Mechanisms underlying necdin-induced regulation of PIAS1 SUMO E3 ligase

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Summary

Necdin is a pleiotropic protein that promotes differentiation and survival of mammalian neurons. Necdin is expressed abundantly in postmitotic neurons and interacts with many nuclear proteins such as E2F family proteins, p53, hypoxia-inducible proteins, Bmi-1, Sirtuin1, and FoxO1. Necdin is a member of MAGE (melanoma antigen) family proteins that share a highly conserved MAGE homology domain. It has previously been reported that several MAGE proteins interact with ubiquitin E3 ligases and modulate their activities. However, it remains unknown whether necdin and its related MAGE family proteins interact with SUMO (small ubiquitin-like modifier) E3 ligases such as PIAS (Protein Inhibitor of Activated STAT) family, Nsmce2/Mms21 and Cbx4/Pc2. In the present study, we investigated whether necdin interacts with these SUMO E3 ligases.

We first examined whether necdin and other MAGE proteins (necdin-like 2, MAGED1, MAGEF1, MAGEL2) interact with three known SUMO E3 ligases PIAS1, Nsmce2, and Cbx4. Co-immunoprecipitation analysis revealed that necdin, MAGED1, MAGEF1 and MAGEL2 bound to PIAS1 but not to Nsmce2 or Cbx4. These SUMO E3 ligases bound to MAGEA1 but failed to interact with necdin-like 2 (MAGEG1). Necdin bound via its hydrophobic pocket domain to PIAS1 central domains including PINIT (Pro-Ile-Asn-Ile-Thr)-motif and RING (Really Interesting New Gene) domains, which are highly conserved among PIAS family proteins and indispensable for the catalytic activity. We then examined the effects of necdin on the SUMOylation activity of PIAS1. Necdin suppressed PIAS1-dependent SUMOylation of the substrates STAT1 and PML (promyelocytic leukemia protein). Moreover, necdin strongly suppressed the effects of PIAS1 on the transcriptional activities of p53 and PML. In co-transfection assays, PIAS1 protein levels were markedly reduced by co-expressed necdin, and the reduction of PIAS1 levels was relieved in the presence of the proteasome inhibitor MG132. Furthermore, necdin markedly promoted degradation of PIAS1 via the ubiquitin-proteasome pathway. In transfected HEK293A cells, N- and C-terminally truncated PIAS1 mutants bound to necdin but failed to undergo necdin-dependent ubiquitination, indicating that the N- and C-terminal domains intramolecularly regulate ubiquitination. Because the N-terminus of PIAS1 contains the SAP (SAF-Acinus-PIAS) domain that interacts with the nuclear matrix, we examined whether N- and C-terminal deletions of PIAS1 affect its association with the nuclear matrix. Both PIAS1 and necdin were associated with the nuclear matrix, to which the PIAS1 terminal deletion mutants failed to localize, implying that the nuclear matrix is indispensable for necdin-dependent ubiquitination of PIAS1. Furthermore, lentivirus-mediated necdin overexpression in H1299 cells reduced endogenous PIAS1 protein levels and decreased proliferation rates.

The present study has demonstrated that necdin suppresses the function of PIAS1 both by inhibiting its SUMO E3 ligase activity and by promoting ubiquitin-dependent degradation. Protein SUMOylation levels are high in neural stem cells, in which PIAS1 expression is upregulated. Expression of necdin is low in neural stem/progenitor cells and upregulated during neuronal differentiation. These findings suggest that necdin suppresses SUMOylation of PIAS1 substrate proteins involved in neuronal differentiation. The present study also provides insights into the

involvement of necdin and other MAGE family proteins in PIAS1-mediated events in various types of cells.

PUBLICATION

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I. INTRODUCTION

I-1. General introduction

I-1.1. Posttranslational modifications with ubiquitin and SUMO

Protein posttranslational modifications include the covalent addition of small chemical groups or proteins, proteolytic cleavage, and degradation of entire proteins. These modifications influence almost all aspects of cellular events under physiological and pathological conditions.

Ubiquitin was first discovered in 1975, as a highly enriched protein in thymic fractions (Goldstein, 1975). Ubiquitin mainly targets the conjugated proteins to proteasomal proteolysis. Several other ubiquitin-like small proteins (UBLs) have been found later to act as mediators of post-translational modifications. These proteins are collectively called ubiquitin-like proteins (UBLs), which include SUMO (small ubiquitin-related modifier), Nedd8, ISG15, Atg8, URM1, FAT10 and MNSFβ (van der Veen and Ploegh, 2012). Unlike ubiquitin, UBLs mediate a wide array of distinct functions of conjugated proteins.

Lys residues are most frequently modified with ubiquitin. There has been no consensus sequence motif for ubiquitination (Finley and Chau, 1991), whereas UBL modification occurs via consensus motifs. Targeted proteins are modified with multiple ubiquitin molecules rather than the attachment of a single moiety (Ciechanover et al., 1980). These multimers or ubiquitin chains are generated by isopeptide linkage between the C-terminus of preceding ubiquitin and the ε-amino group of following ubiquitin. Polyubiquitinated proteins modified in this fashion are detected as high molecular weight smears by Western blot analysis.

Ubiquitin is a 76 amino acid protein that contains seven lysine residues. Polyubiquitination usually occurs via Lys48 or Lys63 (Emmerich et al., 2011; Li and Ye, 2008). Lys48 linkages form more packed polymer structure, whereas Lys63-linked polyubiquitin exhibits an extended configuration (Tenno et al., 2004; Varadan et al., 2004). Lys48-linked ubiquitin chains signal degradation by targeting the proteins to 26S proteasome. Lys48-linked tetra-ubiquitin is the minimal signal for proteasomal targeting and subsequent degradation. In contrast, Lys63-linked polyubiquitination is unable to target the proteins to proteasome, but rather serves as a regulatory posttranslational modification to modulate signaling pathways, ribosomal biogenesis, intracellular trafficking and DNA damage repair (Hochstrasser, 2004; Pickart and Fushman, 2004). Unlike polyubiquitination, monoubiquitination is principally involved in the regulation of key cellular functions such as transcriptional activation (Hicke, 2001) and endocytosis (Haglund et al., 2003).

SUMO family proteins represent the most functionally diverse group of UBLs (Hay, 2005). SUMO proteins have only ~18% sequence identity with ubiquitin but share high degree of three dimensional structure similarity to ubiquitin (Bayer et al., 1998). SUMO, a ~11kDa protein, is highly conserved from yeast to mammals and preferentially conjugated mainly to consensus ψKXE/D motif (ψ represents a bulky hydrophobic residue, X any amino acid, and E/D acidic residues) on target proteins (Gareau and Lima, 2010). Yeast and invertebrates contain a single SUMO gene, whereas vertebrates have three SUMO paralogs: SUMO1, SUMO2 and SUMO3

(Matunis et al., 1996). SUMO1 has only ~50% identity with SUMO2 and SUMO3, and this difference is reflected by the functional diversity of these paralogs. Cells contain large amounts of unconjugated SUMO2 and SUMO3, whereas SUMO1 is mainly present as covalently attached to the major target RanGAP1 (Saitoh and Hinchey, 2000). Although there is little substrate preference for SUMO prologues *in vitro*, there is a considerable level of the substrate selectivity *in vivo* as RanGAP1 is preferentially conjugated with SUMO1, but not with SUMO2 or SUMO3. Several other substrates display similar affinities towards different SUMO prologues. SUMO2 and SUMO3 contain an N-terminal ψKXE/D motif that facilitates generation of polymeric SUMO conjugates or poly-SUMO chains, whereas SUMO1 is conjugated as a monomer (Tatham et al., 2001).

I-1.2. Enzymatic cascades of protein modifications with ubiquitin and SUMO

Protein ubiquitination was first identified as an ATP hydrolysis-related enzymatic process of protein degradation (Ciechanover et al., 1980). Like many of eukaryotic regulatory systems, ubiquitination is regulated through enzymatic cascades. Although ubiquitin shares a homology with other UBLs, the conjugation cascade is mediated by distinct proteins/enzymes. Nascent UBL peptides are inactive and require an activating step before conjugation. UBLs are conjugated to target proteins via their C-terminus diglycine motif. Specific proteases called UBL-specific proteases cleave the C-terminal residues to expose the diglycine motif. The cleaved protein is then processed in the ubiquitin conjugation enzymatic cycle. Cleaved UBL proteins are first catalyzed by the activating enzyme E1. E1 is a heterodimeric protein complex containing an ATP hydrolysis subunit. In enzymatic cycle for ubiquitination, energy derived from ATP hydrolysis is used to form the high energy thioester bond between ubiquitin and the E1 activating enzyme. The thioester-bound ubiquitin is then passed to an intermediary conjugation enzyme E2. Although there is no direct role of conjugation enzyme in the subsequent ligation, these intermediary enzymes reduce the activation energy requirements providing a rapid reaction. In the final step, ubiquitin is conjugated to the target substrate by the action of an E3 protein ligase, which links the carboxy terminus of ubiquitin and the ε-amino group of the target lysine residue by bringing the substrate and E2-conjugated ubiquitin. Compared to the few numbers of E1 and E2 enzymes, the number of mammalian E3 enzymes has been greatly expanded. Mammalian genome encodes ~600 E3 ubiquitin ligases, which contribute to the substrate specificity of ubiquitination of their target proteins (Randow and Lehner, 2009).

SUMO is conjugated to target proteins through an enzymatic cascade analogous to ubiquitin conjugation (**Fig. I.1**). First, C-terminal region of immature SUMO is cleaved, and the diglycine motif is exposed through the catalytic action of SUMO/sentrin-specific protease (SENP). Exposed C-terminal carboxyl group of cleaved SUMO is then adenylated by a heterodimeric enzyme Aos1/Uba2 (also called SAE1/SAE2) and conjugated through utilization of ATP. Activated SUMO is transferred through a thiol linkage to the Cys residue C173 in Uba2, which is transferred to another Cys residue of E2 conjugation enzyme, Ubc9, forming a thioester-SUMO conjugate (Johnson et al., 1997). Ubc9 is the only E2 conjugation enzyme that has been identified to date (Johnson and Blobel, 1997). Besides the

intermediary role in supplying activated SUMO, Ubc9 can directly mediate SUMOylation of target proteins through interaction with the consensus SUMOylation motif on target proteins in contrast to the ubiquitin conjugation where E3 catalytic activity is essential for target conjugation (Bernier-Villamor et al., 2002). SUMO E3 ligases are proteins that catalyze transfer of SUMO to a target lysine residue. SUMO E3 ligases catalyze the attachment of SUMO to target proteins by stabilizing the interaction between the target protein and the E2-SUMO conjugate.

I-1.3. Ubiquitin E3 ligases

Eukaryotes have two distinct classes of ubiquitin E3 ligases with the specific features termed HECT (**h**omologous to **E**6AP **C**-**t**erminus) and RING (**R**eally **I**nteresting **N**ew **G**ene) domains. HECT is a ~350 amino acid domain localized at the C-terminus of the proteins. Human genome encodes for 28 HECT domain E3 ligases (Rotin and Kumar, 2009). HECT proteins are involved in broad biological pathways related to protein trafficking, cellular proliferation, tumorigenesis and immune response (Bernassola et al., 2008). E6AP, also known as UBE3A, is a classic HECT domain-containing E3 ligase and interacts with various viral oncoproteins to facilitate degradation of tumor suppressor proteins such as p53 (Talis et al., 1998) or pRb (Munakata et al., 2007). Deletion of the UBE3A gene, which is paternally imprinted on human chromosome 15, leads to Angelman syndrome, a neurodevelopmental syndrome characterized by seizures, dementia and abnormalities in speech, sleep patterns and movement (Kishino et al., 1997).

The RING domain was first characterized as a Zn^{+2} binding stretch of amino acid sequence (Freemont et al., 1991). The RING domain is characterized by Cys and His residues occurring within a certain spacing dictated by the canonical motif:

$$Cys\text{-}X_2\text{-}Cys\text{-}X_{(9\text{-}39)}\text{-}Cys\text{-}X_{(1\text{-}3)}\text{-}His\text{-}X_{(2\text{-}3)}\text{-}Cys\text{-}X_2\text{-}Cys\text{-}X_{(4\text{-}48)}\text{-}Cys\text{-}X_2\text{-}Cys$$

In this motif, Cys and His residues form the core of the structure to support the coordination of two Zn^{+2} atoms within the domain structure (Deshaies and Joazeiro, 2009). The RING domain varies from the canonical zinc finger (X2-Cys-X2,4-Cys-X12-His-X3,4,5-His) in which cation coordination residues in RING are interspersed within random residues (Borden and Freemont, 1996). RING domain proteins interact with E2 conjugation enzymes that assist terminal E3 ligase reaction (Brzovic et al., 2006; Zheng et al., 2000). The RING domain surface is versatile and well conserved so that RING domain proteins interact with several cognate E2 conjugation enzymes via similar contact residues. Point mutations of the zinc coordination residues in the RING domain induce a strong inhibition of the E3 ligase activity (Deshaies and Joazeiro, 2009). Therefore, these point mutations are widely used as negative controls for ubiquitination assays.

I-1.4. SUMO E3 ligases

Unlike ubiquitination, SUMOylation is regulated by only several E3 SUMO ligases. To date, four SUMO E3 ligases have been well characterized. These SUMO E3 ligases are classified into two groups: RING domain-containing E3 ligases such as PIAS (Protein Inhibitor of Activated STAT) family and Nsmce2 (Nse2, Mms21), and non-RING E3 ligases such as RanBP2 and Cbx4 (chromobox homolog 4, Pc2).

The PIAS family proteins bind to Ubc9 and specific target proteins. Molecular

natures and functions of these family proteins are most extensively characterized among SUMO E3 ligases as described in the next section.

Nsmce2, a mammalian ortholog of yeast Mms21, is a component of Smc5/6 complex (Structural Maintenance of Chromosome5/6) that is involved in homologous recombination, genomic stability and DNA repair (Potts, 2009). Nsmce2 was identified as a SUMO E3 ligase in methyl methanesulfonate (MMS)-sensitive budding yeast mutants (Zhao and Blobel, 2005). Noteworthily, Nsmce1, another non-Smc5/6 component, is a RING-type E3 ubiquitin ligase. Nsmce2 promotes SUMOylation of the Smc5/6 complex components Smc5, Smc6, Nsmce3 and Nsmce4 (Potts and Yu, 2005). Nsmce2 stimulates SUMOylation of itself other than substrate proteins (Andrews et al., 2005). In contrast to other SUMO E3 ligases that promote conjugation with monomeric or dimeric SUMO, Nsmce2 substrates undergo conjugation with polymeric SUMO chains resulting in high molecular weight smears (Andrews et al., 2005).

RanBP2 (also known as Nup358) is a 358-kDa component of nuclear pore complexes. RanBP2 is essential for nuclear protein import (Hutten et al., 2008). RanBP2 forms a stable complex with SUMO-conjugated RanGAP1 to stabilize it. A 30-kDa domain of RanBP2 has SUMO-binding and catalytic activities but does not contain a RING domain.

Cbx4 is a member of Polycomb group (PcG) proteins, which form large multimeric complexes involved in transcriptional repression through forming protein complexes that promote histone methyltransferase activity. Cbx4 contains SUMO E3 ligase activity for the transcriptional corepressor CtBP (Kagey et al., 2003). Cbx4 recruits the CtBP to PcG bodies, and overexpression of Cbx4 leads to the enhancement of CtBP SUMOylation. Cbx4has been suggested to be involved in chromatin remodeling by promoting SUMOylation of several known SUMO targets such as histone deacetylases and topoisomerases.

I-1.5. PIAS family SUMO E3 ligases

The largest group of SUMO E3 ligases is the PIAS family consisting of PIAS1, PIAS2, PIAS3, PIAS4, and their variants (Rytinki et al., 2009). They are identified as the negative regulators of STAT transcriptional activity, and thus named Protein Inhibitor of Activated Stat (PIAS). PIAS E3 ligases have distinct conserved domains. The SAP (SAF-A/Acinus/PIAS) domain at the N-terminus is responsible for subnuclear localization of the protein through binding to AT-rich DNA sequences present in nuclear matrix-attachment regions (Zhou et al., 2008). The PINIT (Pro-Ile-Asn-Ile-Thr) domain is a PIAS signature domain for PIAS1-4 (Rytinki et al., 2009) and required for nuclear retention of PIAS3 (Duval et al., 2003). The catalytic domain of PIAS proteins contains the SP-RING (Siz/PIAS-RING) motif, a variant RING motif lacking two conserved Cys residues in the conventional RING motif (Hochstrasser, 2001). The C-terminal domain of PIAS proteins is the least conserved domain that contains an acidic domain encompassing a SUMO-interacting motif (SIM). Moreover, the C-terminal domain has a Ser/Thr rich region common to all PIAS proteins except PIAS4 (Shuai, 2006). PIAS proteins have been shown to catalyze SUMOylation of ~60 different substrates (Shuai, 2006). Substrates of PIAS proteins are mainly transcription factors and their modifiers such as STAT (Liu et al., 1998), p53 (Kahyo et al., 2001), c-jun (Schmidt and Muller, 2002), Smad2/4 (Yang et al.,

2013), PML (Rabellino et al., 2012) and CEBPβ (Liu et al., 2013). Posttranslational modifications of PIAS affect E3 ligase activities. Phosphorylation of PIAS1 at the C-terminus by MAPK-activated protein kinase 2 stimulates E3 ligase activity towards p53 (Heo et al., 2013). Furthermore, SUMOylation of PIAS4 promotes E3 ligase activity towards TCF4 *in vivo* (Ihara et al., 2005). However, detailed molecular mechanisms underlying regulation of PIAS SUMO E3 ligases remain elusive.

I-2. Specific Introduction

I-2.1. Necdin and MAGE family

Necdin was originally identified in 1991 as a hypothetical protein encoded by an mRNA expressed in neurally differentiated P19 embryonal carcinoma cells (Maruyama et al., 1991). In the same year, a tumor antigen, originally named MZ2-E, which induced a cytotoxic response by autologous T-cells, was discovered (van der Bruggen et al., 1991). MZ2-E was later identified as one of the human 12 *hMAGEA* genes cluster on X-chromosome (De Plaen et al., 1994) and named MAGEA1 (Chomez et al., 2001). Homologous sequences are consequently identified on different clusters and named MAGEB (Muscatelli et al., 1995) and MAGEC (Lucas et al., 1998) gene families. MAGEA, MAGEB and MAGEC proteins are encoded in a single terminal exon. Interestingly expression of MAGEA, MAGEB and MAGEC genes are highly restricted to cells of tumor origin, germ cells, testis and placenta (Barker and Salehi, 2002). These proteins were also called CTA (Cancer-Testis Antigens) (Fratta et al., 2011; Old and Chen, 1998). The restricted expression is due to extensive methylation of the promoter regions of MAGEA, MAGEB and MAGEC gene clusters in normal tissues (De Smet et al., 1995). Although expression of MAGEA, MAGEB and MAGEC proteins has been identified in cancers of different origin, physiological or pathophysiological functions of these MAGE family proteins remain largely unknown.

Necdin and MAGE proteins share a large homologous region known as the MAGE homology domain (MHD). Mammalian MAGE family proteins are divided into two classes based on the sequence similarities of the MHDs and gene expression patterns (Barker and Salehi, 2002; Chomez et al., 2001) (**Fig. I.2A**). Type I MAGEs such as MAGEA, MAGEB, and MAGEC subfamilies are expressed in cancer and male germ cells but not in normal cells, and their genes are located on chromosome X (van der Bruggen et al., 1991). In contrast, Type II MAGEs such as necdin, necdin-like 2 (NDNL2, also known as MAGEG1), MAGED, MAGEE, MAGEF, MAGEH, and MAGEL are expressed in normal cells including neural cells. Type II MAGE proteins are further divided into two groups based on the MHD domain similarities. First subgroup consists of proteins of low molecular weight (<350 residues) with high MHD similarities to necdin including necdin-like 2, MAGEF1 and MAGEH1. The other subgroup includes proteins of higher molecular weight and an extended N-terminal region that share high MAGED1 MHD similarity. This subgroup consists of MAGED1-D4, MAGEE1 and MAGEL2 (originally named necdin-like 1). These two distinct subgroups are suggested to exert distinct functional and biochemical effects. However, biochemical roles of Type II MAGE proteins based on the differences in the overall structure and MHD sequence remain to be elucidated.

Among Type II MAGE proteins, necdin, MAGEL2 and necdin-like 2 (Chibuk et al., 2001) are encoded by the genes that are closely located on human chromosome 15. Human necdin (*NDN*) and *MAGEL2* genes are located at chromosome 15q11-12, a region responsible for the pathogenesis of the classic genomic imprinting-associated neurodevelopmental disorder Prader-Willi syndrome (Jay et al., 1997; Kozlov et al., 2007; Lee et al., 2000; MacDonald and Wevrick, 1997; Nakada et al., 1998). Both necdin and MAGEL2 are expressed only from the paternal alleles and implicated in the neural development based on the phenotypes of gene knockout mice (Gerard et al., 1999; Kuwako et al., 2005; Lee et al., 2000; Muscatelli et al., 2000). In contrast to necdin and its homologous MAGE proteins, there is limited information about biochemical functions of most MAGE family proteins.

I-2.2. Expression and function of necdin

Necdin was first isolated from a subtraction cDNA library of murine P19 embryonal carcinoma cells neurally differentiated by retinoic acid treatment (Maruyama et al., 1991). The necdin gene (*Ndn*) encodes a 325-amino acid protein, which contains a highly conserved core domain (aa 83-292) with 91% identity between mouse and human and a less conserved highly-disordered N-terminus (Uetsuki et al., 1996; Nakada et al., 1998). Necdin is abundantly expressed in postmitotic neurons distributed in the central and peripheral nervous systems of mice (Aizawa et al., 1992). Although it was initially thought that necdin expression was restricted to postmitotic neurons and skeletal muscle cells (Uetsuki et al., 1996), necdin is also expressed in neuroepithelial stem cells or neural stem cells (Huang et al., 2013; Minamide et al., 2014). The human necdin gene (*NDN*) is located on the chromosomal region 15q11, which is implicated in the pathogenesis of the neurodevelopmental Prader-Willi syndrome (PWS). In this region, maternal *NDN* and several other genes are silenced through genomic imprinting (Jay et al., 1997; MacDonald and Wevrick, 1997; Nakada et al., 1998).

Studies on molecular function of necdin have been initiated by screening of cDNA libraries by yeast two-hybrid assay to elucidate biochemical interaction partners of necdin. Necdin interacts with viral transforming proteins Simian virus 40 (SV40) large T antigen and adenovirus E1A, also with cellular transcription factor E2F1 (Taniura et al., 1998). These proteins are known as binding partners of the retinoblastoma (Rb) protein, and necdin interacts with the Rb-binding domain of these proteins, suggesting a functional similarity between necdin and Rb. Furthermore, necdin induces growth suppression on SAOS2 cells deficient in Rb, and necdin suppresses apoptosis by E2F1 in N1E-115 neuroblastoma cells (Kobayashi et al., 2002)(Kobayashi 2002). Necdin also interacts with p53, another growth suppressor, to suppress p53-induced transactivation and apoptosis without affecting the growth suppressive function of p53 (Taniura et al., 1999). These data taken together suggest that necdin is a tumor suppressor-like protein that interacts with viral oncogenes and their cellular targets.

Necdin interacts with p75 neurotrophin receptor, a transmembrane protein to facilitate differentiation (Tcherpakov et al., 2002). It interacts with Msx2 homeodomain protein via MAGED1 and modulates Msx2-dependent transcriptional activities (Kuwajima et al., 2004). Moreover, necdin inhibits apoptosis by suppressing

E2F1-dependent transcription of cyclin-dependent protein kinase cell division cycle 2 (Cdc2, also known as Cdk1) (Kurita et al., 2006) and by facilitating Sirt1-mediated deacetylation of p53 (Hasegawa and Yoshikawa, 2008). Necdin-induced growth suppression occurs not only in transformed cell lines but also in primary preadipocytes (Fujiwara et al., 2012) and neural precursor cells, in which necdin interacts with HIF2α (Huang et al., 2013) and Bmi1 (Minamide et al., 2014). Furthermore, Drosophila MAGE protein (the sole MAGE protein in Drosophila), like necdin, regulates neural precursor cell proliferation (Nishimura et al., 2008). In hematopoietic stem cells, necdin also exerts growth suppressive effect (Kubota et al., 2009). These findings suggest that necdin regulates proliferation of neuronal and non-neuronal stem/progenitor cells by interacting with many proteins involved in the cell cycle and cellular differentiation.

I-2.3. MAGE proteins and posttranslational modifications with ubiquitin

It has been reported that several Type I MAGE proteins interact with RING ubiquitin E3 ligases and that the interaction promotes their E3 ligase catalytic activities (Doyle et al., 2010). MAGE family proteins interact differentially with E3 RING ubiquitin ligases. For example, MAGEA2, MAGEA3, MAGEA6, MAGEC2, and MAGED1 directly bind to TRIM28 E3 RING ubiquitin ligase, whereas MAGEF1 and NDNL2 (MAGEG1) interact with NSE1 (**Fig. I.2B**). The crystal structure of NDNL2-NSE1 provides structural insights into MAGE family proteins and their interaction with E3 RING ubiquitin ligases (Doyle et al., 2010). In addition, these RING E3 ubiquitin ligase-interacting MAGE proteins enhance the ubiquitin ligase activity. For example, a MAGEC2-TRIM28 complex ubiquitinates and targets p53 for proteasomal degradation. Thus, MAGE proteins may serve as modulators of RING domain-containing ubiquitin E3 ligases. In contrast, effects of the MAGE proteins on the regulation of RING type E3 SUMO ligases remain elusive. Because similar roles of MAGE proteins in the regulation of SUMO E3 ligases are suggested, we investigate the roles of MAGE proteins in the regulation of SUMO E3 ligases.

I-3. Figures I.1, 2

Figure I.1 **Schematic description of SUMO conjugation enzymatic cascade.**

Figure I.2. **MAGE family proteins modulate RING ubiquitin E3 ligases differentially.**

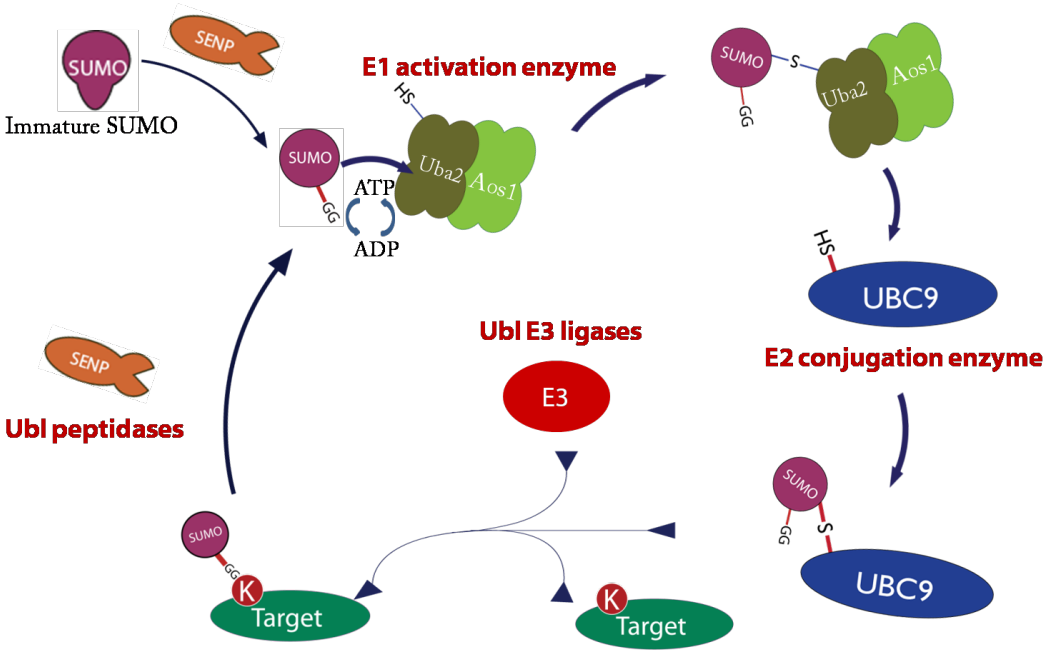
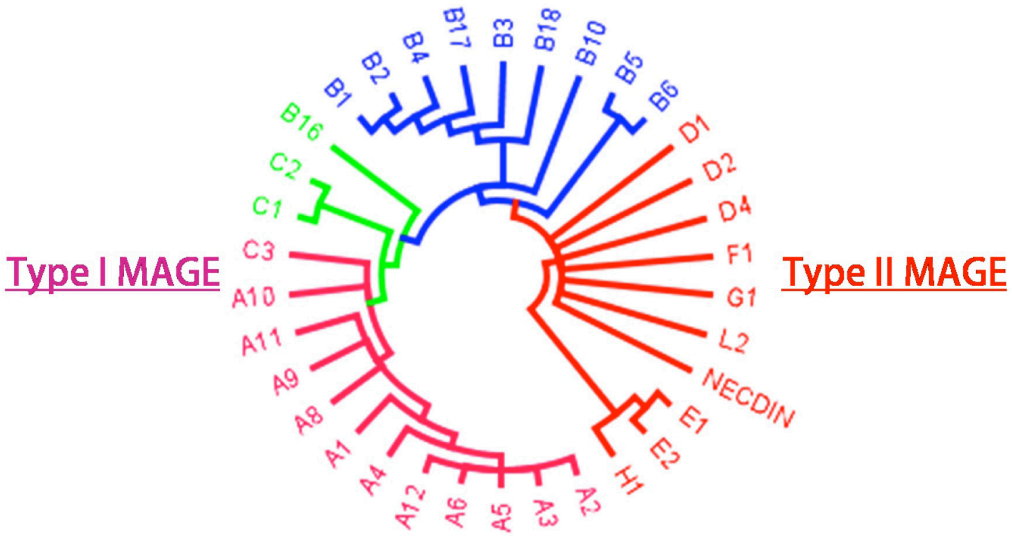


Figure I.1 Schematic description of SUMO conjugation enzymatic cascade.

A



B

Ubiquitin E3 ligase	Human MAGE protein
TRIM28	A2, A3, A6, C2, D1
LNX1	B18
NSE1	F1, G1 (NDNL2)
TRIM27	D4B, F1, L2
Praja-1	D1, G1

Figure I.2. MAGE family proteins modulate RING ubiquitin E3 ligases differentially.
(**A**) Phylogenetic tree presentation of MAGE family proteins. (**B**) Summary of interactions between ubiquitin E3 ligases and human MAGE proteins. Data are taken from Doyle, J. et al. (2010)

II. EXPERIMENTAL PROCEDURES

II-1. cDNAs and plasmids

Full-length cDNAs encoding mouse Nsmce1 (NCBI NM_026330.3), Nsmce2 (NCBI NM_026746.3), Cbx4/Pc2 (NCBI NM_007625.2) and PIAS1 (NCBI NM_019663.3) were cloned from mouse E14.5 forebrain cDNA library and subcloned into 6xMyc(N)-pcDNA3.1(+) vector (Hasegawa et al., 2012). Expression vectors for mouse p53, necdin, and their deletion mutants were constructed as described (Huang et al., 2013; Taniura et al., 2005). cDNAs for mouse MAGED1, necdin-like2 and MAGEL2 were cloned as described (Kuwajima et al., 2004; Kuwako et al., 2004). Human MAGEF1 was cloned from nuclear DNA library of HEK293A cells. Human MAGEA1 cDNA was provided by Dr. Kyogo Itoh (Kurume University School of Medicine). cDNAs encoding PIAS1 aa 1-100, 101-300, 301-400 and 401-652 or deletion mutants aa 101-652 and 1-400 were generated using synthetic oligonucleotide primers and subcloned into the expression vector. cDNAs encoding full-length mouse STAT1 (NCBI NM_001205313.1) and PML (NCBI NM_008884.5) were cloned from cDNA libraries of E14.5 mouse forebrain and adult mouse spleen, respectively, and subcloned into p3xFLAG-CMV10 (Sigma-Aldrich). cDNA encoding mouse SUMO1 was obtained from RIKEN BioResource Center DNA Bank and subcloned into hemagglutinin (HA)-pcDNA3.1(+) carrying the N-terminal HA sequence. All cDNAs used were sequenced for their identities. Experiments were approved by Recombinant DNA Committee of Osaka University (Approval No. 2938-1) and performed in accordance with national and institutional guidelines.

II-2. Co-immunoprecipitation assay

HEK293A cells (1 x 10^6 cells per 60 mm dish) were transfected with combinations of expression vectors, incubated in Dulbecco's Modified Eagle Medium (Life Technologies) supplemented with 10% fetal bovine serum at 5% CO_2 at 37°C, and harvested 24 hrs after transfection as described (Taniura et al., 1998). The total amount of infected DNA was equalized by adding pcDNA3.1(+) vector. Transfected cells were lysed in a lysis buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630 (Sigma-Aldrich), 10% glycerol, and protease inhibitors (Complete, Roche). Lysates (200 μg) were incubated at 4°C for 2 hrs with antibodies to FLAG (M2; Sigma-Aldrich; 1:100), Myc (9E10; 1:10) and necdin (NC243; 1:200)(Niinobe et al., 2000). Proteins bound to antibodies were pelleted with protein A-Sepharose (GE Healthcare), eluted from protein A-Sepharose, separated by 10% SDS-PAGE, and electroblotted onto polyvinylidene difluoride membranes (Immobilon; Millipore). Membranes were incubated with antibodies to Myc (1:10), FLAG (1:500), necdin (1:3000), HA (HA.11; Covance; 1:100), β-tubulin (TUB 2.1; Sigma-Aldrich; 1:1000) and γ-tubulin (GTU-88;Sigma-Aldrich; 1:1000) at room temperature for 1 hr. After incubation with horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgGs (Cappel) for 1 hr at room temperature, proteins were visualized with chemiluminescence reagents (Western Lightning Plus-ECL, PerkinElmer).

II-3. SUMOylation assay

HEK293A cells (1 x 10^6 cells per 60 mm dish) were transfected with combinations of expression vectors for HA-SUMO1, Myc-PIAS1, necdin, FLAG-STAT1 and FLAG-PML and harvested 24 hrs after transfection. For analysis of PML SUMOylation, cells were treated with 10 μM MG132 (Peptide Institute) 4 hrs prior to harvest as described (Rabellino et al., 2012). Cells were lysed in the lysis buffer supplemented with 20 mM N-ethylmaleimide, and the lysates (300 μg) were incubated at 4°C for 2 hrs with anti-FLAG antibody (M2; Sigma-Aldrich). The protein-antibody complexes were pelleted with protein A-Sepharose, separated by 10% SDS-PAGE, and detected by Western blotting as above. Signal intensities of sumoylated proteins were quantitated by densitometry with ImageJ1.48 software. The protein concentration was determined by the Bradford method (Bio-Rad).

II-4. Reporter assay

Luciferase reporter assay for p53-driven transactivation was performed using a 2.4-kilobase pair fragment of human p21/WAF1 promoter inserted into the luciferase reporter vector PGV-G (Toyo Ink) as described previously (Taniura et al., 1999). Combinations of expression vectors for Flag-p53, HA-PIAS1, necdin, and GFP-SUMO1 were transfected along with promoter-reporter into HEK293A cells. Transfectants were harvested 36 hr after transfection, and luciferase activities were measured with a luminometer (Lumat LB9501, Berthold) using Dual-Luciferase Reporter Assay system (Promega). Transfection efficiency was normalized with the activity of co-expressed Renilla luciferase.

II-5. Ubiquitination assay

HEK293A cells (1 x 10^6 cells per 60 mm dish) were transfected with combinations of expression vectors for FLAG-tagged ubiquitin, HA-tagged ubiquitin, Myc-tagged full-length PIAS1, Myc-tagged PIAS1 deletion mutants, necdin, FLAG-tagged necdin, FLAG-tagged MAGEA1, and FLAG-tagged necdin-like 2. Transfected cells were incubated for 20 hrs, treated with 30 μM MG132 or DMSO (vehicle control) for 4 hrs, and harvested. Cells lysates (250 μg) were incubated at 4°C for 2 hrs with the anti-Myc antibody, and the protein-antibody complexes were pelleted with protein A-Sepharose, separated by 10% SDS-PAGE, and detected by Western blotting as above. Signal intensities of ubiquitinated proteins were quantitated by densitometry.

II-6. Immunocytochemistry

HEK293A cells (2-4 x 10^5 cells per 35 mm dish) were cultured on coverslips for 17 hrs, transfected with expression vectors, and incubated for another 24 hrs at 5% CO_2 at 37°C. Cells were fixed with 10% formalin for 20 min at room temperature and permeabilized with methanol for 20 min at room temperature. For proteasome inhibition, transfected cells were incubated in the presence of 30 μM MG132 or DMSO for 4 hrs prior to fixation. For immunostaining of the nuclear matrix-associated

proteins, cells were sequentially treated with 0.5% Triton X100, 25 U/ml RNase-free DNase (RQ1, Promega), 0.25 M ammonium sulfate and 2M NaCl according to the method described (He et al., 1990; Taniura and Yoshikawa, 2002). Fixed cells were treated with PBS-Tween 20 containing 1% BSA (Sigma-Aldrich) for 1 hr at room temperature, incubated with primary antibodies to Myc (Cell Signaling; 1:300), necdin (NC243;1:1000) and NuMA (Ab-1, Oncogene;1:100) overnight at 4ºC, and with Alexa Fluor 488-conjugated anti-mouse (or anti-rabbit) IgG (Life Technologies;1:1000) and cyanine 3-conjugated anti-rabbit (or anti-mouse) IgG (Jackson ImmunoResearch, 1:500). Chromosomal DNA was stained with 3.3 μM Hoechst 33342 (Sigma-Aldrich). Stained cells were observed with a fluorescence microscope (BX-50-34-FLAD1, Olympus), and images were captured with a charge-coupled device camera system (DP-70; Olympus).

II-7. Lentivirus infection

Recombinant lentiviruses were produced in HEK293FT cells by transfecting SIN vector plasmids and two helper plasmids as described previously (Fujiwara et al., 2012; Miyoshi et al., 1998). Necdin cDNAs were subcloned into pENTR1A entry vector (Life Technologies) to construct CSII-EF1α-necdin-IRES-EmGFP, in which Emerald Green Fluorescent Protein (EmGFP) (Life Technologies) was used for an expression indicator (Minamide et al., 2014). The viral titer was measured by serial dilution on HEK293FT cells (Life Technologies) and determined as GFP-positive cell population by immunocytochemistry. Lentivirus vectors for EmGFP (empty vector) or necdin (+EmGFP) were infected into H1299 cells at a multiplicity of infection (MOI) of 12, and the infected cells were incubated for 72 hrs prior to analyses. Expressed proteins were analyzed by Western blotting using antibodies against PIAS1 (EPR2518Y, Abcam; 1:5000), necdin, and γ-tubulin. Signal intensities of proteins were quantitated by densitometry.

II-8. Cell proliferation assay

H1299 cells were plated on coverslips in 12-well plates (1 x 10^5 cells per well), cultured for 24 hrs and infected with lentiviruses expressing EmGFP and necdin. BrdU (10 μM) was added 48 hrs after viral infection, incubated for another 4 hrs. Cells were fixed with 10% formalin for 20 min at room temperature and permeabilized with methanol for 20 min at room temperature. BrdU was detected by fluorescence immunocytochemistry as described in (Fujiwara et al., 2012; Taniura et al., 1999). The proliferation rate was expressed as BrdU-positive cells per total cells, which were counterstained with 5 μM Hoechst 33342.

II-9. Statistics

Statistical significance was tested using Tukey-Kramer multiple comparison method unless otherwise stated. A significance of $p<0.05$ was required for rejection of the null hypothesis.

III. RESULTS

III-1. MAGE proteins differentially interact with SUMO E3 ligases

We first analyzed the interactions of typical SUMO E3 ligases including Nsmce2, Cbx4 and PIAS1 with MAGE proteins including necdin, MAGEA1, MAGED1, MAGEF1, necdin-like 2 and MAGEL2 by co-immunoprecipitation assay. To verify the assay system, we used the RING-type protein (non-SMC element 1) which interacts with necdin-like 2 and MAGEF1 but not with necdin, MAGED1 and MAGEL2 (Doyle et al., 2010). We found that Nsmce1 interacted with MAGEA1, necdin-like 2 and MAGEF1, but not with necdin, MAGED1 or MAGEL2 (**Fig. III. 1A**). We next examined the interactions of the SUMO E3 ligases Nsmce2, Cbx4, and PIAS1 with these MAGE proteins (**Fig. III.1B-D**). The MAGE proteins except MAGEA1 failed to interact with Nsmce2 and Cbx4 (**Fig. III.1B, C**). Intriguingly, PIAS1 interacted with necdin, MAGEA1, MAGED1, MAGEF1 and MAGEL2, but not with necdin-like 2 (**Fig. III.1D**). These data indicate that PIAS1 is a common target of diversified mammalian MAGE proteins.

III-2. Necdin and PIAS1 interact each other through conserved domains

To characterize the interaction between necdin and PIAS1, we constructed deletion mutants of PIAS1 and necdin for co-immunoprecipitation assay using Human Embryonic Kidney 293A (HEK293A) cells (**Fig. III. 2A**). The PIAS1 mutants were designed to contain PIAS-specific motifs: PIAS1 amino acids (aa) 1-100 (NT) containing the SAF-Acinus-PIAS (SAP) domain; PIAS1 aa 101-300 (PD) containing the Pro-Ile-Asn-Ile-Thr (PINIT) domain; PIAS1 aa 301-400 (SR) containing the Siz/PIAS RING (SP-RING) domain; PIAS1 aa 401-651 (CT) containing the SIM (SUMO-interactive motif) and Ser/Thr-rich domains. We constructed necdin mutants such as necdin aa 1-100 (NT) containing N-terminal proline-rich acidic region, necdin aa 101-325 (CT) containing the entire MAGE homology domain (MHD) (aa 116-280) and necdin ΔH4/5 (aa 190-222 deletion) lacking the p53-interacting region (Taniura et al., 2005), which corresponds to helices H4 and H5 region of necdin-like 2 MHD (PDB 3NW0) (Doyle et al., 2010; Hudson et al., 2011). Necdin was co-immunoprecipitated with the PIAS1 fragments containing the PINIT (PD) and SP-RING (SR) domains, and these were conversely co-immunoprecipitated with necdin (**Fig. III.2B**). Necdin failed to interact with the PIAS1 terminal regions. In this assay, we confirmed that necdin bound to p53 (positive control) but not to N-terminally truncated p53 (p53 ΔN, negative control)(Taniura et al., 1999).

We then analyzed the PIAS1-binding region of necdin using HEK293A cells transfected with truncated necdin mutants (**Fig. III.3A**). PIAS1 interacted with full-length necdin and the N-terminally truncated mutant (CT) but not with the N-terminal region (NT). We also found that full-length necdin and CT reduced expression levels of Myc-PIAS1, suggesting that co-expressed necdin destabilizes the PIAS1 protein (**Fig. III.3B**, third panel). To confirm whether the necdin MHD is

required for PIAS1 binding, we used the p53 binding-defective mutant necdinΔH4/5 (**Fig. III.3C**). This mutant failed to bind to PIAS1 or p53 (control), suggesting that the H4/5 region of the MHD is indispensable for the interaction between necdin and PIAS1.

Homology modeling based on the X-ray crystallographic data of necdin-like 2 (Doyle et al., 2010) revealed that the mouse necdin aa 191-222 region corresponds to helices 4 and 5 in the winged-helix motif B (WH-B) (**Fig. III.4A, B**). The H4/5 region in necdin-like 2 is predicted to form a hydrophobic pocket that interacts with NSMCE4A (Hudson et al., 2011). We have previously found that this deletion mutant neither induces cell growth suppression nor associates with the nuclear matrix (Taniura et al., 2005). Because MAGE proteins such as MAGEA1, MAGED1, MAGEF1 and MAGEL2 interact with PIAS1 as shown Fig. III.1D, it is possible that the PIAS1-binding domains of these MAGE proteins take configurations similar to that of necdin. In contrast, necdin-like 2, which shows structural and functional similarities to necdin (Kuwako et al., 2004), failed to interact with PIAS1. Necdin-like 2 bears five amino acid substitutions in the H4/5 region compared with PIAS1-interacting MAGE proteins (**Fig. III.4C**). It is noteworthy that three Thr residues are present in and near the H5 region of necdin-like 2, whereas other MAGE proteins contain only one or no Thr residue in their H4/5 regions. We speculate that necdin-like 2 lacks PIAS1 binding owing to its unique configuration of the H4/5 region.

III-3. Necdin inhibits PIAS1 SUMO E3 ligase activity

Since necdin interacted with the PINIT and SP-RING domains that constitute the catalytic region of PIAS1 (Liang et al., 2004), we investigated whether necdin affects the SUMO E3 ligase activity of PIAS1. We examined the effects of necdin on PIAS1-dependent SUMOylation using HEK293A cells transfected with SUMO1 and the PIAS1 substrates STAT1 (Liu et al., 1998) and PML (Rabellino et al., 2012). Because the PIAS1 protein level was decreased by co-expressed necdin, we used twice the amount of the PIAS1-expressing vector to avoid reducing the PIAS1 enzyme level. In this assay, faint bands of sumoylated STAT1 were detected at ~95 kDa in the absence of PIAS1, and PIAS1 promoted STAT1 SUMOylation to 5.2 times the control level (**Fig. III.5A, B**). Co-expression of necdin suppressed the PIAS1-promoted STAT1 SUMOylation level by 57%. Similarly, PIAS1 markedly increased the level of sumoylated PML (6.6 times the control level) detected as smeared bands at >150 kDa, and co-transfection of necdin decreased the PML SUMOylation level by 54% (**Fig. III.6A, B**). We also found that PIAS1-dependent SUMOylation of p53 was reduced by co-expression of necdin (**Fig. III.7**). We then examined the effect of necdin on PIAS-mediated activation of p53-dependent transcription using the human p21/WAF1 promoter containing the p53-binding motif and luciferase reporter system (**Fig. III.8**). Both PIAS1 and SUMO1 enhanced p53-mediated transcription activities, and necdin markedly reduced the enhancement. These data taken together suggest that necdin suppresses the SUMO E3 ligase activity of PIAS1.

III-4. Necdin promotes proteasomal degradation of PIAS1

In the preceding experiments, PIAS1 protein levels were reduced when necdin was co-expressed. Thus, we examined whether necdin affects the stability of PIAS1 using transfected HEK293A cells (**Fig. III.9A**). Necdin reduced the PIAS1 level in a dose-dependent manner. To examine whether necdin degrades the PIAS1 protein via the ubiquitin-proteasomal pathway, we used the proteasome inhibitor MG132. Treatment with MG132 protected PIAS1 from necdin-promoted degradation (**Fig. III.9B**), suggesting that necdin promotes PIAS1 degradation in the proteasome.

We then examined whether necdin promotes PIAS1 ubiquitination by immunoprecipitation assay using HEK293A cells transfected with ubiquitin cDNA (**Fig. III.9C**), Ubiquitinated PIAS1 was detected as multiple bands at 100-250 kDa. Although the ubiquitinated PIAS1 level was reduced, presumably owing to the degradation of the PIAS1 protein, in the absence of MG132, necdin significantly promoted PIAS1 ubiquitination in the presence of MG132 (**Fig. III.9D**). These data indicate that necdin promotes PIAS1 degradation in the ubiquitin-proteasome system. To analyze the specificity of necdin in PIAS1 degradation, we examined the effects of MAGEA1 and necdin-like 2 on PIAS1 ubiquitination in the presence of MG132. MAGEA1 promoted ubiquitination of PIAS1 to a significant but lesser extent than necdin, whereas necdin-like 2 exerted little or no ubiquitination-promoting effect (**Fig. III.10A, B**). We also found that the necdin ΔH4/5 mutant failed to promote PIAS1 ubiquitination (**Fig. III.10C, D**).

We next examined the subcellular localization of PIAS1 and necdin by immunocytochemistry using transfected HEK293A cells. PIAS1 was localized exclusively in the nucleus, whereas necdin was in both the nucleus and the cytoplasm (**Fig. III.11A**). Although co-expression of necdin and PIAS1 did not alter their subcellular localization patterns, co-expressed necdin markedly reduced nuclear PIAS1 signals. We then examined the effects of MG132 on the distribution and expression levels of PIAS1 (**Fig. III.11B**). MG132 *per se* had little or no effect on the localization of PIAS1 or necdin, but suppressed the necdin-induced reduction of nuclear PIAS1 levels. We classified PIAS1(Myc)-expressing cells into three groups by relative fluorescence intensities; undetectable or barely detectable, low, and high (**Fig. III.11C, D**). The PIAS1 immunoreactivity in 55% of necdin-positive cells was undetectable or barely detectable in the absence of MG132, whereas 52% of necdin-immunopositive cells had high fluorescence intensities in the presence of MG132, suggesting that necdin promotes degradation of nuclear PIAS1 in the proteasome.

III-5. PIAS1 N- and C-terminal regions are required for necdin-dependent ubiquitination

To determine the PIAS1 regions responsible for necdin-dependent degradation, we transfected HEK293A cells with PIAS1 mutants lacking the N-terminus (ΔNT) and C-terminus (ΔCT)(**Fig. III.12A**). As expected, these truncated mutants, which contain the central conserved domains, bound to necdin (**Fig. III.12B**). Although necdin significantly reduced the full-length PIAS1 level, PIAS1ΔNT and ΔCT mutants were resistant to necdin-promoted degradation (**Fig. III.13A, B**). We also examined whether these mutants undergo necdin-dependent ubiquitination (**Fig. III.13C**).

PIAS1ΔNT was resistant to necdin-promoted ubiquitination, whereas PIAS1ΔCT underwent robust ubiquitination in a necdin-independent manner. These results suggest that these PIAS1 terminal regions mediate the susceptibility of PIAS1 to necdin-dependent ubiquitination.

III-6. Necdin alters subcellular localization of PIAS1

To investigate the mechanism whereby the terminal truncation of PIAS1 affects necdin-dependent ubiquitination, we analyzed the subcellular localization of ectopically expressed PIAS1 mutants in HEK293A cells. The full-length PIAS1 and PIAS1ΔNT were distributed in the nucleus, whereas PIAS1ΔCT localized predominantly in the cytoplasm (**Fig. III.14A**). All of the full-length and mutant PIAS1 (Myc)-transfected cells showed similar distribution patterns. PIAS1ΔNT partially accumulated as nuclear speckles that were immunopositive for PML (**Fig. III. 14B**), consistent with the previous observation that a PIAS1 mutant lacking the SAP domain localizes to the PML bodies (Sudharsan and Azuma, 2012). Because PIAS4 localizes in the nuclear matrix via its N-terminal SAP domain (Sachdev et al., 2001), we immunocytochemically examined the localization of these mutants in the nuclear matrix (**Fig. III.15**). Necdin localized in the nuclear matrix and accumulated as distinct speckles, consistent with the previous findings (Taniura and Yoshikawa, 2002). Full-length PIAS1 localized in the nuclear matrix of all of the transfected immunopositive cells, whereas PIAS1 deletion mutants were totally undetectable in the nuclear matrix, suggesting that the N- and C-terminal regions of PIAS1 are indispensable for its nuclear matrix localization.

III-7. Necdin suppressed endogenous expression of PIAS1

To assess the physiological role of the interaction between necdin and PIAS1, we determined whether necdin induces degradation of the PIAS1 protein in an assay system where stable expression system is employed.

To examine whether ectopic expression of necdin promotes degradation of endogenous PIAS1, we infected H1299 cells, a human non-small cell lung carcinoma cell line that endogenously expresses PIAS1 at a relatively high level (Rabellino et al., 2012), with necdin-expressing lentivirus vector and analyzed the PIAS1 levels (**Fig. III.16**). Under the conditions where most of H1299 cells were infected with the control EmGFP vector (**Fig. III.16A**), necdin overexpression significantly reduced the endogenous PIAS1 levels (**Fig. III.16B, C**). We also examined the effect of necdin on the proliferation of stably necdin-expressing H1299 cells by BrdU incorporation assay. We observed that necdin overexpression significantly reduced the number of BrdU-positive dividing cells (**Fig. III.16D, E**).

III-8. Figures III.1-16

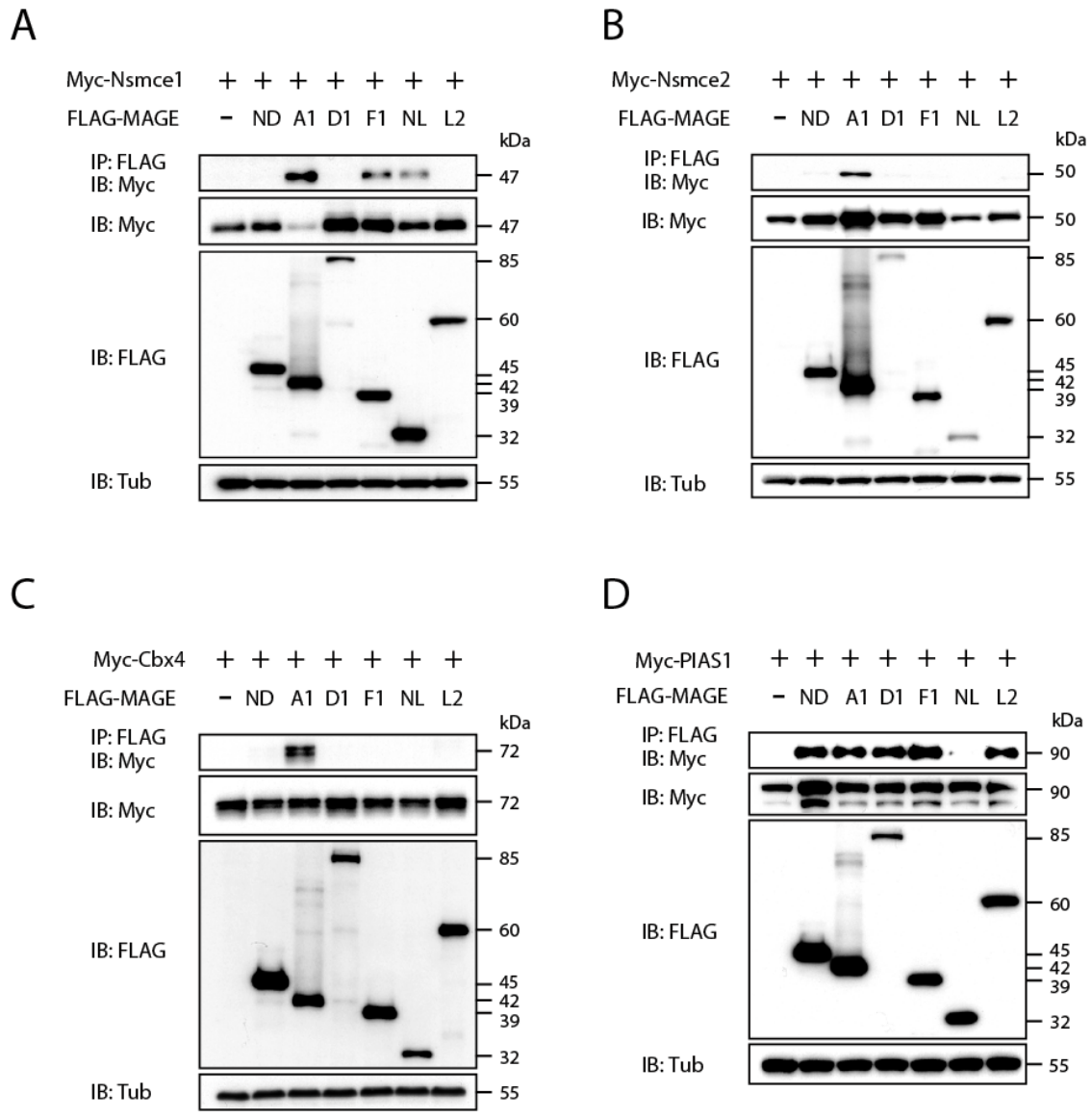


Figure III.1 MAGE proteins differentially interact with Nsmce1, Nsmce2, Cbx4 and PIAS1. (**A-D**) Interactions of MAGE proteins with Nsmce1, Nsmce2, Cbx4, and PIAS1. Expression vectors for FLAG-tagged necdin (ND, 2 μg), MAGEA1 (A1, 1 μg), MAGED1 (D1, 8 μg), MAGEF1 (F1, 4 μg), necdin-like 2 (NL, 1 μg), and MAGEL2 (L2, 6 μg) were co-transfected with expression vectors for Myc-tagged Nsmce1 (Myc-Nsmce1, 3 μg)(**A**)**,** Nsmce2 (Myc-Nsmce2, 3 μg)(**B**), Cbx4 (Myc-Cbx4, 3 μg)**(C)**, and PIAS1 (Myc-PIAS1, 6 μg)**(D)** into HEK293A cells. Proteins were immunoprecipitated (IP) with anti-FLAG antibody (FLAG) and immunoblotted (IB) with anti-Myc (Myc) (top panels, **A**-**D**). Expressed proteins were immunoblotted (IB) with antibodies to Myc, FLAG and β-tubulin (Tub). Molecular sizes are in kilodaltons (kDa).

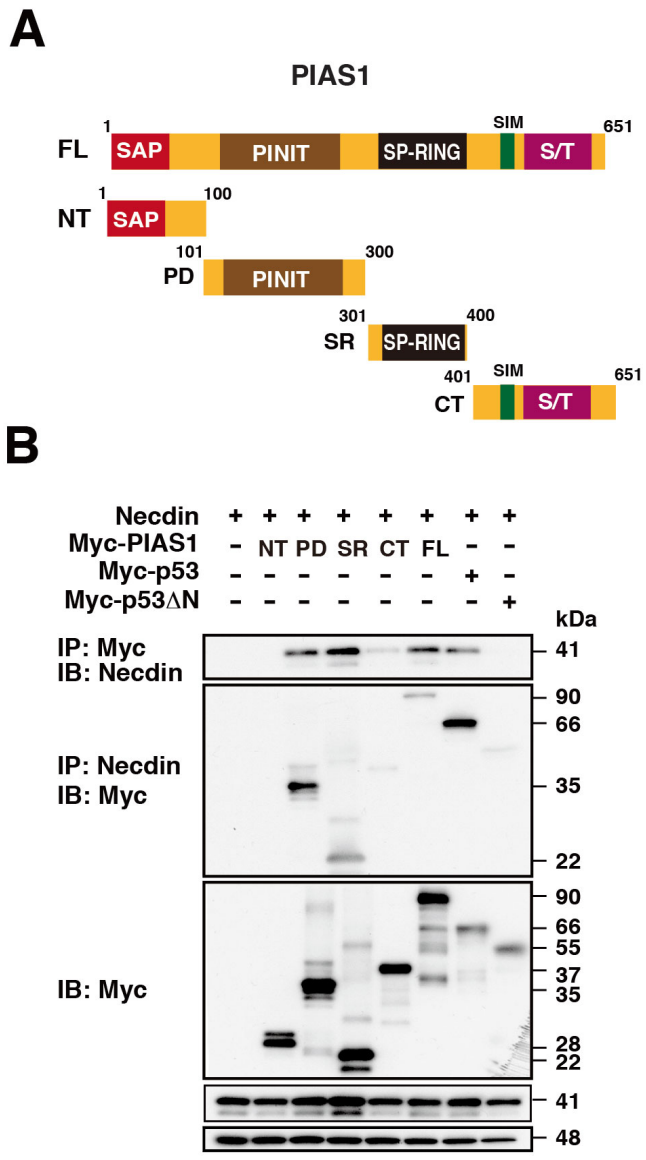


Figure III.2 Necdin interacts with PIAS1 central conserved domains.
(**A**) Domain structure of mouse PIAS1 Abbreviations: FL, full-length; NT, N-terminus; CT, C-terminus; SAP, SAF-Acinus-PIAS; PD, PINIT motif-containing domain; SR, SP-RING; SIM, SUMO-interacting motif; S/T, Ser/Thr-rich.
(**B**) Interactions of necdin with PIAS1 deletion mutants. HEK293A cells were transfected with combinations of expression vectors for necdin (3 μg), and Myc-tagged PIAS1 mutants (NT, PD, SR, CT, FL; 3 μg each). Cell lysates were immunoprecipitated (IP) and immunoblotted (IB) with antibodies to Myc and necdin (top two panels).

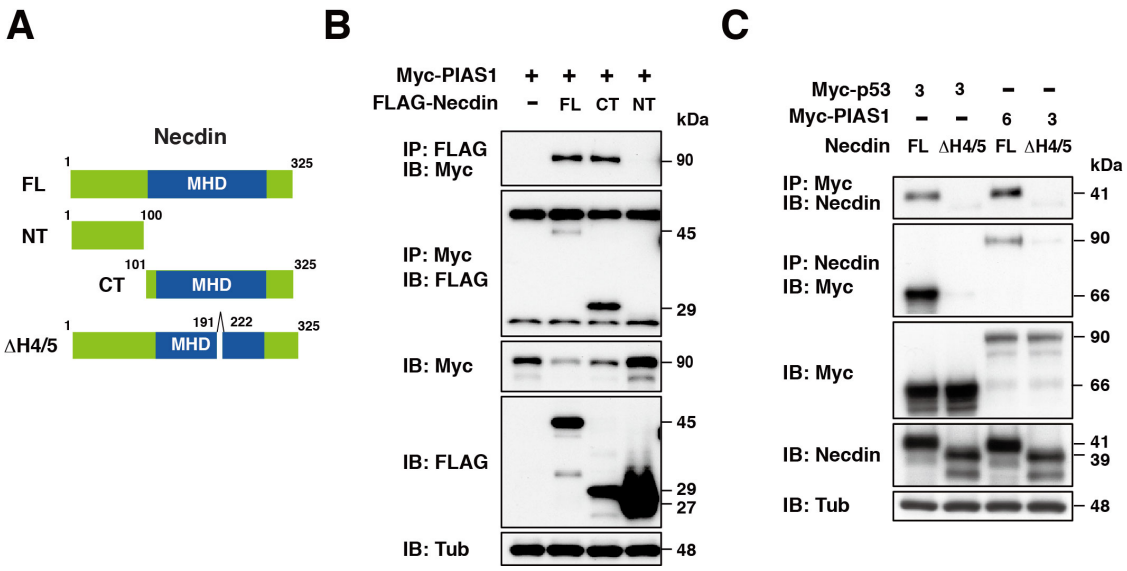


Figure III.3 Necdin interacts with PIAS1 via conserved amino acid stretch on MHD.
(**A**) Domain structure of mouse necdin. Abbreviations:MHD, MAGE homology domain; ΔH4/5, aa 191-222 deletion.
(**B**, **C**) Interactions of PIAS1 with necdin deletion mutants. HEK293A cells were transfected with expression vectors for Myc-tagged PIAS1 (Myc-PIAS1, 3 μg), FLAG-tagged necdin FL (1 μg), CT (1 μg), NT (8 μg) (**B**); Myc-p53 (3 μg), Myc-PIAS1 (3 or 6 μg), Necdin FL (1 μg), ΔH4/5 (1 μg) (**C**). Immunoprecipitated proteins were analyzed using antibodies to Myc (**B**, **C**), FLAG (**B**) and necdin (**C**)(top two panels). Expressed proteins were immunoblotted with antibodies to Myc, necdin, FLAG and γ-tubulin (Tub).

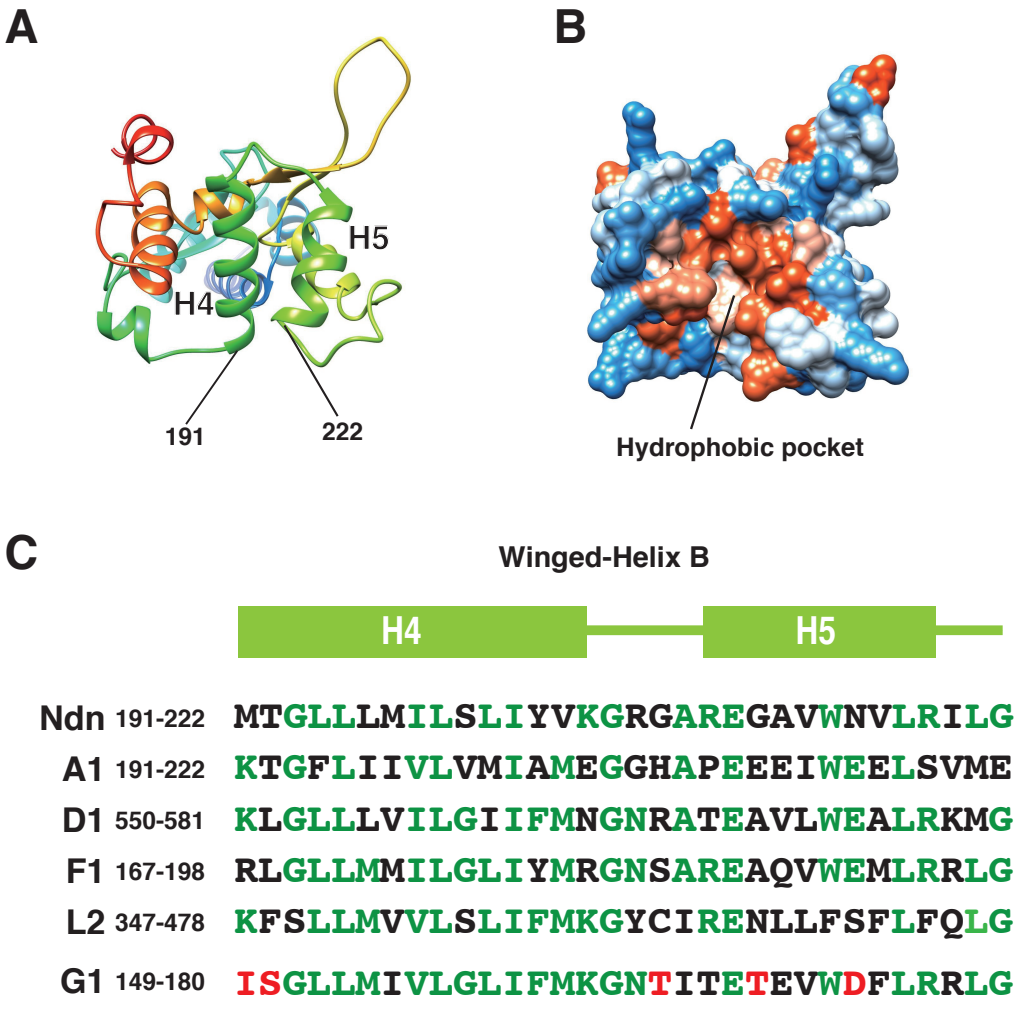


Figure III.4 Necdin interacts with PIAS1 via a hydrophobic pocket region of MHD.
(**A, B**) Structure models of the necdin H4/5 region. A structural model of the necdin MHD was created by Spanner homology modeling program using the crystal structure data of necdin-like 2 (PDB 3NW0). A view focused on the H4/5 region (necdin aa 191-222) in the winged-helix B area is presented. Helices 4 (H4) and 5 (H5), positions of 191(M) and 222 (G) (A), the hydrophobic pocket consisting of surface hydrophobic (red) and hydrophilic (blue) residues (B) are indicated.
(**C**) Sequence alignment of the H4/5 region. Consensus amino acid residues (≥3 identical)(green), the residues unique to necdin-like 2 (red), and Thr (T)(dot) are shown. Abbreviations: ND, necdin; A1, MAGEA1; D1, MAGED1; F1, MAGEF1; L2, MAGEL2; NL, necdin-like 2.

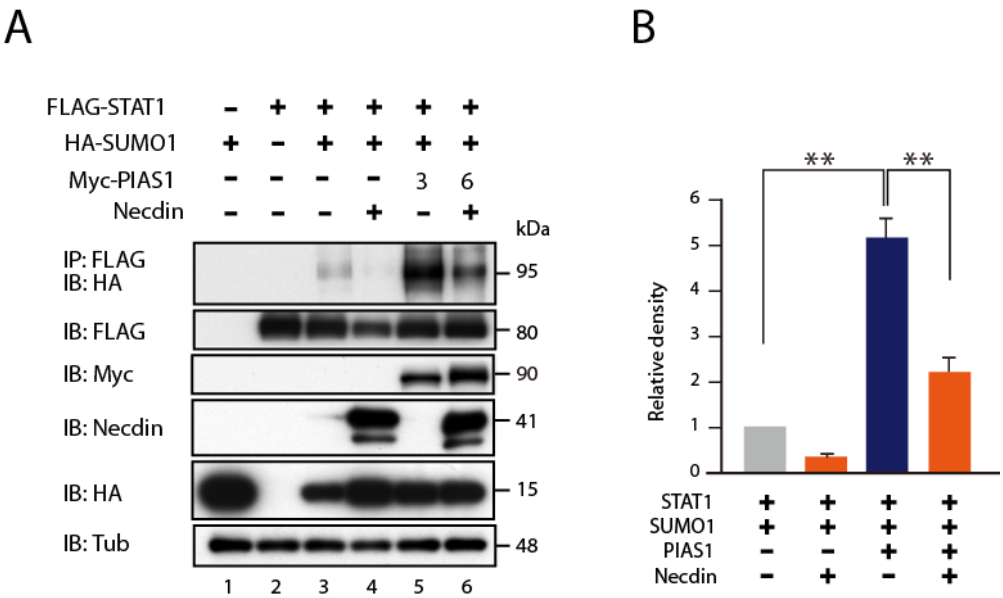


Figure III.5 Necdin inhibits PIAS1-dependent sumoylation of STAT1.
(**A**, **B**) Inhibition of PIAS1-dependent STAT1 sumoylation by necdin. HEK293A cells were transfected with combinations of expression vectors for FLAG-tagged STAT1 (FLAG-STAT1, 3 μg) (**A**), HA-tagged SUMO1 (HA-SUMO1, 10 μg), Myc-tagged PIAS1 (Myc-PIAS1, 3 or 6 μg) and necdin (Necdin, 1 μg). Cell lysates were immunoprecipitated (IP) with anti-FLAG antibody (FLAG) and immunoblotted (IB) with anti-HA antibody (HA). Expressed proteins were immunoblotted with antibodies to FLAG, Myc, necdin, HA, and γ-tubulin (Tub). Sumoylated STAT1 signals were quantified by densitometry and normalized with STAT1 (second panels) and PIAS1 levels (third panels)(**B**). Values (**B**) represent the mean ± SD ($n = 3$). $^{**}p<0.01$.

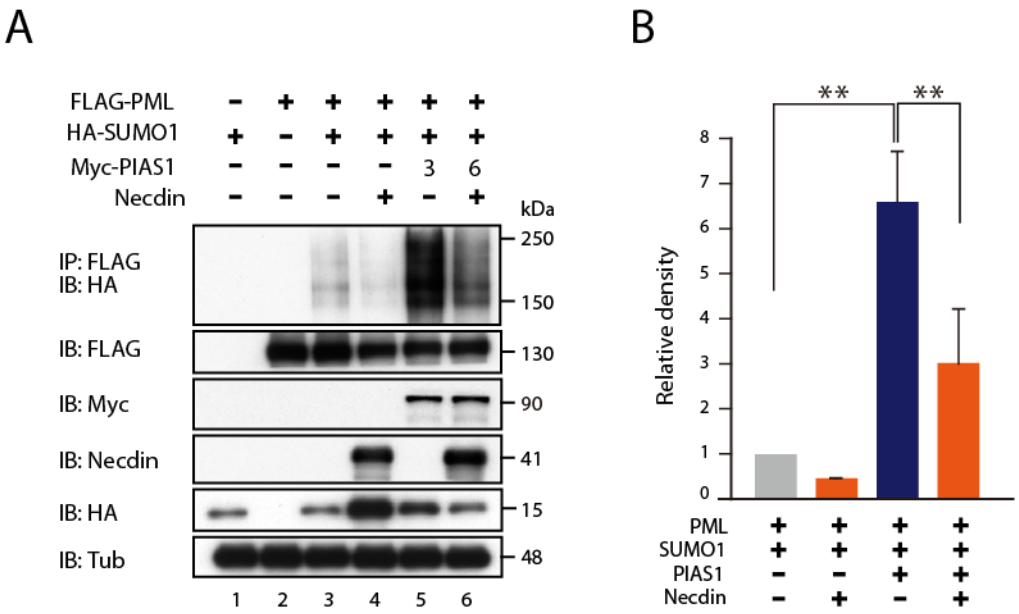


Figure III.6 Necdin inhibits PIAS1-dependent sumoylation of PML.
(**A**, **B**) Inhibition of PIAS1-dependent PML sumoylation by necdin. HEK293A cells were transfected with combinations of expression vectors for FLAG-tagged PML (FLAG-PML, 3 μg), HA-tagged SUMO1 (HA-SUMO1, 4 μg), Myc-tagged PIAS1 (Myc-PIAS1, 3 or 6 μg) and necdin (Necdin, 1 μg). PML sumoylation was analyzed and quantified as Fig. III.5. Values (**B**) represent the mean ± SD (n = 3). $^{**}p<0.01$.

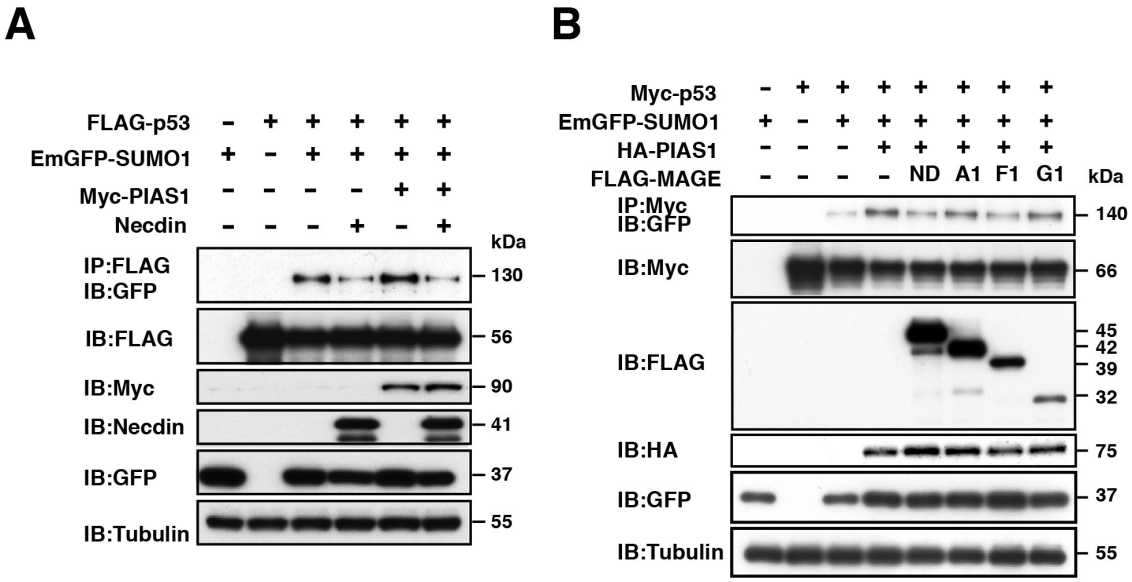


Figure III.7 Necdin inhibits PIAS1-dependent sumoylation of p53.
(**A**) Inhibition of PIAS1-dependent p53 sumoylation by necdin. HEK293A cells were transfected with combinations of expression vectors for FLAG-tagged p53 FLAG-p53, 3 μg), GFP-tagged SUMO1 (GFP-SUMO1, 4 μg), Myc-tagged PIAS1 (Myc-PIAS1, 3 or 6 μg) and necdin (Necdin, 1 μg). Cell lysates were immunoprecipitated (IP) with anti-FLAG antibody (FLAG) and immunoblotted (IB) with anti-GFP antibody. Expressed proteins were immunoblotted with antibodies to FLAG, Myc, necdin, GFP, and γ-tubulin (Tub).
(**B**) Analysis of PIAS1-dependent p53 sumoylation by MAGE proteins. HEK293A cells were transfected with combinations of expression vectors for FLAG-tagged MAGE(Necdin, MAGE-A1, MAGE-F1 and MAGE-G1), Myc-tagged p53 Myc-p53, 3 μg), GFP-tagged SUMO1 (GFP-SUMO1, 4 μg), HA-tagged PIAS. p53 sumoylation was analyzed as above.

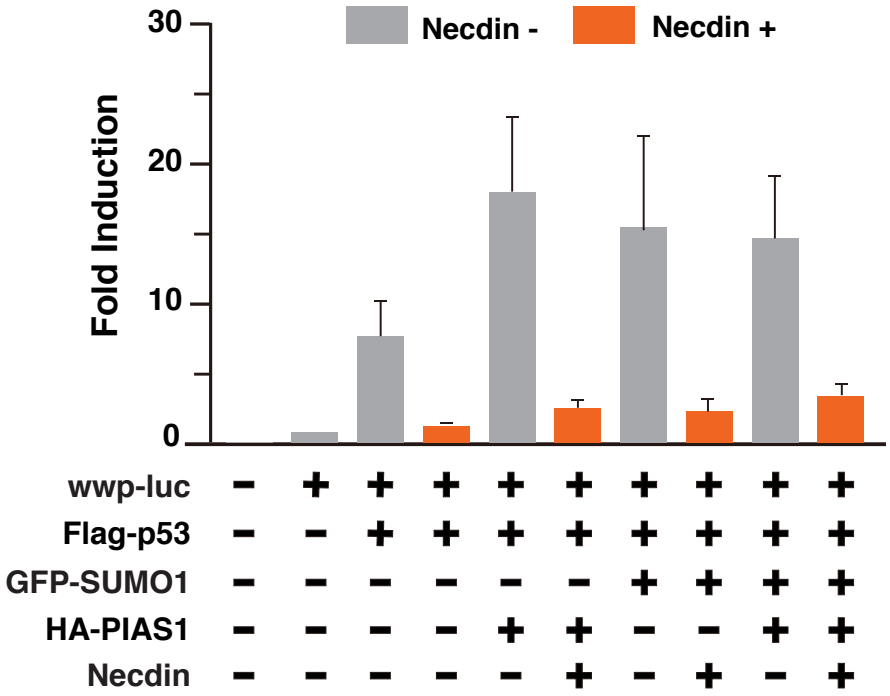


Figure III.8 Necdin cancels PIAS1-dependent stimulation of p53 transcriptional activity.

HEK293A cells were transfected with the wwp-luc promoter plasmid and combinations of plasmids encoding Flag-tagged p53 (Flag-p53), GFP-tagged SUMO1 (GFP-SUMO1), HA-tagged PIAS1 (HA-PIAS1), and necdin. Luciferase activities were measured 24 hrs post transfection. Values represent the mean ± SD (n = 2).

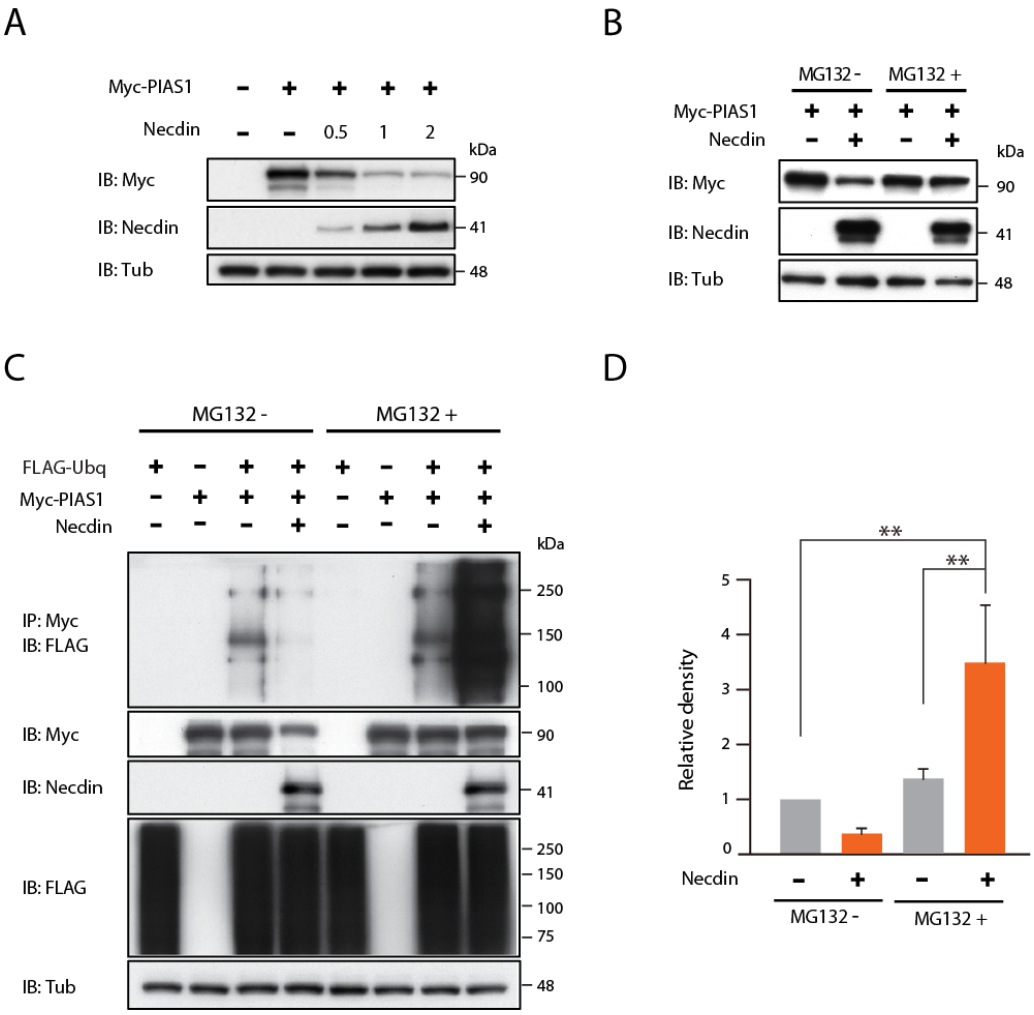


Figure III.9 Necdin promotes PIAS1 degradation via the ubiquitin-proteasome pathway.
(**A**) Necdin-dependent PIAS1 degradation. HEK293A cells were transfected with expression vectors for Myc-tagged PIAS1 (Myc-PIAS1, 3 μg) and necdin (0.5, 1, 2 μg).
(**B**) Proteasome-mediated degradation of PIAS1. HEK293A cells were transfected with expression vectors for Myc-PIAS1 (3 μg) and necdin (1 μg), incubated for 20 hrs, treated with 30 μM MG132 (MG132+) or DMSO (MG132-) for 4 hrs, and harvested. Expression of Myc-PIAS1 (Myc), necdin and γ-tubulin (Tub) was analyzed by immunoblotting (**A**, **B**).
(**C, D**) Necdin-promoted PIAS1 ubiquitination. HEK293A cells were transfected with combinations of expression vectors for FLAG-tagged ubiquitin (FLAG-Ubq, 4 μg)(**C**), Myc-PIAS1 (3 μg), necdin (1 μg) (**C**). Transfected cells were incubated for 20 hrs, treated with 30 μM MG132 for 4 hrs, and harvested. Cell lysates were immunoprecipitated (IP) with anti-Myc antibody (Myc) and immunoblotted (IB) with antibodies to FLAG. Expression of Myc-PIAS1 (Myc), necdin, FLAG-tagged proteins (FLAG), and γ-tubulin (Tub) was analyzed by immunoblotting. Ubiquitinated PIAS1 levels were determined by densitometry of immunoblots (**D**). Values (**B**) represent the mean ± SD (n = 3). **p<0.01.

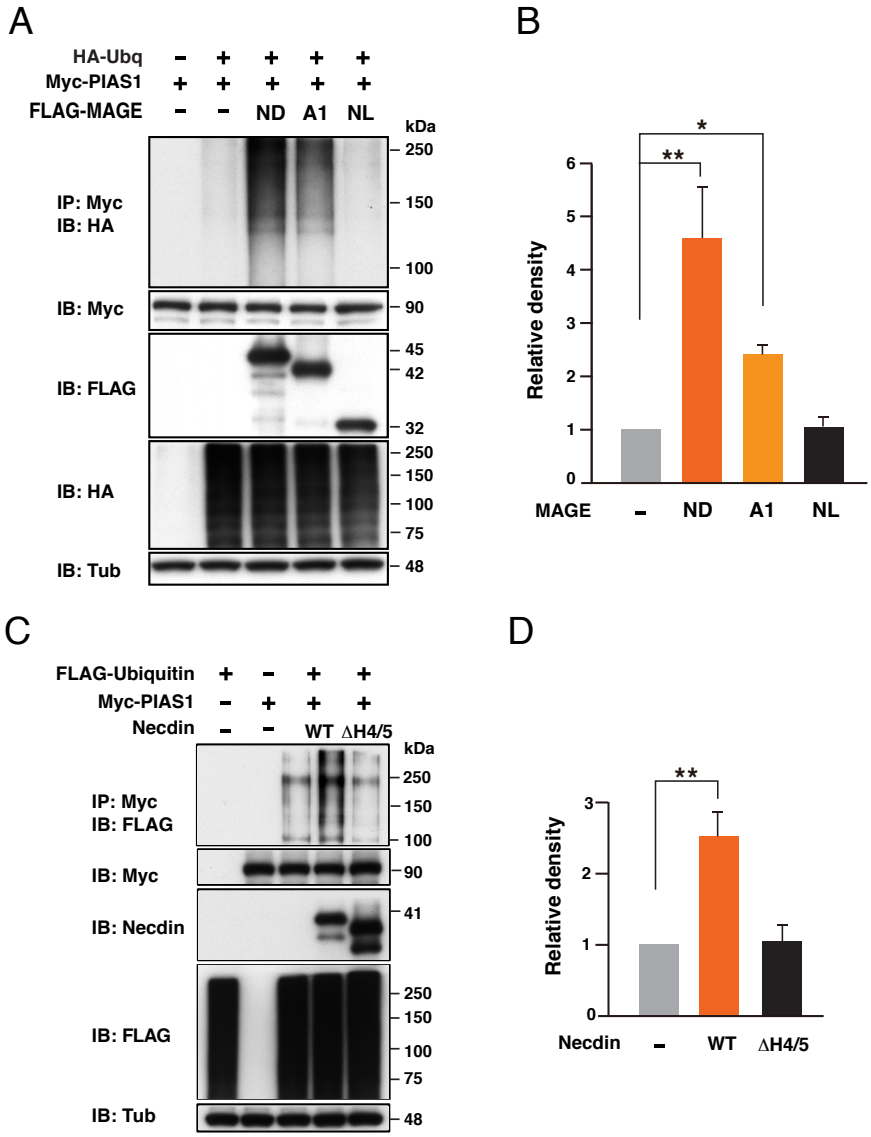


Figure III.10 Necdin specifically promotes PIAS1 ubiquitination.
(A-D) Necdin-promoted PIAS1 ubiquitination. HEK293A cells were transfected with combinations of expression vectors for HA-tagged ubiquitin (HA-Ubq, 8 μg)(**A**), FLAG-tagged ubiquitin (FLAG-Ubq, 4 μg)(**C**), Myc-PIAS1 (3 μg), FLAG-tagged necdin (ND, 2 μg), MAGEA1 (A1, 2 μg), and necdin-like 2 (NL, 1 μg) (**A**) and FLAG-tagged necdin (WT, ND, 2 μg),and necdinΔH4/5(ΔH4/5, 5 μg) (**C**). Transfected cells were incubated for 20 hrs, treated with 30 μM MG132 for 4 hrs, and harvested. Cell lysates were immunoprecipitated (IP) with anti-Myc antibody (Myc) and immunoblotted (IB) with antibodies to HA (**A**) and FLAG (**C**). Expression of Myc-PIAS1 (Myc), necdin, FLAG-tagged proteins (FLAG), HA-Ubiquitin (HA) and γ-tubulin (Tub) was analyzed by immunoblotting. Uquibitinated PIAS1 levels were determined by densitometry of immunoblots (**B**, **D**). Values represent the mean ± SD (n = 3). *p<0.05, **p<0.01. Abbreviations (**A**): ND, necdin; A1, MAGEA1; NL, necdin-like 2.

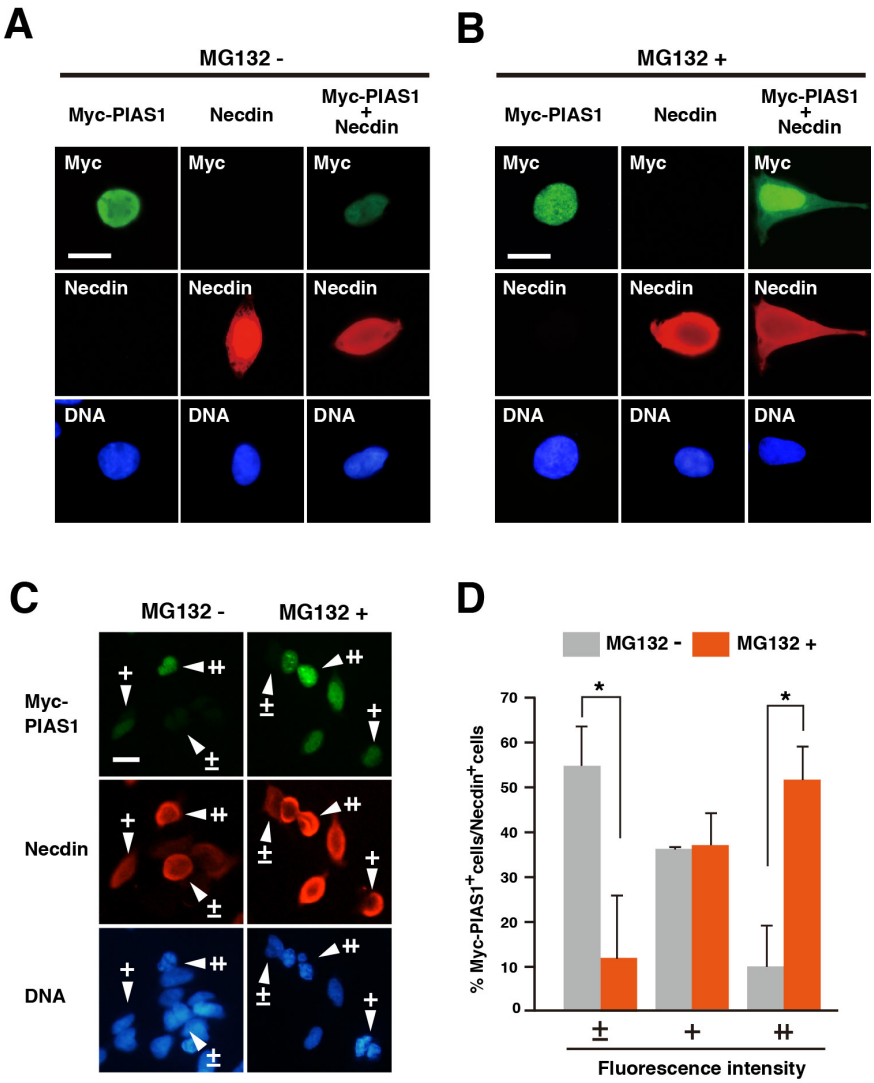


Figure III.11 Necdin reduces nuclear PIAS1 levels in transfected cells.
(**A**, **B**) Localization of PIAS1 and necdin in transfected cells. HEK293A cells were transfected with expression vectors for Myc-PIAS1 (Myc-PIAS1, 3 μg), necdin (Necdin, 2 μg) or both (Myc-PIAS1+Necdin) and fixed 24 hrs later (**A**). Transfected cells were treated with 30 μM MG132 for 4 hrs prior to harvest (**B**). Cells were immunostained with antibodies to Myc (top panels) and necdin (middle panels). Chromosomal DNA (DNA) was stained with Hoechst 33342 (bottom panels).
(**C, D**) Quantification of PIAS1-expressing cells. Fluorescence intensities of PIAS1-expressing cells cotransfected with necdin were classified into three groups; undetectable or barely detectable (±), low (+), and high (++). Arrowheads point to representative cells (**C**). Classified PIAS1-expressing cells were quantified as % of necdin-expressing cells (**D**). Transfection efficiency (mean ± SD, $n = 3$): (MG132-) PIAS1, 24 ± 4%; Necdin, 32 ± 5%; PIAS1+Necdin, 32 ± 5%; (MG132+) PIAS1, 27 ± 1%; Necdin, 33 ± 3%; PIAS1+Necdin, 39 ± 4%. Values (**D**) represent the mean ± SD ($n = 3$). *$p<0.05$. Scale bars (**A**-**C**), 20 μm.

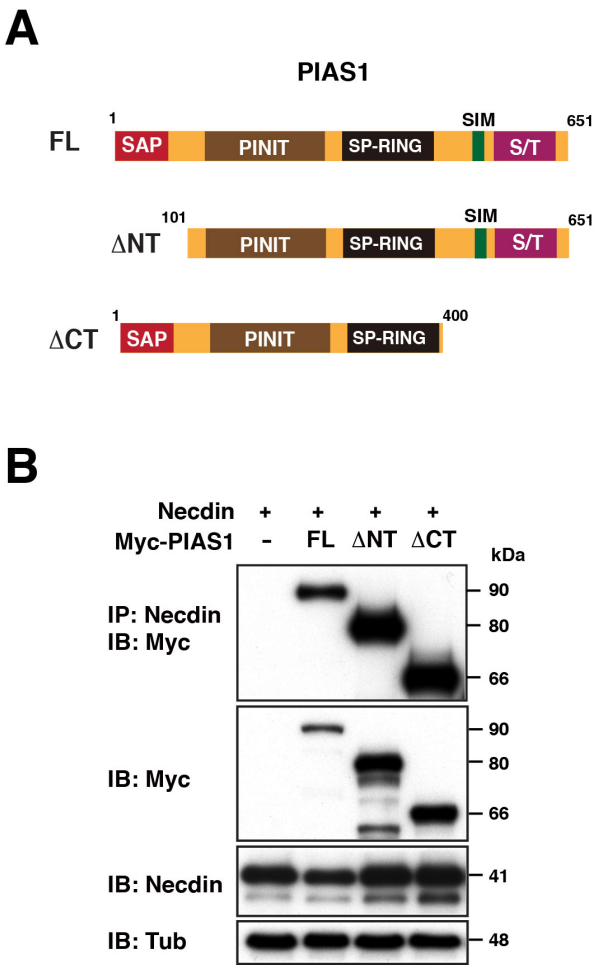


Figure III.12 PIAS1 terminal-truncated mutants interact with necdin.
(**A**) Diagrams of PIAS1 terminal-truncated mutants. Abbreviations: FL, full-length; ΔNT, N-terminal deletion mutant; ΔCT, C-terminal deletion mutant. PIAS domain names are as in Fig. III.2A.
(**B**) Interactions of necdin with PIAS1 mutants. HEK293A cells were transfected with expression vectors for necdin (1 μg), Myc-tagged PIAS1 FL (6 μg), ΔNT (4 μg) and ΔCT (8 μg). Cell lysates were immunoprecipitated (IP) with anti-necdin antibody and immunoblotted (IB) with anti-Myc antibody. Expression levels of Myc-PIAS1 mutants (Myc), necdin (Necdin), FLAG-ubiquitin (FLAG) and γ-tubulin (Tub) were analyzed by immunoblotting .

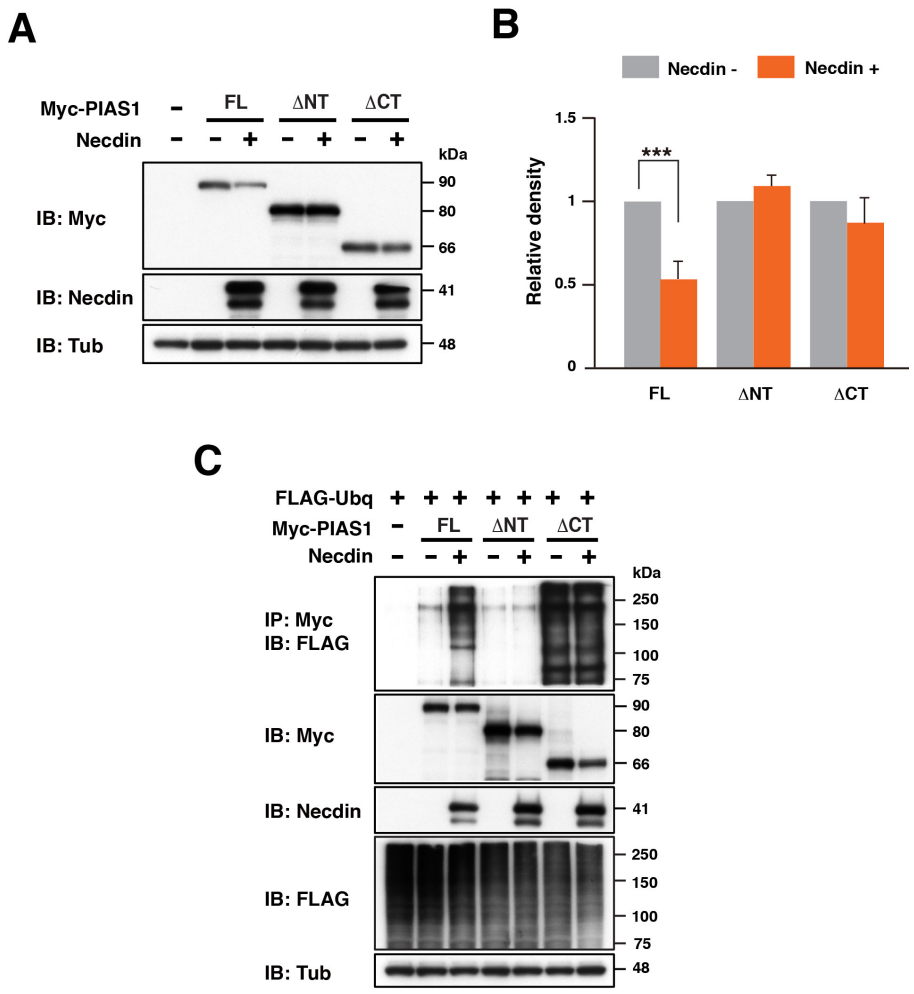


Figure III.13 PIAS1 terminal-truncated mutants lack necdin-dependent ubiquitination.

(**A, B**) Protein levels of PIAS1 terminal-truncated mutants. HEK293A cells were transfected with expression vectors for Myc-tagged PIAS1 mutants (FL, ΔNT, ΔCT; 3 μg each) and necdin (1 μg)(**A**). Expression levels of Myc-PIAS1 mutants (Myc) were quantitated (**B**). Values represent the mean ± SD ($n = 3$). ***$p<0.001$.

(**C**) Ubiquitination of PIAS1 terminal-truncated mutants. HEK293A cells were transfected with expression vectors for FLAG-tagged ubiquitin (FLAG-Ubq, 4 μg), Myc-tagged PIAS1 mutants (3 μg), and necdin (1 μg), cultured for 20 hrs, and treated with 30 μM MG132 for 4 hrs prior to harvest. Cell lysates were immunoprecipitated (IP) with anti-Myc antibody (Myc) and immunoblotted (IB) with anti-FLAG antibody. Expression levels of Myc-PIAS1 mutants (Myc), necdin (Necdin), FLAG-ubiquitin (FLAG) and γ-tubulin (Tub) were analyzed by immunoblotting.

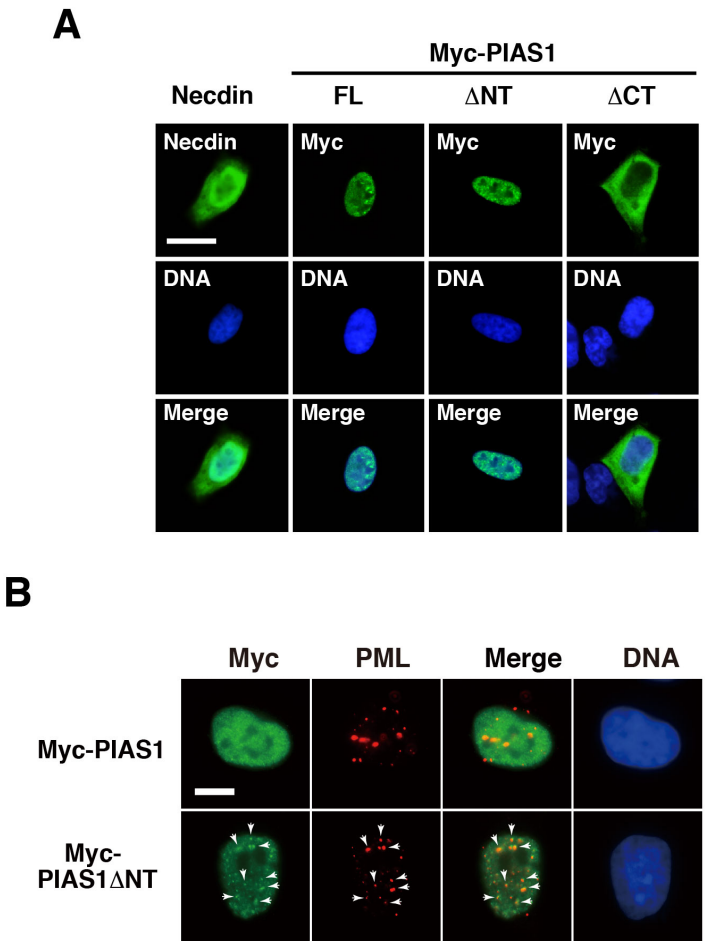


Figure III.14 Subcellular localization of PIAS1-truncated mutants.
(**A**) Subcellular localization of necdin and PIAS1 terminal-truncated mutants. HEK293A cells were transfected with expression vectors for necdin (2 μg), Myc-tagged full-length PIAS1 and truncated mutants (FL, ΔNT, ΔCT; 3 μg each), fixed 24 hrs later, and immunostained with antibodies to necdin and Myc (top panels). Chromosomal DNA (DNA) was stained with Hoechst 33342 for nuclear location (middle panels). Images of expressed proteins and nuclear DNA are merged (bottom panels). Transfection efficiency (mean ± SD, n = 3): Necdin, 20 ± 3%; FL,15 ± 4%; ΔNT, 17 ± 3%; ΔCT, 7 ± 8%.
(**B**) HEK293A cells were transfected with expression vectors for Myc-tagged full-length PIAS1 (Myc-PIAS1, 3 μg) and N-terminal-truncated PIAS1 mutant (PIAS1ΔNT, 3 μg), fixed 24 hrs later, and double-stained for Myc-PIAS1 (Myc) and PML. Images of Myc and PML are merged (Merge). Arrows point to Myc-PIAS1$^+$/PML$^+$ speckles. Chromosomal DNA (DNA) was stained with Hoechst 33342. Scale bar, 10 μm.

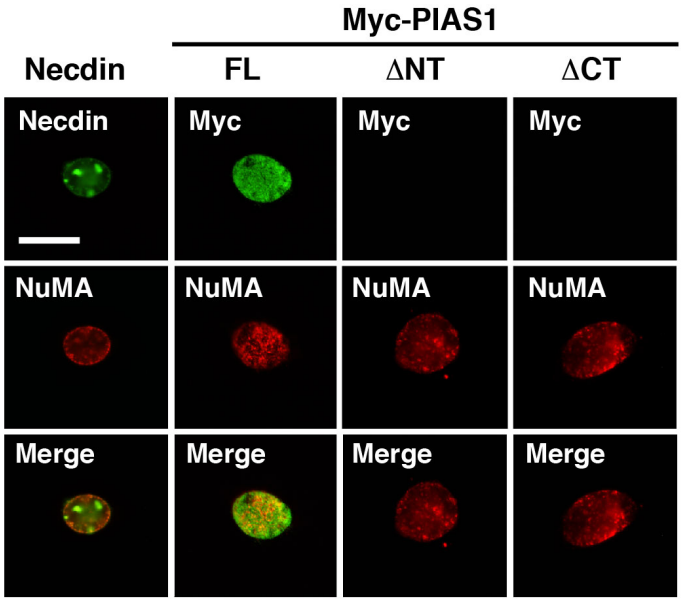


Figure III.15 PIAS1 terminal-truncated mutants dissociate from the nuclear matrix. Localization of necdin and PIAS1 terminal-truncated mutants in the nuclear matrix. The nuclear matrix of HEK293 cells was prepared 24 hrs after transfection. Proteins associated with the nuclear matrix were double-stained for necdin (or Myc)(top panels) and the nuclear matrix protein NuMA (middle panels), and their images are merged (bottom panels). Scale bars 20 μm.

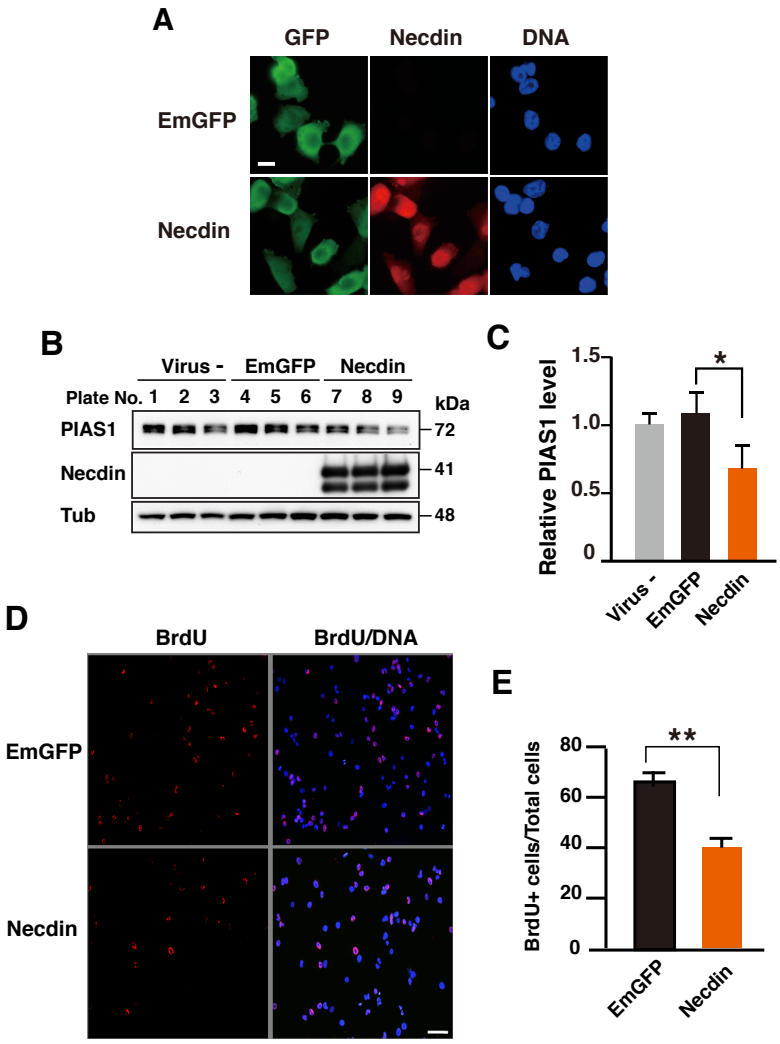


Figure III.16 Lentivirus- mediated expression of necdin reduces endogenous PIAS1 levels.

(**A-C**) H1299 cells infected with lentiviruses. Lentivirus vectors for EmGFP and necdin (+EmGFP) were infected into H1299 cells. Infected cells were grown on coverslips and fixed for immunocytochemistry using antibodies to GFP and necdin. Chromosomal DNA (DNA) was stained with Hoechst 33342. Scale bar, 20 μm. (**B**) Western blot analysis. Cell lysates of uninfected (Virus-, Plate Nos. 1-3) and infected cells expressing EmGFP (EmGFP, Plate Nos. 4-6) and necdin (Necdin, Plate Nos. 7-9) were analyzed 72 hrs after infection by immunoblotting using antibodies to PIAS1, necdin and γ-tubulin (Tub). **(C)** Quantification of the PIAS1 protein levels. Signal intensities of PIAS1 and γ-tubulin were quantitated by densitometry, and PIAS1 values were normalized with those of γ-tubulin.

(**D,E**) Effect of necdin on H1299 proliferation. Proliferation of lentivirus-infected H1299 cells was determined by BrdU incorporation assay. Infected cells were stained for BrdU incorporated into nuclear DNA (**A**), and BrdU-positive cells were counted after counterstaining with Hoechst 33342 (**B**). Values represent the mean ± SD (n = 3). *p<0.05, **p<0.03.

IV. DISCUSSION

IV-1. Characterization of MAGE proteins with different responses to SUMO E3 ligases

The present study has demonstrated that necdin interacts with PIAS1 SUMO E3 ligase and suppresses its catalytic activity. Other MAGE proteins, except necdin-like 2, also bound to PIAS1, whereas Nsmce2 and Cbx4 SUMO E3 ligases bound only to MAGEA1. Necdin interacts with RanBP2, another well-studied SUMO E3 ligase associated with the nuclear pore complex (K Fujiwara and K Yoshikawa, unpublished observations). Several MAGE proteins bind to RING-type ubiquitin E3 ligases such as TRIM27 and TRIM28 to promote ubiquitination of their substrates, but necdin is unable to interact with these ubiquitin E3 ligases (Doyle et al., 2010; Yang et al., 2007). We confirmed the previous findings (Doyle et al., 2010; Taylor et al., 2008) that Nsmce1, the ubiquitin E3 ligase in the SMC5/6 complex, interacts with necdin-like 2 and MAGEF1 but not with necdin. Necdin-like 2 shows biochemical and functional characteristics similar to those of necdin (Kuwako et al., 2004). Additionally, chicken and Drosophila MAGE proteins, which resemble mammalian necdin-like 2, exhibit functional similarities to necdin (Lopez-Sanchez et al., 2007; Nishimura et al., 2008). In contrast to these similarities among MAGE family proteins, it is likely that mammalian MAGE proteins are diversified to interact with their specific ubiquitin and SUMO E3 ligases (**Fig. IV.1**).

IV-2. Necdin-dependent ubiquitin-proteasomal degradation of PIAS1

The present study has also shown that necdin promotes degradation of PIAS1 via the ubiquitin-proteasome pathway. Necdin is likely to enhance PIAS1 ubiquitination by interacting with endogenous ubiquitin E3 ligases. PIAS1 is ubiquitinated by the ubiquitin E3 ligase hSiah2 (human homologues of seven in absentia) (Depaux et al., 2007). Necdin binds to the ubiquitin E3 ligase Mdm2 and promotes degradation of the proapoptotic protein CCAR1/CARP1 (cell cycle apoptosis regulatory protein) (Francois et al., 2012). Thus, we examined whether hSiah2 and Mdm2 mediate necdin-promoted PIAS1 ubiquitination. However, we found that these ubiquitin E3 ligases were unable to ubiquitinate PIAS1 in a necdin-dependent manner (Gur and Yoshikawa, unpublished observations). Although detailed molecular mechanisms underlying necdin-promoted PIAS1 ubiquitination remain to be elucidated, we assume that necdin, like other MAGE proteins (Doyle et al., 2010), serves as an adaptor to form a necdin-ubiquitin E3 ligase complex for PIAS1 ubiquitination. We found that PIAS1 promoted PML SUMOylation, consistent with the previous report (Rabellino et al., 2012). PIAS1-dependent PML SUMOylation may promote PML degradation by RNF4, a SUMO-directed E3 ubiquitin ligase (Lallemand-Breitenbach et al., 2008; Rabellino et al., 2012; Tatham et al., 2008). Thus, it is possible that necdin prevents PML degradation by suppressing PIAS1-dependent PML SUMOylation. Because PML regulates cell fate during neocortical development (Regad et al., 2009), we speculate that necdin stabilizes PML by suppressing endogenous PIAS1 during neuronal differentiation.

IV-3. Molecular mechanisms of ubiquitin-proteasomal degradation of PIAS1

The necdin-interacting region of PIAS1 consists of two known domains: the PINIT domain that contains the Pro-Ile-Asn-Ile-Thr motif in the middle of the PIAS-conserved sequence stretch (Duval et al., 2003) and the SP-RING domain similar to the RING-finger domain found in many ubiquitin E3 ligases (Hochstrasser, 2001; Shuai and Liu, 2005). These two domains are highly conserved among PIAS family members (Rytinki et al., 2009). Although PIAS1 interacts with SUMO-modified substrates via its distinct regions throughout the whole molecule (Shuai and Liu, 2005), the SUMO ligase activity relies solely on these central domains (Liang et al., 2004). As expected, necdin inhibited PIAS1-dependent SUMOylation of STAT1 (Liu et al., 1998) and PML (Rabellino et al., 2012). Thus, it is likely that necdin-mediated suppression of the PIAS1 catalytic activity is attributed to its specific binding region in PIAS1.

The N-terminal deletion of PIAS1 lost both ubiquitination and responsiveness to necdin. The N-terminal domain contains a highly conserved SAP domain that mediates association with the nuclear matrix. Thus, PIAS1 is likely to interact with the nuclear matrix via the SAP domain. We found that the PIAS1 N-terminal deletion mutant was dissociated from the nuclear matrix and translocated to the PML bodies, consistent with the previous report (Sudharsan and Azuma, 2012). We have previously shown that necdin localizes in the nuclear matrix and interacts with the nuclear matrix-associated protein SAF-A/hnRNP U (Taniura and Yoshikawa, 2002). These findings suggest that necdin and PIAS1 are colocalized in the nuclear matrix, where necdin modulates PIAS1 ubiquitination. Intriguingly, PIAS1 C-terminal deletion enhanced ubiquitination in a necdin-independent manner, implying that the PIAS1 C-terminus negatively controls necdin-promoted ubiquitination. Because C-terminally truncated PIAS1 was translocated to the cytoplasm, the C-terminus of PIAS1 is likely to contribute to the nuclear retention. We speculate that these terminal regions are required for the translocation of PIAS1 to the nuclear matrix that contributes to the necdin-dependent ubiquitination (**Fig. IV.2**).

IV-4. Physiological roles of necdin-induced regulation of PIAS1

Necdin plays anti-apoptotic and pro-survival roles in various cell types such as neurons and neural stem/progenitor cells through interactions with various proteins (Hasegawa and Yoshikawa, 2008; Huang et al., 2013; Kobayashi et al., 2002; Kurita et al., 2006; Kuwako et al., 2005; Minamide et al., 2014; Takazaki et al., 2002; Taniura et al., 1999). Previous studies have shown that PIAS1 promotes apoptosis in response to various stimuli (Liu and Shuai, 2001; Sudharsan and Azuma, 2012; Yang et al., 2013). These observations suggest that necdin targets PIAS1 to suppress its proapoptotic activities. Because the necdin-binding region in PIAS1 is highly conserved among PIAS family members, it is possible that necdin also interacts with other PIAS family proteins to regulate their functions. Although information on the regulation of PIAS family proteins during neuronal development is currently limited, we speculate that necdin interacts with PIAS family proteins to modulate SUMOylation of their target

proteins involved in mammalian neuron development.

Necdin is expressed abundantly in terminally differentiated neurons, whereas its protein levels are reduced in undifferentiated neural stem/progenitor cells (Huang et al., 2013). SUMOylation plays key regulatory roles in the course of embryonic development.

Ubc9$^{-/-}$ knockout mice exhibit embryonic lethal phenotype, indicating that SUMOylation is essential for embryonic development. Embryonic stem cells derived from the inner cell mass of Ubc9$^{-/-}$ mice display severe apoptosis and fail to proliferate. Furthermore, nuclear organization and molecular events concerning cell division, such as chromosomal segregation, is aberrant in embryonic cells derived from Ubc9$^{-/-}$ mice (Nacerddine et al., 2005). These results imply that SUMOylation plays a key role in the maintenance of stem/progenitor cells in the undifferentiated state. SUMO-conjugated proteins are abundant in neural stem cells at early stages of brain development but their levels decrease during neural differentiation (our unpublished observations). Collectively, our results shed light onto the molecular mechanism underlying necdin-mediated SUMOylation and its involvement in the control of neuronal differentiation.

IV-5. Figures IV.1, 2

Figure IV.1 **Differential binding of MAGE proteins to SUMO and ubiquitin E3 ligases.**

Figure IV.2 **Necdin-mediated effects on PIAS1 are dependent on subcellular localization.**

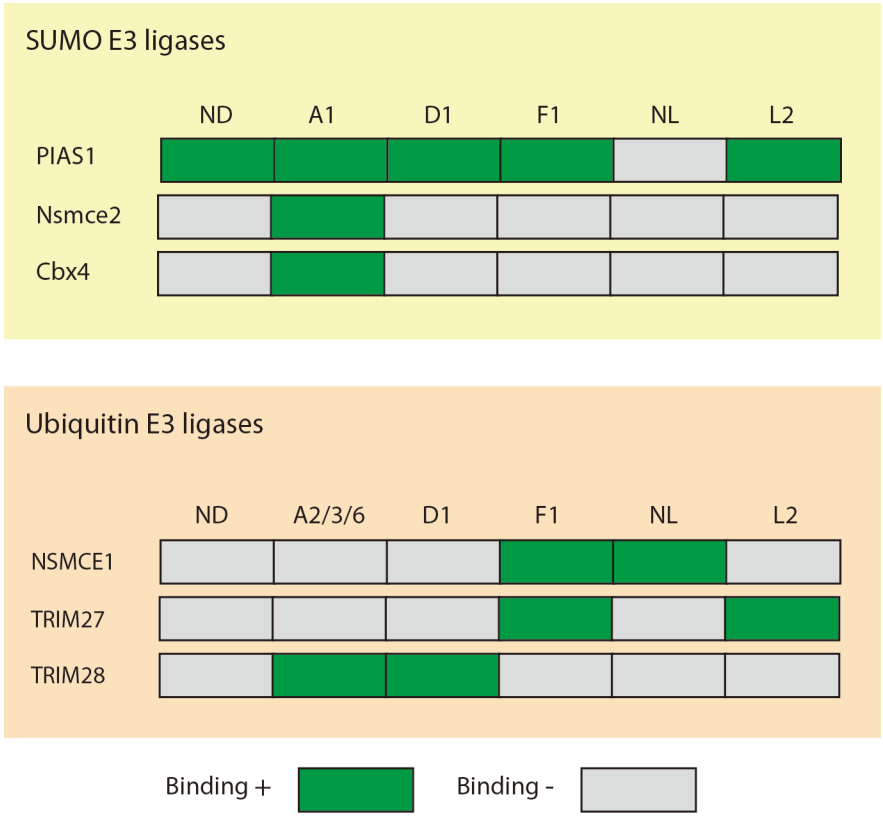


Figure IV.1 Differential binding of MAGE proteins to SUMO and ubiquitin E3 ligases. The interactions between MAGE proteins and SUMO E3 ligases are schematically presented (upper panel). Information on the interactions between MAGE proteins and ubiquitin E3 ligases was taken from the report by Doyle et al. (2010)(lower panel). Abbreviations: ND, necdin; A1, MAGEA1; D1, MAGED1; F1, MAGEF1; NL, necdin-like 2; L2, MAGEL2; A2/3/6, MAGEA2/MAGEA3/MAGEA6.

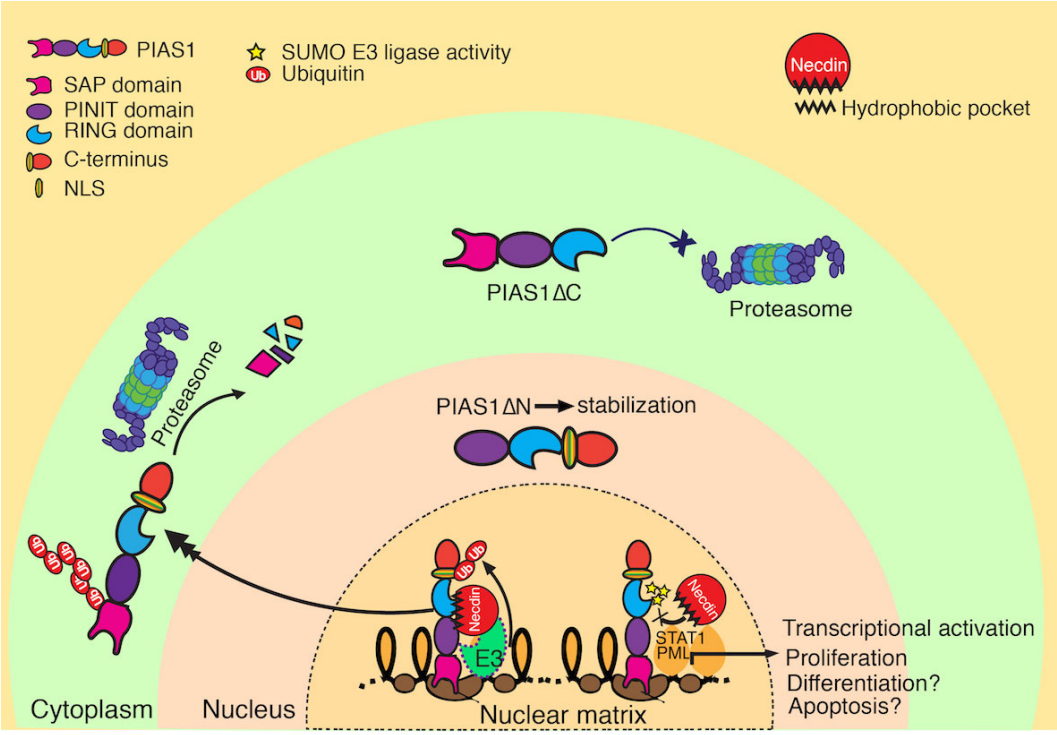


Figure IV.2 Necdin-mediated effects on PIAS1 are dependent on subcellular localization. For details, see Discussion.

V. REFERENCES

- Aizawa, T., Maruyama, K., Kondo, H., and Yoshikawa, K. (1992). Expression of necdin, an embryonal carcinoma-derived nuclear protein, in developing mouse brain. Brain Res Devel Brain Res *68*, 265-274.
- Andrews, E.A., Palecek, J., Sergeant, J., Taylor, E., Lehmann, A.R., and Watts, F.Z. (2005). Nse2, a component of the Smc5-6 complex, is a SUMO ligase required for the response to DNA damage. Mol Cell Biol *25*, 185-196.
- Barker, P.A., and Salehi, A. (2002). The MAGE proteins: emerging roles in cell cycle progression, apoptosis, and neurogenetic disease. J Neurosci Res *67*, 705-712.
- Bayer, P., Arndt, A., Metzger, S., Mahajan, R., Melchior, F., Jaenicke, R., and Becker, J. (1998). Structure determination of the small ubiquitin-related modifier SUMO-1. J Mol Biol *280*, 275-286.
- Bernassola, F., Karin, M., Ciechanover, A., and Melino, G. (2008). The HECT family of E3 ubiquitin ligases: multiple players in cancer development. Cancer Cell *14*, 10-21.
- Bernier-Villamor, V., Sampson, D.A., Matunis, M.J., and Lima, C.D. (2002). Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1. Cell *108*, 345-356.
- Borden, K.L., and Freemont, P.S. (1996). The RING finger domain: a recent example of a sequence-structure family. Curr Opin Struct Biol *6*, 395-401.
- Brzovic, P.S., Lissounov, A., Christensen, D.E., Hoyt, D.W., and Klevit, R.E. (2006). A UbcH5/ubiquitin noncovalent complex is required for processive BRCA1-directed ubiquitination. Mol Cell *21*, 873-880.
- Chibuk, T.K., Bischof, J.M., and Wevrick, R. (2001). A necdin/MAGE-like gene in the chromosome 15 autism susceptibility region: expression, imprinting, and mapping of the human and mouse orthologues. BMC Genet *2*, 22.
- Chomez, P., De Backer, O., Bertrand, M., De Plaen, E., Boon, T., and Lucas, S. (2001). An overview of the MAGE gene family with the identification of all human members of the family. Cancer Res *61*, 5544-5551.
- Ciechanover, A., Heller, H., Elias, S., Haas, A.L., and Hershko, A. (1980). ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. Proc Natl Acad Sci USA *77*, 1365-1368.
- De Plaen, E., Traversari, C., Gaforio, J., Szikora, J.-P., De Smet, C., Brasseur, F., van der Bruggen, P., Lethé, B., Lurquin, C., Chomez, P., *et al*. (1994). Structure, chromosomal localization, and expression of 12 genes of the MAGE family. Immunogenetics *40*, 360-369.
- De Smet, C., Courtois, S.J., Faraoni, I., Lurquin, C., Szikora, J.P., De Backer, O., and Boon, T. (1995). Involvement of two Ets binding sites in the transcriptional activation of the MAGE1 gene. Immunogenetics *42*, 282-290.
- Depaux, A., Regnier-Ricard, F., Germani, A., and Varin-Blank, N. (2007). A crosstalk between hSiah2 and Pias E3-ligases modulates Pias-dependent activation. Oncogene *26*, 6665-6676.

- Deshaies, R.J., and Joazeiro, C.A. (2009). RING domain E3 ubiquitin ligases. Annu Rev Biochem *78*, 399-434.
- Doyle, J.M., Gao, J., Wang, J., Yang, M., and Potts, P.R. (2010). MAGE-RING protein complexes comprise a family of E3 ubiquitin ligases. Mol Cell *39*, 963-974.
- Duval, D., Duval, G., Kedinger, C., Poch, O., and Boeuf, H. (2003). The 'PINIT' motif, of a newly identified conserved domain of the PIAS protein family, is essential for nuclear retention of PIAS3L. FEBS Lett *554*, 111-118.
- Emmerich, C.H., Schmukle, A.C., and Walczak, H. (2011). The emerging role of linear ubiquitination in cell signaling. Sci Signal *4*, re5.
- Finley, D., and Chau, V. (1991). Ubiquitination. Annu Rev Cell Biol *7*, 25-69.
- Francois, S., D'Orlando, C., Fatone, T., Touvier, T., Pessina, P., Meneveri, R., and Brunelli, S. (2012). Necdin enhances myoblasts survival by facilitating the degradation of the mediator of apoptosis CCAR1/CARP1. PloS One *7*, e43335.
- Fratta, E., Coral, S., Covre, A., Parisi, G., Colizzi, F., Danielli, R., Nicolay, H.J., Sigalotti, L., and Maio, M. (2011). The biology of cancer testis antigens: putative function, regulation and therapeutic potential. Mol Oncol *5*, 164-182.
- Freemont, P.S., Hanson, I.M., and Trowsdale, J. (1991). A novel cysteine-rich sequence motif. Cell *64*, 483-484.
- Fujiwara, K., Hasegawa, K., Ohkumo, T., Miyoshi, H., Tseng, Y.H., and Yoshikawa, K. (2012). Necdin controls proliferation of white adipocyte progenitor cells. PloS One *7*, e30948.
- Gareau, J.R., and Lima, C.D. (2010). The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. Nat Rev Mol Cell Biol *11*, 861-871.
- Gerard, M., Hernandez, L., Wevrick, R., and Stewart, C.L. (1999). Disruption of the mouse necdin gene results in early post-natal lethality. Nat Genet *23*, 199-202.
- Goldstein, G. (1975). The isolation of thymopoietin (thymin). Annals New York Acad Sci *249*, 177-185.
- Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P.P., and Dikic, I. (2003). Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol *5*, 461-466.
- Hasegawa, K., Kawahara, T., Fujiwara, K., Shimpuku, M., Sasaki, T., Kitamura, T., and Yoshikawa, K. (2012). Necdin controls FoxO1 acetylation in hypothalamic arcuate neurons to modulate the thyroid axis. J Neurosci *32*, 5562-5572.
- Hasegawa, K., and Yoshikawa, K. (2008). Necdin regulates p53 acetylation via Sirtuin1 to modulate DNA damage response in cortical neurons. J Neurosci *28*, 8772-8784.
- Hay, R.T. (2005). SUMO: a history of modification. Molecular cell *18*, 1-12.
- He, D.C., Nickerson, J.A., and Penman, S. (1990). Core filaments of the nuclear matrix. J Cell Biol *110*, 569-580.
- Heo, K.S., Chang, E., Takei, Y., Le, N.T., Woo, C.H., Sullivan, M.A., Morrell, C., Fujiwara, K., and Abe, J. (2013). Phosphorylation of protein inhibitor of activated STAT1 (PIAS1) by MAPK-activated protein kinase-2 inhibits endothelial inflammation via increasing both PIAS1 transrepression and SUMO E3 ligase activity. Arterioscler Thromb Vasc Biol *33*, 321-329.

- Hicke, L. (2001). Protein regulation by monoubiquitin. Nat Rev Mol Cell Biol *2*, 195-201.
- Hochstrasser, M. (2001). SP-RING for SUMO: new functions bloom for a ubiquitin-like protein. Cell *107*, 5-8.
- Hochstrasser, M. (2004). Ubiquitin signalling: what's in a chain? Nat Cell Biol *6*, 571-572.
- Huang, Z., Fujiwara, K., Minamide, R., Hasegawa, K., and Yoshikawa, K. (2013). Necdin controls proliferation and apoptosis of embryonic neural stem cells in an oxygen tension-dependent manner. J Neurosci *33*, 10362-10373.
- Hudson, J.J., Bednarova, K., Kozakova, L., Liao, C., Guerineau, M., Colnaghi, R., Vidot, S., Marek, J., Bathula, S.R., Lehmann, A.R., *et al*. (2011). Interactions between the Nse3 and Nse4 components of the SMC5-6 complex identify evolutionarily conserved interactions between MAGE and EID families. PloS One *6*, e17270.
- Hutten, S., Flotho, A., Melchior, F., and Kehlenbach, R.H. (2008). The Nup358-RanGAP complex is required for efficient importin alpha/beta-dependent nuclear import. Mol Biol Cell *19*, 2300-2310.
- Ihara, M., Yamamoto, H., and Kikuchi, A. (2005). SUMO-1 modification of PIASy, an E3 ligase, is necessary for PIASy-dependent activation of Tcf-4. Mol Cell Biol *25*, 3506-3518.
- Jay, P., Rougeulle, C., Massacrier, A., Moncla, A., Mattei, M.G., Malzac, P., Roeckel, N., Taviaux, S., Lefranc, J.L., Cau, P., *et al*. (1997). The human necdin gene, NDN, is maternally imprinted and located in the Prader-Willi syndrome chromosomal region. Nat Genet *17*, 357-361.
- Johnson, E.S., and Blobel, G. (1997). Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p. J Biol Chem *272*, 26799-26802.
- Johnson, E.S., Schwienhorst, I., Dohmen, R.J., and Blobel, G. (1997). The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. EMBO J *16*, 5509-5519.
- Kagey, M.H., Melhuish, T.A., and Wotton, D. (2003). The polycomb protein Pc2 is a SUMO E3. Cell *113*, 127-137.
- Kahyo, T., Nishida, T., and Yasuda, H. (2001). Involvement of PIAS1 in the SUMOylation of tumor suppressor p53. Mol Cell *8*, 713-718.
- Kishino, T., Lalande, M., and Wagstaff, J. (1997). UBE3A/E6-AP mutations cause Angelman syndrome. Nat Genet *15*, 70-73.
- Kobayashi, M., Taniura, H., and Yoshikawa, K. (2002). Ectopic expression of necdin induces differentiation of mouse neuroblastoma cells. J Biol Chem *277*, 42128-42135.
- Kozlov, S.V., Bogenpohl, J.W., Howell, M.P., Wevrick, R., Panda, S., Hogenesch, J.B., Muglia, L.J., Van Gelder, R.N., Herzog, E.D., and Stewart, C.L. (2007). The imprinted gene Magel2 regulates normal circadian output. Nat Genet *39*, 1266-1272.
- Kubota, Y., Osawa, M., Jakt, L.M., Yoshikawa, K., and Nishikawa, S. (2009). Necdin restricts proliferation of hematopoietic stem cells during hematopoietic regeneration. Blood *114*, 4383-4392.

- Kurita, M., Kuwajima, T., Nishimura, I., and Yoshikawa, K. (2006). Necdin downregulates CDC2 expression to attenuate neuronal apoptosis. J Neurosci *26*, 12003-12013.
- Kuwajima, T., Taniura, H., Nishimura, I., and Yoshikawa, K. (2004). Necdin interacts with the Msx2 homeodomain protein via MAGE-D1 to promote myogenic differentiation of C2C12 cells. J Biol Chem *279*, 40484-40493.
- Kuwako, K., Hosokawa, A., Nishimura, I., Uetsuki, T., Yamada, M., Nada, S., Okada, M., and Yoshikawa, K. (2005). Disruption of the paternal necdin gene diminishes TrkA signaling for sensory neuron survival. J Neurosci *25*, 7090-7099.
- Kuwako, K., Taniura, H., and Yoshikawa, K. (2004). Necdin-related MAGE proteins differentially interact with the E2F1 transcription factor and the p75 neurotrophin receptor. J Biol Chem *279*, 1703-1712.
- Lallemand-Breitenbach, V., Jeanne, M., Benhenda, S., Nasr, R., Lei, M., Peres, L., Zhou, J., Zhu, J., Raught, B., and de The, H. (2008). Arsenic degrades PML or PML-RARalpha through a SUMO-triggered RNF4/ubiquitin-mediated pathway. Nat Cell Biol *10*, 547-555.
- Lee, S., Kozlov, S., Hernandez, L., Chamberlain, S.J., Brannan, C.I., Stewart, C.L., and Wevrick, R. (2000). Expression and imprinting of MAGEL2 suggest a role in Prader-willi syndrome and the homologous murine imprinting phenotype. Human molecular genetics *9*, 1813-1819.
- Li, W., and Ye, Y. (2008). Polyubiquitin chains: functions, structures, and mechanisms. Cell Mol Life Sci *65*, 2397-2406.
- Liang, M., Melchior, F., Feng, X.H., and Lin, X. (2004). Regulation of Smad4 SUMOylation and transforming growth factor-beta signaling by protein inhibitor of activated STAT1. J Biol Chem *279*, 22857-22865.
- Liu, B., Liao, J., Rao, X., Kushner, S.A., Chung, C.D., Chang, D.D., and Shuai, K. (1998). Inhibition of Stat1-mediated gene activation by PIAS1. Proc Natl Acad Sci USA *95*, 10626-10631.
- Liu, B., and Shuai, K. (2001). Induction of apoptosis by protein inhibitor of activated Stat1 through c-Jun NH2-terminal kinase activation. J Biol Chem *276*, 36624-36631.
- Liu, Y., Zhang, Y.D., Guo, L., Huang, H.Y., Zhu, H., Huang, J.X., Liu, Y., Zhou, S.R., Dang, Y.J., Li, X., *et al*. (2013). Protein inhibitor of activated STAT 1 (PIAS1) is identified as the SUMO E3 ligase of CCAAT/enhancer-binding protein beta (C/EBPbeta) during adipogenesis. Mol Cell Biol *33*, 4606-4617.
- Lopez-Sanchez, N., Gonzalez-Fernandez, Z., Niinobe, M., Yoshikawa, K., and Frade, J.M. (2007). Single mage gene in the chicken genome encodes CMage, a protein with functional similarities to mammalian type II Mage proteins. Physiol Genomics *30*, 156-171.
- Lucas, S., De Smet, C., Arden, K.C., Viars, C.S., Lethe, B., Lurquin, C., and Boon, T. (1998). Identification of a new MAGE gene with tumor-specific expression by representational difference analysis. Cancer Res *58*, 743-752.
- MacDonald, H.R., and Wevrick, R. (1997). The Necdin Gene is Deleted in Prader-Willi Syndrome and is Imprinted in Human and Mouse. Hum Mol Genet *6*, 1873-1878.

- Maruyama, K., Usami, M., Aizawa, T., and Yoshikawa, K. (1991). A novel brain-specific mRNA encoding nuclear protein (necdin) expressed in neurally differentiated embryonal carcinoma cells. Biochem Biophys Res Commun *178*, 291-296.
- Matunis, M.J., Coutavas, E., and Blobel, G. (1996). A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. J Cell Biol *135*, 1457-1470.
- Minamide, R., Fujiwara, K., Hasegawa, K., and Yoshikawa, K. (2014). Antagonistic interplay between necdin and Bmi1 controls proliferation of neural precursor cells in the embryonic mouse neocortex. PloS One *9*, e84460.
- Miyoshi, H., Blomer, U., Takahashi, M., Gage, F.H., and Verma, I.M. (1998). Development of a self-inactivating lentivirus vector. J Virol *72*, 8150-8157.
- Munakata, T., Liang, Y., Kim, S., McGivern, D.R., Huibregtse, J., Nomoto, A., and Lemon, S.M. (2007). Hepatitis C virus induces E6AP-dependent degradation of the retinoblastoma protein. PLoS Pathog *3*, 1335-1347.
- Muscatelli, F., Abrous, D.N., Massacrier, A., Boccaccio, I., Le Moal, M., Cau, P., and Cremer, H. (2000). Disruption of the mouse Necdin gene results in hypothalamic and behavioral alterations reminiscent of the human Prader-Willi syndrome. Hum Mol Genet *9*, 3101-3110.
- Muscatelli, F., Walker, A.P., De Plaen, E., Stafford, A.N., and Monaco, A.P. (1995). Isolation and characterization of a MAGE gene family in the Xp21.3 region. Proc Natl Acad Sci USA *92*, 4987-4991.
- Nacerddine, K., Lehembre, F., Bhaumik, M., Artus, J., Cohen-Tannoudji, M., Babinet, C., Pandolfi, P.P., and Dejean, A. (2005). The SUMO pathway is essential for nuclear integrity and chromosome segregation in mice. Dev Cell *9*, 769-779.
- Nakada, Y., Taniura, H., Uetsuki, T., Inazawa, J., and Yoshikawa, K. (1998). The human chromosomal gene for necdin, a neuronal growth suppressor, in the Prader-Willi syndrome deletion region. Gene *213*, 65-72.
- Niinobe, M., Koyama, K., and Yoshikawa, K. (2000). Cellular and subcellular localization of necdin in fetal and adult mouse brain. Dev Neurosci *22*, 310-319.
- Nishimura, I., Sakoda, J.Y., and Yoshikawa, K. (2008). Drosophila MAGE controls neural precursor proliferation in postembryonic neurogenesis. Neuroscience *154*, 572-581.
- Old, L.J., and Chen, Y.T. (1998). New paths in human cancer serology. J Exp Med *187*, 1163-1167.
- Pickart, C.M., and Fushman, D. (2004). Polyubiquitin chains: polymeric protein signals. Curr Opin Chem Biol *8*, 610-616.
- Potts, P.R. (2009). The Yin and Yang of the MMS21-SMC5/6 SUMO ligase complex in homologous recombination. DNA repair *8*, 499-506.
- Potts, P.R., and Yu, H. (2005). Human MMS21/NSE2 is a SUMO ligase required for DNA repair. Mol Cell Biol *25*, 7021-7032.
- Rabellino, A., Carter, B., Konstantinidou, G., Wu, S.Y., Rimessi, A., Byers, L.A., Heymach, J.V., Girard, L., Chiang, C.M., Teruya-Feldstein, J., *et al*. (2012). The SUMO E3-ligase PIAS1 regulates the tumor suppressor PML and its oncogenic counterpart PML-RARA. Cancer Res *72*, 2275-2284.

- Randow, F., and Lehner, P.J. (2009). Viral avoidance and exploitation of the ubiquitin system. Nat Cell Biol *11*, 527-534.
- Regad, T., Bellodi, C., Nicotera, P., and Salomoni, P. (2009). The tumor suppressor Pml regulates cell fate in the developing neocortex. Nat Neurosci *12*, 132-140.
- Rotin, D., and Kumar, S. (2009). Physiological functions of the HECT family of ubiquitin ligases. Nat Rev Mol Cell Biol *10*, 398-409.
- Rytinki, M.M., Kaikkonen, S., Pehkonen, P., Jaaskelainen, T., and Palvimo, J.J. (2009). PIAS proteins: pleiotropic interactors associated with SUMO. Cell Mol Life Sci *66*, 3029-3041.
- Sachdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F., and Grosschedl, R. (2001). PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. Genes Dev *15*, 3088-3103.
- Saitoh, H., and Hinchey, J. (2000). Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. J Biol Chem *275*, 6252-6258.
- Schmidt, D., and Muller, S. (2002). Members of the PIAS family act as SUMO ligases for c-Jun and p53 and repress p53 activity. Proc Natl Acad Sci USA *99*, 2872-2877.
- Shuai, K. (2006). Regulation of cytokine signaling pathways by PIAS proteins. Cell Res *16*, 196-202.
- Shuai, K., and Liu, B. (2005). Regulation of gene-activation pathways by PIAS proteins in the immune system. Nat Rev Immunol *5*, 593-605.
- Sudharsan, R., and Azuma, Y. (2012). The SUMO ligase PIAS1 regulates UV-induced apoptosis by recruiting Daxx to SUMOylated foci. J Cell Sci *125*, 5819-5829.
- Takazaki, R., Nishimura, I., and Yoshikawa, K. (2002). Necdin is required for terminal differentiation and survival of primary dorsal root ganglion neurons. Exp Cell Res *277*, 220-232.
- Talis, A.L., Huibregtse, J.M., and Howley, P.M. (1998). The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells. J Biol Chem *273*, 6439-6445.
- Taniura, H., Kobayashi, M., and Yoshikawa, K. (2005). Functional domains of necdin for protein-protein interaction, nuclear matrix targeting, and cell growth suppression. J Cell Biochem *94*, 804-815.
- Taniura, H., Matsumoto, K., and Yoshikawa, K. (1999). Physical and functional interactions of neuronal growth suppressor necdin with p53. J Biol Chem *274*, 16242-16248.
- Taniura, H., Taniguchi, N., Hara, M., and Yoshikawa, K. (1998). Necdin, a postmitotic neuron-specific growth suppressor, interacts with viral transforming proteins and cellular transcription factor E2F1. J Biol Chem *273*, 720-728.
- Taniura, H., and Yoshikawa, K. (2002). Necdin interacts with the ribonucleoprotein hnRNP U in the nuclear matrix. J Cell Biochem *84*, 545-555.
- Tatham, M.H., Jaffray, E., Vaughan, O.A., Desterro, J.M., Botting, C.H., Naismith, J.H., and Hay, R.T. (2001). Polymeric chains of SUMO-2 and SUMO-3 are

conjugated to protein substrates by SAE1/SAE2 and Ubc9. J Biol Chem *276*, 35368-35374.

- Taylor, E.M., Copsey, A.C., Hudson, J.J., Vidot, S., and Lehmann, A.R. (2008). Identification of the proteins, including MAGEG1, that make up the human SMC5-6 protein complex. Mol Cell Biol *28*, 1197-1206.
- Tcherpakov, M., Bronfman, F.C., Conticello, S.G., Vaskovsky, A., Levy, Z., Niinobe, M., Yoshikawa, K., Arenas, E., and Fainzilber, M. (2002). The p75 neurotrophin receptor interacts with multiple MAGE proteins. J Biol Chem *277*, 49101-49104.
- Tenno, T., Fujiwara, K., Tochio, H., Iwai, K., Morita, E.H., Hayashi, H., Murata, S., Hiroaki, H., Sato, M., Tanaka, K., et al. (2004). Structural basis for distinct roles of Lys63- and Lys48-linked polyubiquitin chains. Genes Cells *9*, 865-875.
- Uetsuki, T., Takagi, K., Sugiura, H., and Yoshikawa, K. (1996). Structure and expression of the mouse necdin gene. Identification of a postmitotic neuron-restrictive core promoter. J Biol Chem *271*, 918-924.
- van der Veen, A.G., and Ploegh, H.L. (2012). Ubiquitin-like proteins. Annu Rev Biochem *81*, 323-357.
- Varadan, R., Assfalg, M., Haririnia, A., Raasi, S., Pickart, C., and Fushman, D. (2004). Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling. J Biol Chem *279*, 7055-7063.
- Yang, B., O'Herrin, S.M., Wu, J., Reagan-Shaw, S., Ma, Y., Bhat, K.M., Gravekamp, C., Setaluri, V., Peters, N., Hoffmann, F.M., *et al*. (2007). MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. Cancer Res *67*, 9954-9962.
- Yang, N., Zhao, B., Rasul, A., Qin, H., Li, J., and Li, X. (2013). PIAS1-modulated Smad2/4 complex activation is involved in zinc-induced cancer cell apoptosis. Cell Death Dis *4*, e811.
- Zhao, X., and Blobel, G. (2005). A SUMO ligase is part of a nuclear multiprotein complex that affects DNA repair and chromosomal organization. Proc Natl Acad Sci USA *102*, 4777-4782.
- Zheng, N., Wang, P., Jeffrey, P.D., and Pavletich, N.P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell 102, 533-539.
- Zhou, S., Si, J., Liu, T., and DeWille, J.W. (2008). PIASy represses CCAAT/enhancer-binding protein delta (C/EBPdelta) transcriptional activity by sequestering C/EBPdelta to the nuclear periphery. J Biol Chem *283*, 20137-20148.

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