his tag and performed the CDNN program to quantify spectral data (Fig. 6). Over the wavelength region of 210-250nm, we estimated the relative ratio of secondary structure elements of $Daxx_{625-740}$ included 4.0% α-helix, 45.1% antiparallel, 5.3% parallel, 19.3% β-sheet and 35.3% random coil. And the relative ratio of secondary structure elements of $Daxx_{625-740}$ mutant ($Daxx_{K631-633A}$) included 4.2% α-helix, 44.4% antiparallel, 5.3% parallel, 19.3% β-sheet and 35.3% random coil (Table 1). The wild type and mutant showed similar CD spectral patterns. Thus, we confirmed that the mutant structures were not altered by mutagenesis and the change of its interaction with sumoylated Smad4 was due to mutation of three important resides for substrate recognition.

Identification of the substrate recognition motif on Daxx for interacting with sumoylated Smad4 and PML *in vivo*

To further demonstrate the interplay between Daxx KSR residues and sumoylated substrates, Smad4 and PML, their interactions were determined *in vivo*. 293T cells were co-transfected with pRK5-HA-Smad4, EGFP-Daxx/ its mutant and mCherry-Sumo1 to produce high amount of sumoylated Smad4. The cell lysate was immunoprecipitated with HA beads and the western blot analysis revealed that higher amount of wild-type Daxx

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was precipitated by sumoylated Smad4 at K159, compared to sumoylated Smad4 at K113 (Smad4 K159R) (Fig. 7). However, the amount of $Daxx_{K631-633A}$ mutant that sumoylated Smad4 precipitated was lower than $Daxx_{1-732}$, which was as a negative control. Also, when Smad4 lysine 159 was mutated into arginine, the co-IP results indicated that Daxx and its mutant still could be precipitated by sumoylated Smad4 at lysine 113.

Furthermore, to determine the interplay between Daxx KSR residues and sumoylated PML, 293T cells were co-transfected with HA-NLS-PML_{1-394} (cover RBBCC motif)/ HA-NLS-PML_{1-394} K65R/ HA-NLS-PML_{1-394} K160R, EGFP-Daxx/its mutant and mCherry-Sumo1. The cell lysate was immunoprecipitated with HA beads and the western blot analysis revealed that there were two sumoylation sites on HA-NLS-PML_{1-394}, lysine 65 and lysine 160 indicated as black star and black arrow, respectively (Fig. 8). As predicted, wild-type Daxx could be precipitated by sumoylated PML. However, the amount of $Daxx_{K631-633A}$ mutant and $Daxx_{1-732}$ mutant that sumoylated Smad4 precipitated was low. Furthermore, when PML lysine 160 and lysine 65 were mutated into arginine, their interaction with wild-type Daxx also decreased (Fig. 8, right panel). These results demonstrated that $Daxx_{631-633}$ was important for the interaction of Smad4 and PML *in vivo*.

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$Daxx_{K631-633A}$ mutant abolished Daxx-induced transcriptional repression of Smad4

Daxx interacted with Smad4 in SUMO-dependent pathway and could repress Smad4 transcriptional activity. In the Mv1Lu mink lung cells and MDA-MB-468 cells carrying exogenous Smad4, it was found that Daxx could suppress TGF-β induced reporter activity in dose-dependent [19]. Since $Daxx_{K631-633A}$ mutant weakened the interaction with sumoylated Smad4, we next asked whether $Daxx_{K631-633A}$ mutant still remained the ability of repressing Smad4 transcriptional activity. Mv1Lu cells were transfected with Daxx along with the reporter SBE-4, containing four repeats of Smad4 binding elements and followed by TGF-β treatment. In the reporter assay, wild-type Daxx could repress reporter activity in dose-dependent manner and $Daxx_{1-732}$ failed to repress Smad4 transcriptional activation. Compared to 200ng of wild-type Daxx, 200ng of $Daxx_{K631-633A}$ mutant possessed weak repression activity. However, 350ng of $Daxx_{K631-633A}$ mutant has higher repressive activity than wild-type Daxx. These results indicated that three amino acid residues, KSR, residing in the substrate recognition motif may affect Daxx to repress Smad4-mediated transcriptional activity in specific protein expression.

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The association of PML nuclear body formation and the recruitment of Daxx

Precious study indicated that Daxx SIM controlled Daxx to target sumoylated PML and located at PODs. Next, we tested whether sumoylated PML could recruit the $Daxx_{K631-633A}$ mutant into POD. MCF Daxx +/- cells were transiently transfected with HA-Daxx/ HA-$Daxx_{K631-633A}$ and PML-mCherry. Immunofluorescence analysis revealed that wild-type Daxx was colocalized with PML-NBs (Fig. 10A). $Daxx_{K631-633A}$ mutant was expressed both in the cytoplasm and nucleus. In some cells, $Daxx_{K631-633A}$ mutant was not colocalized with PML and affects the formation of PML-NBs (Fig. 10B). Furthermore, we also see the localization of endogenous PML. MCF Daxx +/- cells were transfected GFP-Daxx/GFP-$Daxx_{K631-633A}$ and stained the endogenous PML to visualize their localization. By the immunofluorescence staining, as a control, overexpression of wild-type Daxx resulted in the recruitment of Daxx to PML-NBs (Fig. 10C), while SIM deletion $Daxx_{1-732}$ mutant could not concentrate to endogenous PML-NBs (Fig. 10E). Overexpression of $Daxx_{K631-633A}$ mutant demonstrated that Daxx was present both in the nucleus and cytoplasm since three residues was involved in the nucleus localization sequence (Fig. 10D). However, when $Daxx_{K631-633A}$ mutant was expressed in the nucleus, the majority of them condensed into PML-NBs and formed puncta colocalizing with endogenous PML-NBs but part of them did not. The colocalization rate of red

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fluorescence (endogenous PML-NBs) and green fluorescence (GFP-Daxx) was quantified by MetaMorph software and we defined the colocalization was calculated by yellow dots (merge) devided by green dots (GFP-Daxx). The quantification results showed the localization rate of wild-type Daxx with PML-NBs was around 62% and the localization rate of $Daxx_{K631\text{-}633A}$ with PML-NBs was around 17% (F). To sum up, $Daxx_{KSR}$ residues were important for the interaction of sumoylated PML and also recruitment of Daxx into PML-NBs.

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Chapter IV Discussion

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In the thesis, we have found that besides SIM, a substrate recognition motif on Daxx provides specificity for interacting with sumoylated substrates, including Smad4 and PML. We have identified three amino acid residues, KSR on Daxx may reside in substrate recognition motif. Mutation of these three residues weakens Daxx interaction with purified sumoylated Smad4 and PML *in vitro*. Similarly, the immunoprecipitation assay showed that $Daxx_{K631\text{-}633A}$ mutant had reduced interaction with sumoylated Smad4 and PML. Moreover, mutation also mildly inhibits Daxx to repress TGF-β/Smad4-mediated transcriptional activity. Additionally, when Daxx is mutated, the recruitment of Daxx into PML-NBs is also affected. Since we have observed that not all of the Daxx is not colocalized with PML-NBs by quantification. Taken together, these finding demonstrates that KSR residues are important not only for determining Daxx to recognize its substrates but also for regulating the role of Daxx as a transcriptional repressor and its cellular localization.

Nucleus localization signal was mutated on $Daxx_{K631\text{-}633A}$

Daxx contains two NLS, NLS1 and NLS2 which locates from a.a. 227 to a.a. 231 and from a.a. 627 to a.a. 637, respectively. NLS2 plays a major role in mediating Daxx in translocating into the nucleus by interacting with importin alpha3 [48]. Since site-

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directed mutation KSR residues are involved in NLS2, we observed that Daxx is expressed both in the cytoplasm and the nucleus by immunofluorescence staining. Moreover, previous study showed that due to the mutation of NLS, the transcription repression capacity of Daxx on glucocorticoid receptor (GR) was also compromised [48]. Thus, $Daxx_{K631\text{-}633A}$ mutant unable to repress Smad4-mediate transcription may be due to a loss of its ability to translocating into the nucleus. The consensus sequence of NLS is K-(K/R)-X-(K/R) [49] and lysine and arginine residues are important for NLS activity. To decrease the influence of the lysine residue in KSR mutation, we can mutate SR residues and see its interaction with sumoylated substrates in the future. Currently, it is possible that KSR residues act not only as a NLS but also take part in recognizing specific sumoylated proteins.

The potential problems of Smad4-mediated SBE4 reporter assay

Previous study showed that Daxx but not SIM deletion mutant repressed Smad4-mediated transcription in dose-dependent manner. However, in this thesis, we cannot repeat the previous results and only get high-variation data. The reason may be due to the quality of DNA, low value of luminescent signals and different cell culture condition. Furthermore, reporter assay revealed that when we overexpress 350ng $Daxx_{KSR}$ mutant,

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its repression effect on reporter assay is stronger than wild-type Daxx with the same dose. It can be explained that higher Daxx mutant protein expression level can compensate the effect that mutation of KSR residues causes. Unsurprisingly, it seems that the specificity of KSR residues contributes to is dose-dependent. In low dose of Daxx mutant, we can see its effect.

The interaction of Smad4 with Daxx *in vivo*

Previously, yeast two-hybrid screen showed that Daxx preferred to interact with sumoylated Smad4 at K159 since when Smad4 K113R did not abolish its interaction with Daxx but Smad4 K159R did [19]. However, our co-immunoprecipitation results showed that Daxx can interact with sumoylated Smad4 at K113. Compared to each Daxx mutants, the interaction between wild-type Daxx and sumoylated Smad4 at K113 did not show significant difference. This implied that the binding between sumoylated Smad4 at K113 and Daxx is regulated by other recognition mechanism rather than by which we see in the interaction of Daxx and sumoylated Smad4 at K159. Besides, co-immunoprecipitation results also showed that Smad4 binds better with Daxx SIM deletion mutant than $Daxx_{K631\text{-}633A}$ mutant. The reason may because the partial cytoplasm localization of $Daxx_{K631\text{-}633A}$ mutant decreases its contact with Smad4. Furthermore, in addition to SIM

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at $Daxx_{732-740}$, there is another SIM locating at N terminus [50], which may possess ability to recognize sumoylated Smad4. This may explain why SIM deletion $Daxx_{1-732}$ can bind more sumoylated Smad4 than $Daxx_{K631-633A.}$ Here, again demonstrated that bipartite interaction of Daxx SIM and substrate recognition motif are both important for interacting with sumoylated Smad4 at K159.

The interaction of PML and PML K65R/K160R with Daxx

It has been found that mutation of PML K160 completely abolished recruitment of Daxx into PML-NBs, which implies that K160 sumoylation may be important for its interaction with Daxx [51]. However, our co-immunoprecipitation results demonstrated that both sumoylation mutation of PML K160 and K65 reduce the interaction of PML and Daxx. Thus, we cannot rule out the possibility that Daxx interacts with PML via K160 and K65 sumoylation.

Previously, our lab found that when PML lysine 160 was mutated into arginine, not only K160 sumoylation but also K65 sumoylation was abolished. In addition to exogeneous mCherry-Sumo1, PML is still modified by endogenous Sumo1. Thus, when PML K160R mutant is overexpressed, we cannot see endogenous and exogenous

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sumoylated PML at K160 and K65, but other minor sumoylation band. This implied that sumoylated PML at other sites may contribute to its interaction with Daxx.

Other post translational modifications on KSR residues regulates Daxx function

Daxx are reported to involve in several posttranslational modifications, including phosphorylation, ubiquitination and sumoylation. The phosphorylation of Daxx at serine 669 by HIPK1 regulates its localization to the cytoplasm, which not only modulates the transcriptional repression activity of Daxx in specific reporters but also activates downstream Fas-Daxx-ASK1-JNK1 pathway to induce apoptosis [52]. Besides phosphorylation, lysine 630 and 631 are essential in the sumoylation of Daxx [53]. When K630/631 of Daxx are mutated, nuclear localization of Daxx are not affected and can still be recruited into PML-NBs. However, other study indicates that the sumoylation of Daxx at K630/631 increases nuclear localization and suppresses cell growth [54]. It implies that owing to the sumoylation defect of $Daxx_{K631\text{-}633A}$ mutant, it cannot be translocated to the nucleus and recruited into PML-NBs. Nevertheless, GST-pull down assay indicated the direct interaction between Daxx and sumoylated substrates so we can prove that the interplay between them is without the interference of other factors.

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The interaction between Daxx and sumoylated substrates

In previous study, we identified the dissociation constant of SUMO-1 and Daxx SIM representing by peptide composed of $Daxx_{721-740}$ is 55 μM [55]. In addition to qualitative analysis, we further need to quantified the binding affinity between Daxx and other Sumo-modified substrates and define dissociation constant. We have tried isothermal titration calorimetry (ITC) binding experiments. However, Daxx itself has an issue of monomer-dimer equilibrium in the high concentration, which makes it difficult to measure dissociation constant. In the future, other methods should be tried, such as SPR and MST. Moreover, we propose that Daxx specifically recognizes some sumoylated substrates by the bipartite interaction of SIM and substrate recognition motif. Interestingly, Daxx cannot bind to unmodified Smad4 in the *in vitro* pull assay (data not shown). This implies that Daxx SIM plays a major and prior role in interacting with SUMO modification and Daxx substrate recognition motif then follows and recognizes different substrates. However, we do not know whether there is a similar sequence on the different substrates that substrate recognition motif recognizes.

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Chapter V Figures

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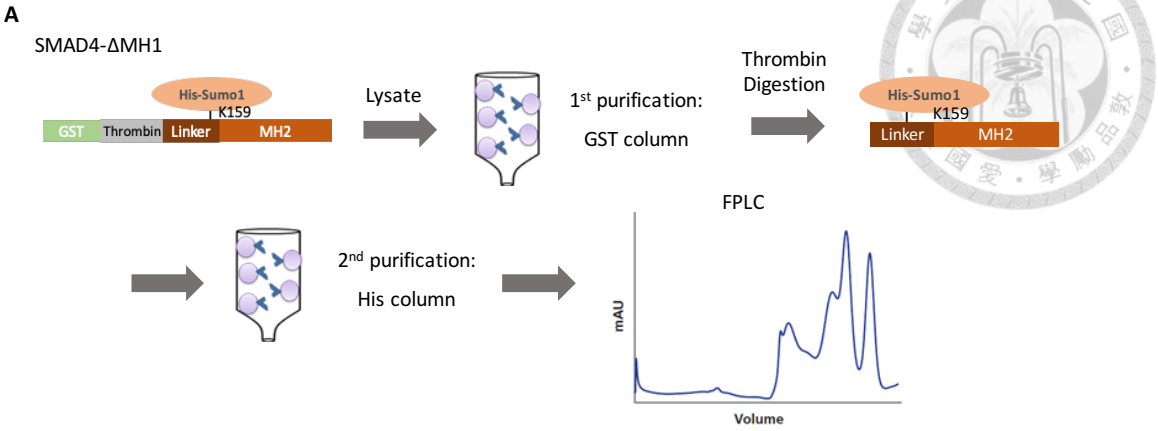

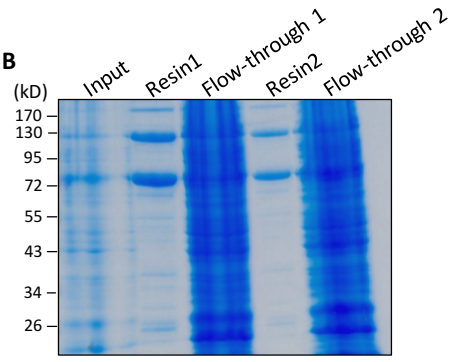

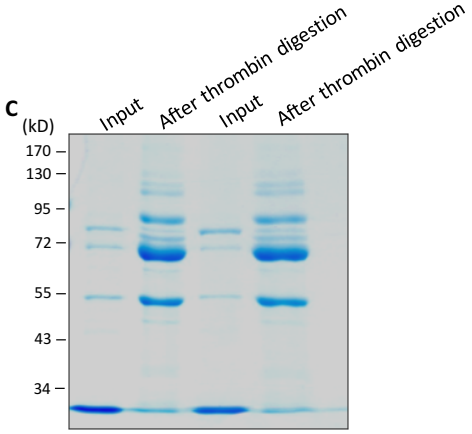

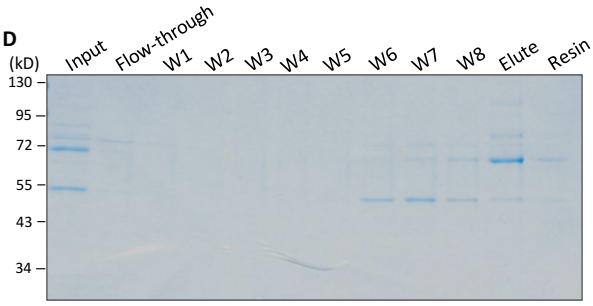

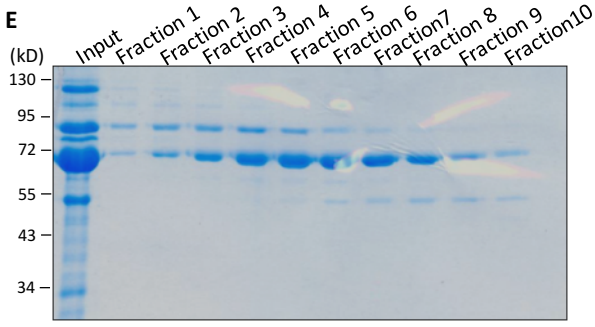

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Figure. 1 Purification of sumoylated Smad4-ΔMH1 at K159

(A) Schematic representation of steps involved in sumoylated Smad4 purification

(B) SDS-PAGE analysis of the protein fractions of sumoylated GST-Smad4-ΔMH1 purified by GST resin

(C) SDS-PAGE of sumoylated Smad4-ΔMH1 after thrombin digestion

(D) SDS-PAGE of sumoylated Smad4-ΔMH1 purified by Nickle resin

(E) SDS-PAGE of sumoylated Smad4-ΔMH1 after FPLC analysis

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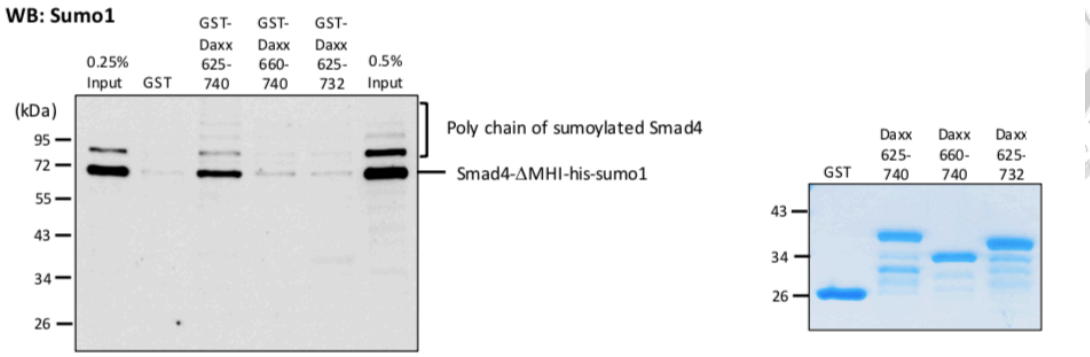

Figure.2 Interaction of deletion Daxx mutant with sumoylated Smad4 *in vitro*

Ten microgram of recombinant GST, GST-Daxx$_{625\text{-}740}$, GST-Daxx$_{660\text{-}740}$ and GST-Daxx$_{625\text{-}732}$ proteins prepared from bacteria were incubated with recombinant sumoylated Smad4. The reaction was subjected to electrophoresis on 10% SDS-PAGE and followed by Coomassie blue staining for GST fusion protein quantification (right panel). The other sample was transferred to nitrocellular membrane and probed with Sumo antibody (left panel).

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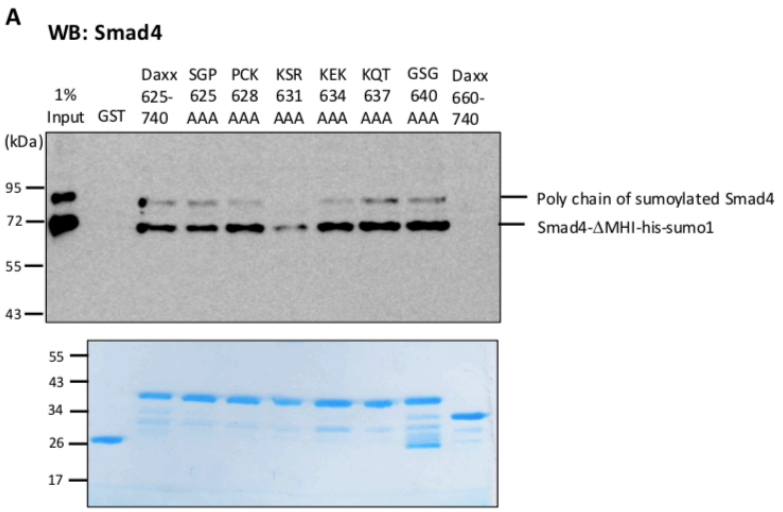

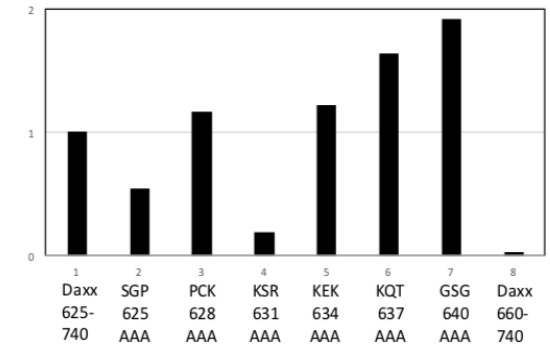

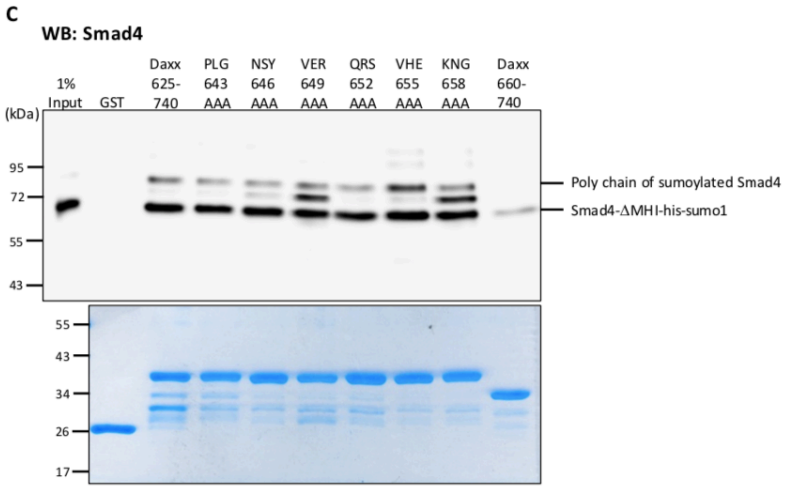

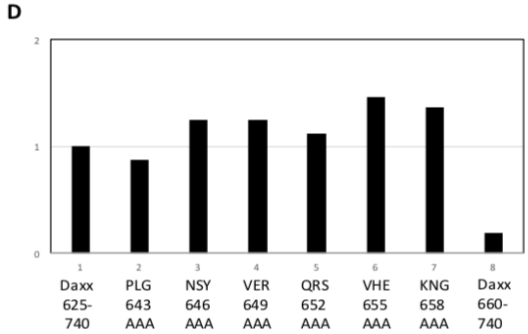

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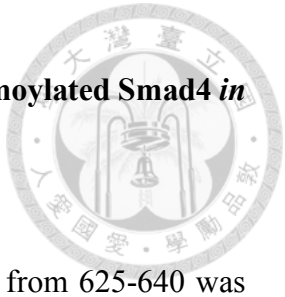

Figure. 3 Interaction of Daxx substrate recognition motif with sumoylated Smad4 *in vitro*

(A) Recombinant GST-Daxx$_{625\text{-}740}$ mutated every three amino acids from 625-640 was incubated with sumoylated Smad4. The reaction was subjected to electrophoresis on 10% SDS-PAGE and followed by Coomassie blue staining for GST fusion protein quantification (bottom panel). The other samples were transferred to nitrocellular membrane and probed with Smad4 antibody (top panel).

(B) The amount of each Daxx band was quantified by ImageJ and relative to the amount of wild-type Daxx$_{625\text{-}740}$.

(C) Recombinant GST-Daxx$_{625\text{-}740}$ mutated every three amino acids from 643-660 was incubated with sumoylated Smad4. The reaction was subjected to electrophoresis on 10% SDS-PAGE and followed by Coomassie blue staining for GST fusion protein quantification (bottom panel). The other samples were transferred to nitrocellular membrane and probed with Smad4 antibody (top panel).

(D) The amount of each Daxx band was quantified by ImageJ and relative to the amount of wild-type Daxx$_{625\text{-}740}$.

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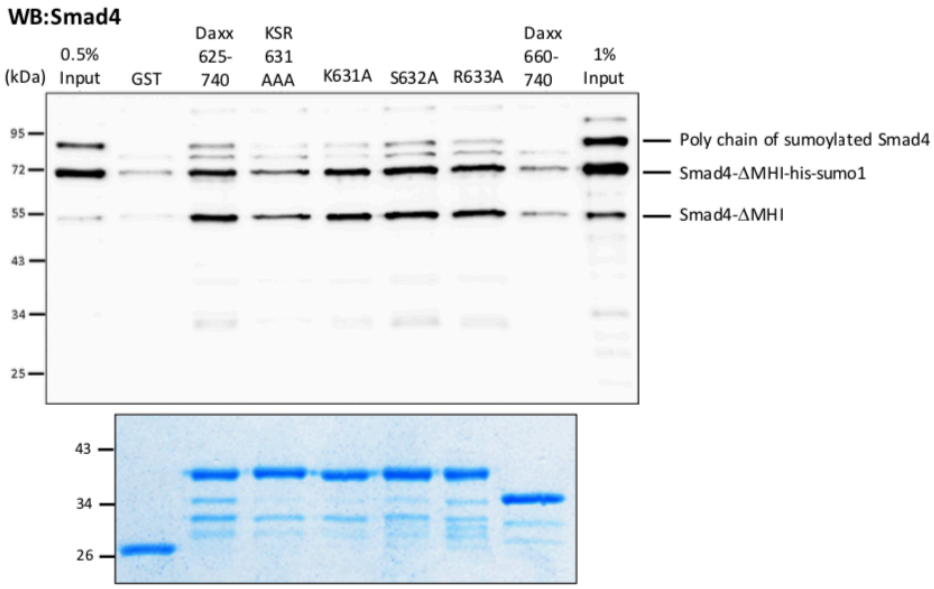

Figure. 4 Interaction of Daxx singe mutant with sumoylated Smad4 *in vitro*

Recombinant GST-Daxx$_{625\text{-}740}$ and single-site mutation of GST-Daxx$_{625\text{-}740}$ were incubated with sumoylated Smad4. The reaction was subjected to electrophoresis on 10% SDS-PAGE and followed by Coomassie blue staining for GST fusion protein quantification (bottom panel). The other samples were transferred to nitrocellular membrane and probed with Smad4 antibody (top panel).

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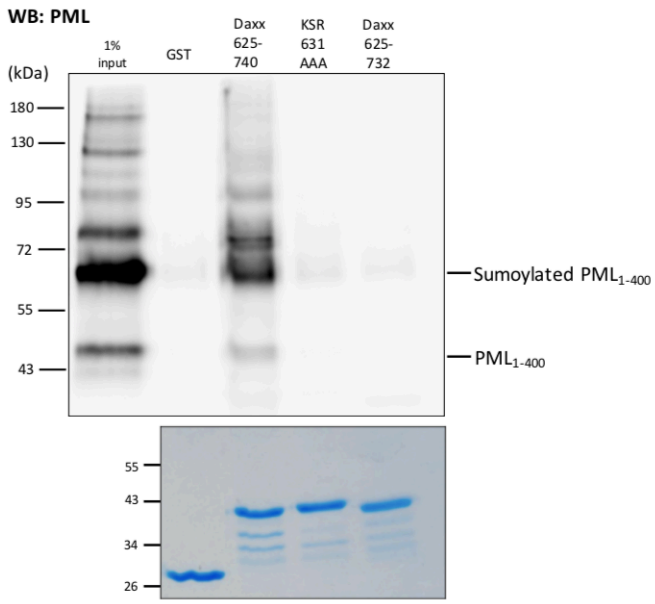

Figure. 5 Interaction of Daxx substrate recognition motif with sumoylated PML *in vitro*

Recombinant GST-Daxx mutant was incubated with sumoylated PML_{1-400} at K160. The reaction was subjected to electrophoresis on 10% SDS-PAGE and followed by Coomassie blue staining for GST fusion protein quantification (bottom panel). The other samples were transferred to nitrocellular membrane and probed with PML antibody (top panel).

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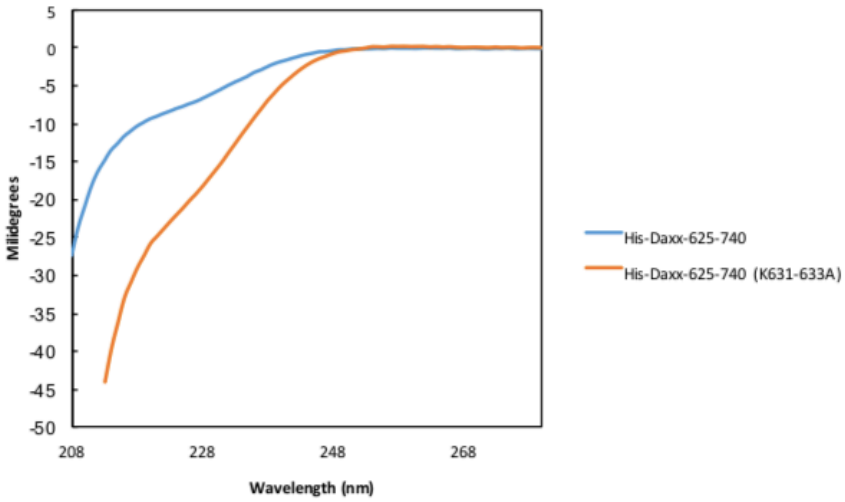

Figure. 6 Circular dichroism spectra of His-Daxx$_{625\text{-}740}$ and His-Daxx$_{K631\text{-}633A}$ mutant

Circular dichroism spectra of His-Daxx$_{625\text{-}740}$ (blue line) and His-Daxx$_{K631\text{-}633A}$ mutant (orange line) in far UV region (195–250 nm); each protein was taken in a concentration of 0.5 mg/ml in buffer containing 20 mM HEPES and 100 mM NaCl, pH 7.0. All spectra were obtained at 25 °C. Pathlength 1 mm, scan rate 1 mm/s averaged per spectrum.

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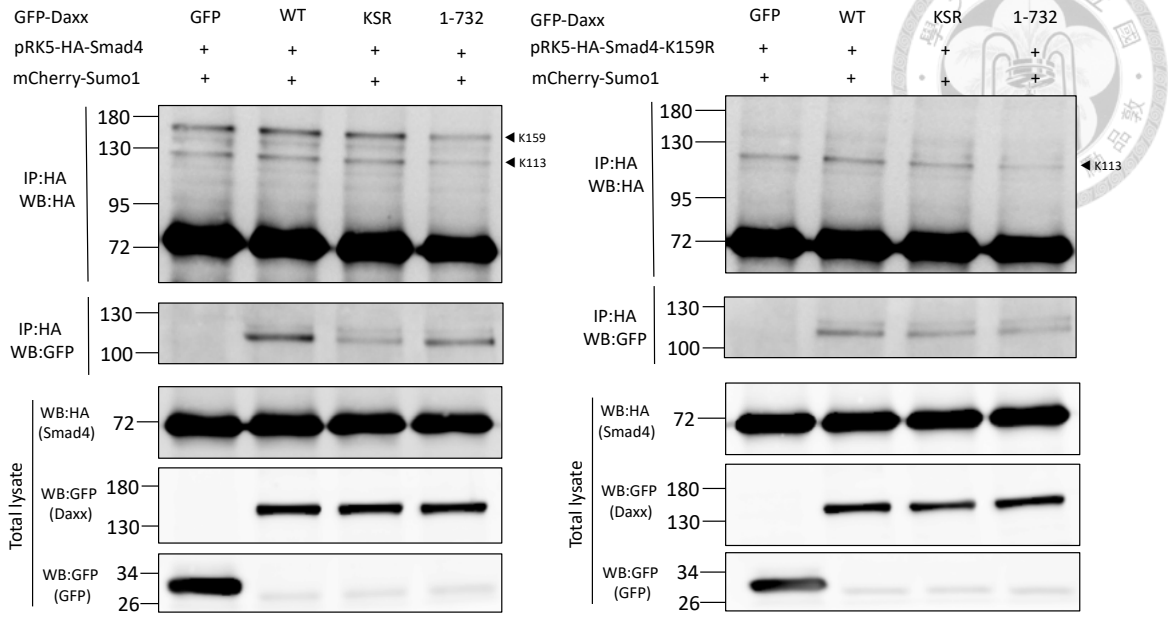

Figure 7. The interaction of Daxx mutant and Smad4 *in vivo*

293T cells were transiently transfected with expressing constructs for pRK5-HA-Smad4 / pRK5-HA-Smad4 K159R, EGFP-Daxx/its mutant and mCherry-Sumo1. The cells were then lysed in the RIPA buffer in the presence of 20 mM NEM. Equal amounts of lysates were immunoprecipitated with anti-HA beads followed by immunoblotting with anti-GFP or anti-HA antibody, respectively. The arrow indicates the SUMO-1 modified at K113 and K159, respectively.

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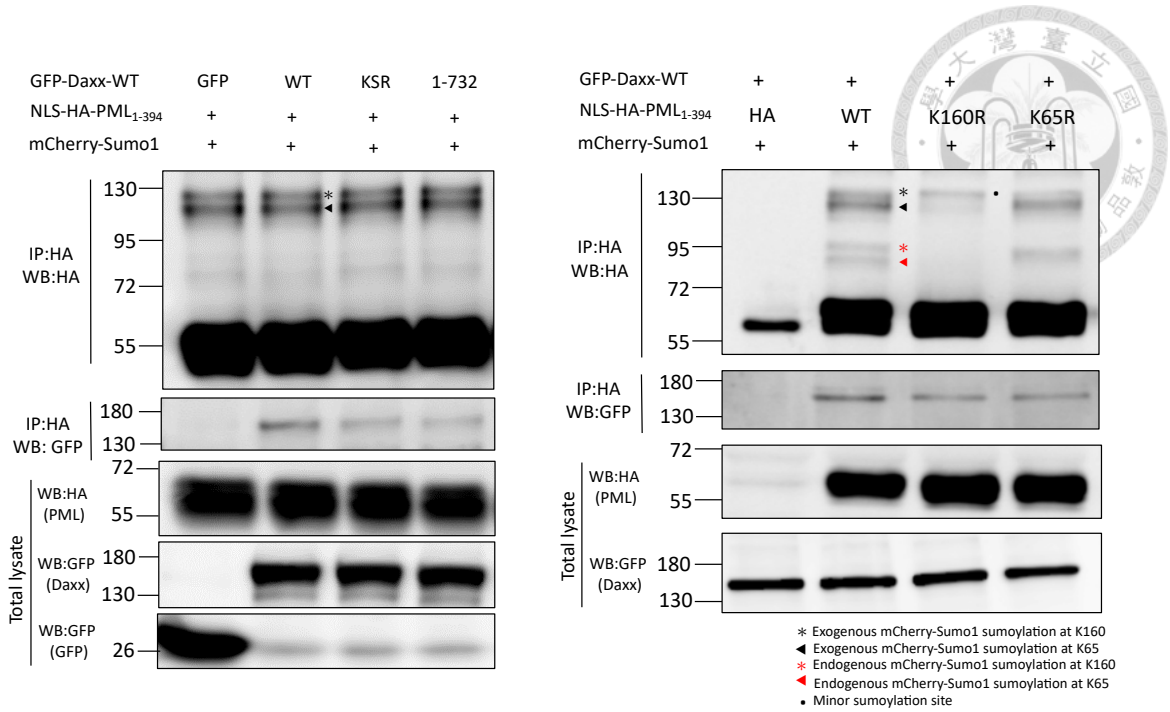

Figure 8. The interaction of Daxx mutant and PML *in vivo*

293T cells were transiently transfected with expressing constructs for HA-NLS-PML$_{1-394}$ / HA-NLS-PML$_{1-394}$ K65R/ HA-NLS-PML$_{1-394}$ K160R, EGFP-Daxx/ its mutant and mCherry-Sumo1, respectively. The cells were then lysed in the RIPA buffer in the presence of 20 mM NEM. Equal amounts of lysates were immunoprecipitated with anti-HA beads followed by immunoblotting with anti-GFP or anti-HA antibody, respectively. The black arrow indicates exogenous mCherry-SUMO-1 modified PML at K160 and the black star indicated exogenous mCherry-SUMO-1 modified PML at K65. The red arrow indicates endogenous SUMO-1 modified PML at K160 and the red star indicated endogenous SUMO-1 modified PML at K65. Other minor sumoylation site indicated as black dot.

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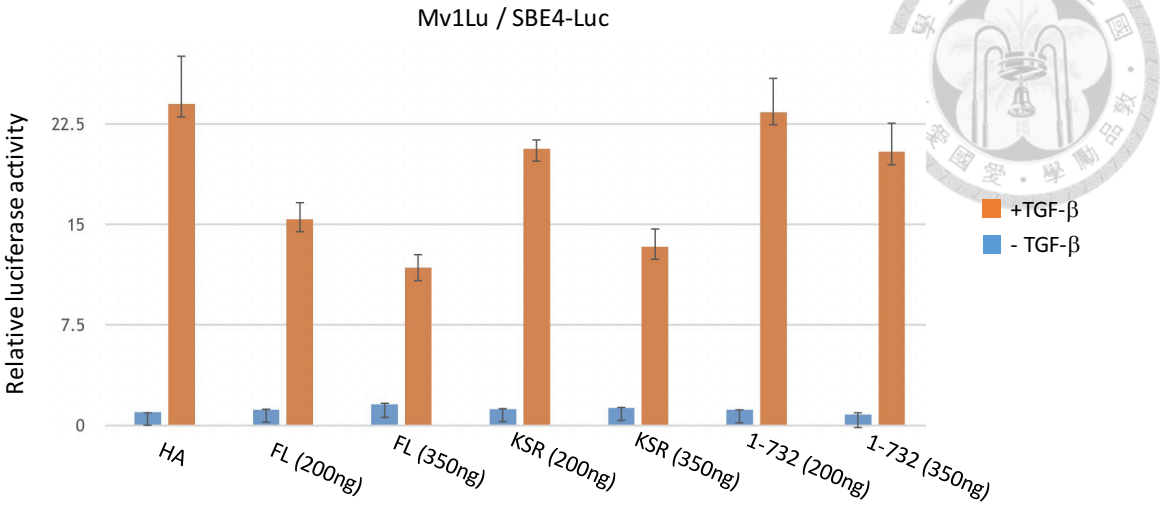

Figure 9. Mutation of Daxx substrate recognition motif potentiates Daxx-dependent repression of Smad4 transcriptional activity

Mv1Lu cells were transiently transfected with SBE4-Luc reporter construct along with increasing amount of HA-Daxx plasmids and its mutant as indicated. After transfection, the cells were starved for 12 h and stimulated with or without TGF-β treatment for 18 h. The cells were harvested and harvested for luciferase reporter assays.

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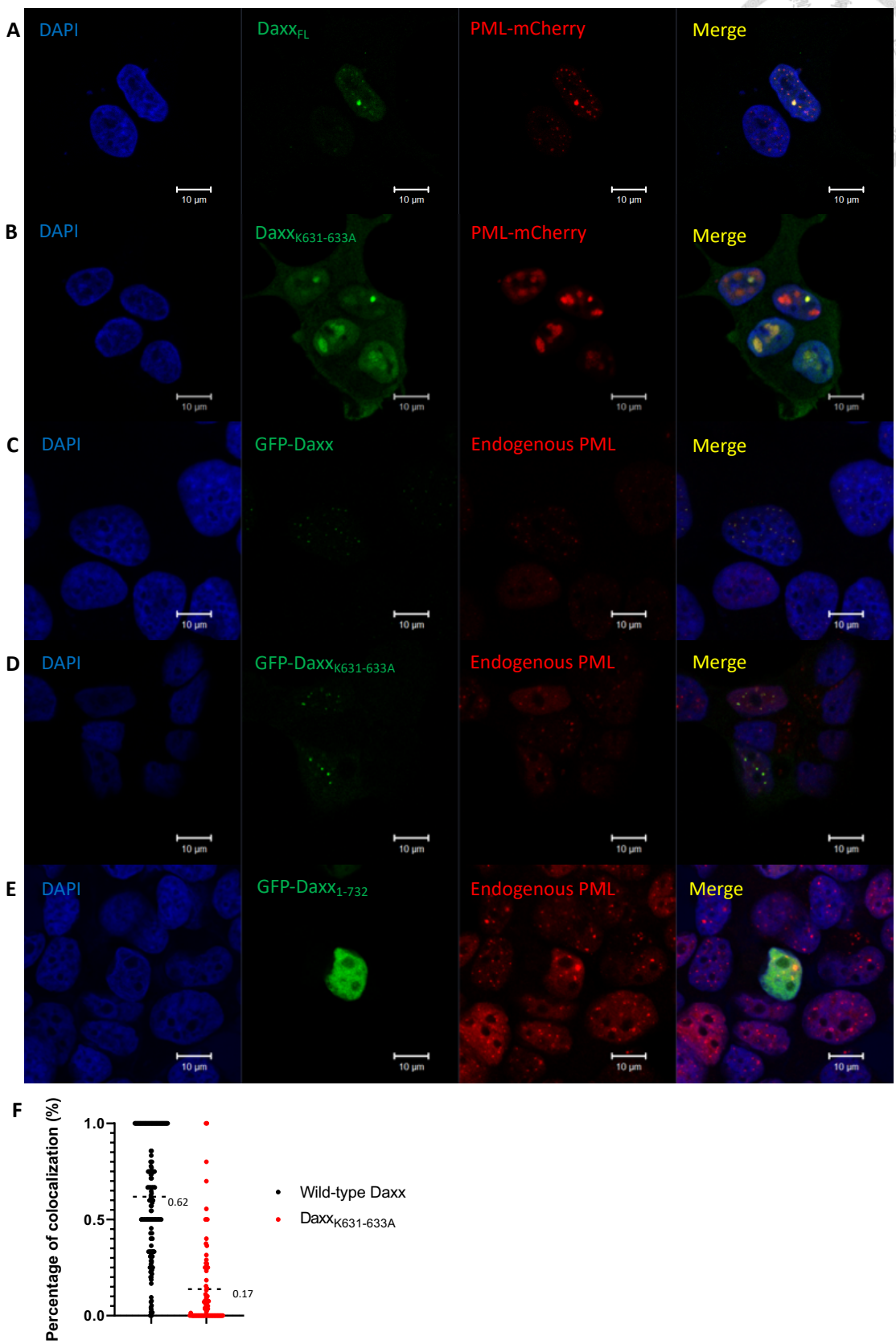

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Figure 10. Mutation of Daxx substrate recognition motif affected the localization of Daxx in PML-NBs

MCF7 (Daxx +/-) cells were transfected with plasmid expressing HA-Daxx (A)/ HA-$Daxx_{K631-633A}$ (B) and PML-mCherry. After 48hr, the cells were immunostained with anti-HA antibody (green) overnight followed by 4',6'-diamidino-2-phenylindole staining (blue). MCF7 (Daxx +/-) cells were transfected with plasmid expressing EGFP-Daxx (C), EGFP-$Daxx_{K631-633A}$ (D) and $Daxx_{1-732}$ (E). After 48hr, the cells were immunostained with anti-PML antibody (red) overnight followed by 4',6'-diamidino-2-phenylindole staining (blue). The cells were examined by confocal microscopy. The overlay of the green (GFP) and red (endogenous PML) images was shown in yellow (merge).

(F) Quantification of percentage of colocalization was defined by the ratio of green puncta (Daxx) and yellow puncta (Merge) and hundred cells were quantified by MetaMorph software.

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Chapter VI Tables

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	Forward primer	Reverse primer
$Daxx_{SGP625AAA}$	5′-CCGGAATTCCCGATATCGCAGCAG CCCCCTGCAAAAAATCTC-3′	5′-GAGATTTTTTGCAGGGGGCTG CTGCGATATCGGGAATTCCGG-3′
$Daxx_{PCK628AAA}$	5′-CCGATATCTCTGGTCCCGCAGCC GCCAAATCTCGGAAGGAGAAG-3′	5′-CTTCTCCTTCCGAGATTTGGCG GCTGCGGGACCAGAGATATCGG-3′
$Daxx_{KSR631AAA}$	5′-CTGGTCCCCCCTGCAAAGCAGC AGCGAAGGAGAAGAAGCAAAC-3′	5′-GTTTGCTTCTTCTCCTTCGCTGC TGCTTTGCAGGGGGGACCAG-3′
$Daxx_{KEK634AAA}$	5′-CCCTGCAAAAAATCTCGGGCCG CCGCGAAGCAAACAGGATCAG-3′	5′-CTGATCCTGTTTGCTTCGCGGC GGCCCGAGATTTTTTGCAGGG-3′
$Daxx_{KQT637AAA}$	5′-AATCTCGGAAGGAGAAGGCAG CAGCAGGATCAGGGCCAT-3′	5′-ATGGCCCTGATCCTGCTGCTGC CTTCTCCTTCCGAGATT-3′
$Daxx_{GSG640AAA}$	5′-GAGAAGAAGCAAACAGCAGCAG CGCCATTAGGAAACAGC-3′	5′-GCTGTTTCCTAATGGCGCTGCT GCTGTTTGCTTCTTCTC-3′
$Daxx_{PLG643AAA}$	5′-AAACAGGATCAGGGGCCGCGGC GAACAGCTATGTGGAAAGGC-3′	5′-GCCTTTCCACATAGCTGTTCGCC GCGGCCCCTGATCCTGTTT-3′
$Daxx_{NSY646AAA}$	5′-ATCAGGGCCATTAGGAGCCGCCG CTGTGGAAAGGCAAAGGTC-3′	5′-GACCTTTGCCTTTCCACAGCGG CGGCTCCTAATGGCCCTGAT-3′
$Daxx_{VER649AAA}$	5′-ATTAGGAAACAGCTATGCCGCCG CGCAAAGGTCAGTGCATG-3′	5′-CATGCACTGACCTTTGCGCGGC GGCATAGCTGTTTCCTAAT-3′
$Daxx_{QRS652AAA}$	5′-AACAGCTATGTGGAAAGGGCCGC CGCAGTGCATGAGAAGAA-3′	5′-TTCTTCTCATGCACTGCGGCGG CCCTTTCCACA TAGCTGTT-3′
$Daxx_{VHE655AAA}$	5′-GGAAAGGCAAAGGTCAGCCGCCG CGAAGAATGGGAAAAAGA-3′	5′-TCTTTTTCCCATTCTTCGCGGCG GCTGACCTTTGCCTTTCC-3′
$Daxx_{KNG658AAA}$	5′-AGGTCAGTGCATGAGGCCGCCGC GAAAAAGATATGTACCCTG-3′	5′-CAGGGTACATATCTTTTTCGCGG CGGCCTCATGCACTGACCT-3′

Table1. Primer pairs used for site-direct mutagenesis

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	α-helix	β–sheet	antiparallel	parallel	random coil
$Daxx_{625-740}$	4.0%	19.3%	45.1%	5.3%	35.3%
$Daxx_{K631-633A}$	4.2%	19.3%	44.4%	5.3%	35.3%

Table2. Circular dichroism spectra of His-$Daxx_{625-740}$ and His-$Daxx_{K631-633A}$ mutant analyzed by CDNN

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References

1. Tomanov, K., et al., *Sumoylation and phosphorylation: hidden and overt links.* J Exp Bot, 2018. **69**(19): p. 4583-4590.

2. Lamoliatte, F., et al., *Uncovering the SUMOylation and ubiquitylation crosstalk in human cells using sequential peptide immunopurification.* Nat Commun, 2017. **8**: p. 14109.

3. Biggar, K.K. and S.S. Li, *Non-histone protein methylation as a regulator of cellular signalling and function.* Nat Rev Mol Cell Biol, 2015. **16**(1): p. 5-17.

4. Han, Z.J., et al., *The post-translational modification, SUMOylation, and cancer (Review).* Int J Oncol, 2018. **52**(4): p. 1081-1094.

5. Cremona, C.A., P. Sarangi, and X. Zhao, *Sumoylation and the DNA damage response.* Biomolecules, 2012. **2**(3): p. 376-88.

6. Sternsdorf, T., K. Jensen, and H. Will, *Evidence for covalent modification of the nuclear dot-associated proteins PML and Sp100 by PIC1/SUMO-1.* J Cell Biol, 1997. **139**(7): p. 1621-34.

7. Wilkinson, K.A. and J.M. Henley, *Mechanisms, regulation and consequences of protein SUMOylation.* Biochem J, 2010. **428**(2): p. 133-45.

8. Olsen, S.K., et al., *Active site remodelling accompanies thioester bond formation*

doi:10.6342/NTU201901601

in the SUMO E1. Nature, 2010. **463**(7283): p. 906-12.

9. Rodriguez, M.S., C. Dargemont, and R.T. Hay, *SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting.* J Biol Chem, 2001. **276**(16): p. 12654-9.

10. Melchior, F., M. Schergaut, and A. Pichler, *SUMO: ligases, isopeptidases and nuclear pores.* Trends Biochem Sci, 2003. **28**(11): p. 612-8.

11. Minty, A., et al., *Covalent modification of p73alpha by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1-interacting proteins and a SUMO-1 interaction motif.* J Biol Chem, 2000. **275**(46): p. 36316-23.

12. Hecker, C.M., et al., *Specification of SUMO1- and SUMO2-interacting motifs.* J Biol Chem, 2006. **281**(23): p. 16117-27.

13. Song, J., et al., *Small ubiquitin-like modifier (SUMO) recognition of a SUMO binding motif: a reversal of the bound orientation.* J Biol Chem, 2005. **280**(48): p. 40122-9.

14. Lin, D.Y., et al., *Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors.* Mol Cell, 2006. **24**(3): p. 341-54.

15. Yang, X., et al., *Daxx, a novel Fas-binding protein that activates JNK and*

doi:10.6342/NTU201901601

apoptosis. Cell, 1997. **89**(7): p. 1067-76.

16. Perlman, R., et al., *TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation.* Nat Cell Biol, 2001. **3**(8): p. 708-14.

17. Chen, L.Y. and J.D. Chen, *Daxx silencing sensitizes cells to multiple apoptotic pathways.* Mol Cell Biol, 2003. **23**(20): p. 7108-21.

18. Lin, D.Y., et al., *Negative modulation of androgen receptor transcriptional activity by Daxx.* Mol Cell Biol, 2004. **24**(24): p. 10529-41.

19. Chang, C.C., et al., *Daxx mediates the small ubiquitin-like modifier-dependent transcriptional repression of Smad4.* J Biol Chem, 2005. **280**(11): p. 10164-73.

20. Kuo, H.Y., et al., *SUMO modification negatively modulates the transcriptional activity of CREB-binding protein via the recruitment of Daxx.* Proc Natl Acad Sci U S A, 2005. **102**(47): p. 16973-8.

21. Kim, E.J., J.S. Park, and S.J. Um, *Identification of Daxx interacting with p73, one of the p53 family, and its regulation of p53 activity by competitive interaction with PML.* Nucleic Acids Res, 2003. **31**(18): p. 5356-67.

22. Li, H., et al., *Sequestration and inhibition of Daxx-mediated transcriptional repression by PML.* Mol Cell Biol, 2000. **20**(5): p. 1784-96.

doi:10.6342/NTU201901601

23. Tang, J., et al., *A novel transcription regulatory complex containing death domain-associated protein and the ATR-X syndrome protein.* J Biol Chem, 2004. **279**(19): p. 20369-77.

24. Ishov, A.M., O.V. Vladimirova, and G.G. Maul, *Heterochromatin and ND10 are cell-cycle regulated and phosphorylation-dependent alternate nuclear sites of the transcription repressor Daxx and SWI/SNF protein ATRX.* J Cell Sci, 2004. **117**(Pt 17): p. 3807-20.

25. Massague, J. and D. Wotton, *Transcriptional control by the TGF-beta/Smad signaling system.* EMBO J, 2000. **19**(8): p. 1745-54.

26. Blobe, G.C., W.P. Schiemann, and H.F. Lodish, *Role of transforming growth factor beta in human disease.* N Engl J Med, 2000. **342**(18): p. 1350-8.

27. Massague, J., *How cells read TGF-beta signals.* Nat Rev Mol Cell Biol, 2000. **1**(3): p. 169-78.

28. Hahn, S.A., et al., *DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1.* Science, 1996. **271**(5247): p. 350-3.

29. Heldin, C.H., K. Miyazono, and P. ten Dijke, *TGF-beta signalling from cell membrane to nucleus through SMAD proteins.* Nature, 1997. **390**(6659): p. 465-71.

doi:10.6342/NTU201901601

30. Zawel, L., et al., *Human Smad3 and Smad4 are sequence-specific transcription activators.* Mol Cell, 1998. **1**(4): p. 611-7.

31. Chacko, B.M., et al., *Structural basis of heteromeric smad protein assembly in TGF-beta signaling.* Mol Cell, 2004. **15**(5): p. 813-23.

32. Qin, B., S.S. Lam, and K. Lin, *Crystal structure of a transcriptionally active Smad4 fragment.* Structure, 1999. **7**(12): p. 1493-503.

33. Roelen, B.A., et al., *Phosphorylation of threonine 276 in Smad4 is involved in transforming growth factor-beta-induced nuclear accumulation.* Am J Physiol Cell Physiol, 2003. **285**(4): p. C823-30.

34. Moren, A., et al., *Differential ubiquitination defines the functional status of the tumor suppressor Smad4.* J Biol Chem, 2003. **278**(35): p. 33571-82.

35. Lee, P.S., et al., *Sumoylation of Smad4, the common Smad mediator of transforming growth factor-beta family signaling.* J Biol Chem, 2003. **278**(30): p. 27853-63.

36. Liang, M., et al., *Regulation of Smad4 sumoylation and transforming growth factor-beta signaling by protein inhibitor of activated STAT1.* J Biol Chem, 2004. **279**(22): p. 22857-65.

37. Melnick, A. and J.D. Licht, *Deconstructing a disease: RARalpha, its fusion*

doi:10.6342/NTU201901601

partners, and their roles in the pathogenesis of acute promyelocytic leukemia. Blood, 1999. **93**(10): p. 3167-215.

38. Jensen, K., C. Shiels, and P.S. Freemont, *PML protein isoforms and the RBCC/TRIM motif.* Oncogene, 2001. **20**(49): p. 7223-33.

39. Li, C., et al., *C-terminal motifs in promyelocytic leukemia protein isoforms critically regulate PML nuclear body formation.* J Cell Sci, 2017. **130**(20): p. 3496-3506.

40. Kamitani, T., et al., *Identification of three major sentrinization sites in PML.* J Biol Chem, 1998. **273**(41): p. 26675-82.

41. Percherancier, Y., et al., *Role of SUMO in RNF4-mediated promyelocytic leukemia protein (PML) degradation: sumoylation of PML and phospho-switch control of its SUMO binding domain dissected in living cells.* J Biol Chem, 2009. **284**(24): p. 16595-608.

42. Sahin, U., et al., *Oxidative stress-induced assembly of PML nuclear bodies controls sumoylation of partner proteins.* J Cell Biol, 2014. **204**(6): p. 931-45.

43. Ascoli, C.A. and G.G. Maul, *Identification of a novel nuclear domain.* J Cell Biol, 1991. **112**(5): p. 785-95.

44. Zhong, S., et al., *Role of SUMO-1-modified PML in nuclear body formation.*

doi:10.6342/NTU201901601

Blood, 2000. **95**(9): p. 2748-52.

45. Lallemand-Breitenbach, V., et al., *Role of promyelocytic leukemia (PML) sumolation in nuclear body formation, 11S proteasome recruitment, and As2O3-induced PML or PML/retinoic acid receptor alpha degradation.* J Exp Med, 2001. **193**(12): p. 1361-71.

46. Lin, D.Y., et al., *Promyelocytic leukemia protein (PML) functions as a glucocorticoid receptor co-activator by sequestering Daxx to the PML oncogenic domains (PODs) to enhance its transactivation potential.* J Biol Chem, 2003. **278**(18): p. 15958-65.

47. Borden, K.L., *Pondering the promyelocytic leukemia protein (PML) puzzle: possible functions for PML nuclear bodies.* Mol Cell Biol, 2002. **22**(15): p. 5259-69.

48. Yeung, P.L., et al., *Daxx contains two nuclear localization signals and interacts with importin alpha3.* J Cell Biochem, 2008. **103**(2): p. 456-70.

49. Lange, A., et al., *Classical nuclear localization signals: definition, function, and interaction with importin alpha.* J Biol Chem, 2007. **282**(8): p. 5101-5.

50. Escobar-Cabrera, E., et al., *Characterizing the N- and C-terminal Small ubiquitin-like modifier (SUMO)-interacting motifs of the scaffold protein DAXX.*

doi:10.6342/NTU201901601

J Biol Chem, 2011. **286**(22): p. 19816-29.

51. Zhu, J., et al., *A sumoylation site in PML/RARA is essential for leukemic transformation.* Cancer Cell, 2005. **7**(2): p. 143-53.

52. Ecsedy, J.A., J.S. Michaelson, and P. Leder, *Homeodomain-interacting protein kinase 1 modulates Daxx localization, phosphorylation, and transcriptional activity.* Mol Cell Biol, 2003. **23**(3): p. 950-60.

53. Tang, J., et al., *Daxx is reciprocally regulated by Mdm2 and Hausp.* Biochem Biophys Res Commun, 2010. **393**(3): p. 542-5.

54. Muromoto, R., et al., *Sumoylation of Daxx regulates IFN-induced growth suppression of B lymphocytes and the hormone receptor-mediated transactivation.* J Immunol, 2006. **177**(2): p. 1160-70.

55. Chang, C.C., et al., *Structural and functional roles of Daxx SIM phosphorylation in SUMO paralog-selective binding and apoptosis modulation.* Mol Cell, 2011. **42**(1): p. 62-74.

doi:10.6342/NTU201901601