SUMOylation of the ING1b tumour suppressor regulates gene transcription.

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Running title: ING1b SUMOylation regulates gene transcription

Abstract

The INhibitor of Growth (ING) proteins are encoded as multiple isoforms in five *ING* genes (*ING1-5*) and act as type II tumor suppressors. They are growth inhibitory when overexpressed and are frequently mislocalized or down-regulated in several forms of cancer. ING1 and ING2 are stoichiometric members of histone deacetylase (HDAC) complexes while ING3-5 are stoichiometric components of different histone acetyl-transferase (HAT) complexes. The INGs target these complexes to histone marks, thus acting as epigenetic regulators. ING proteins affect angiogenesis, apoptosis, DNA repair, metastasis and senescence, but how the proteins themselves are regulated is not yet clear. Here we find a small ubiquitin like modification (SUMOylation) of the ING1b protein and identify lysine 193 (K193) as the preferred ING1b SUMO acceptor site. We also show that PIAS4 is the E3 SUMO ligase responsible for ING1b SUMOylation on K193. Sequence alignment reveals that the SUMO consensus site on ING1b contains a phosphorylation-dependent SUMOylation motif (PDSM) and our data indicate that the SUMOylation on K193 is enhanced by the S199D phosphomimic mutant. Using an ING1b protein mutated at the major SUMOylation site (ING1b E195A), we further demonstrate that ING1b SUMOylation regulates the binding of ING1b to the *ISG15* and *DGCR8* promoters, consequently regulating *ISG15 and DGCR8* transcription. These results suggest a role for ING1b SUMOylation in the regulation of gene transcription.

Summary: Here we show that the ING1b tumor suppressor is SUMOylated on lysine 193 by the PIAS4 E3 SUMO ligase. SUMOylation regulates binding of ING1 to the ISG15 and DGCR8 promoters, implicating SUMOylation of ING1b in transcriptional regulation.Keywords: ING1b, SUMO1, PDSM, ISG15, DGCR8, PIAS4

Introduction

The first member of the Inhibitor of Growth (ING) family of epigenetic regulators, *ING1b*, was isolated using a technique based on subtractive hybridization followed by an *in vivo* screen for genes with characteristics of tumor suppressors (1). Subsequent analyses revealed loss of ING1b expression in 44% of breast cancer tissues and in 10 of 10 breast cancer cell lines examined, further supporting its role as a tumor suppressor (2). Subsequently, four other members of this family, *ING2-5*, were identified by homology search (3-6). Phylogenetic and structural analyses revealed the presence of a highly conserved plant homeodomain (PHD), which binds lysine 4 of histone 3 (H3K4) in a methylation dependent manner with the highest affinity being for H3K4me3 (7,8). ING1b is a stoichiometric component of Sin3a-HDAC1/2 complexs (9) and also binds SIRT1 (10). ING1b recruits SIRT1 and this interaction results in the inhibition of Sin3a-HDAC mediated transctiptional repression (11). ING1b interacts with the Sin3a-HDAC complex through its N-terminus to recruit these complexes onto chromatin to regulate gene transcription. Although ING1b and ING2 function as the targeting modules of the Sin3a-HDAC1/2 complexes, they also play additional roles in the cell through regulating small noncoding RNA expression by regulating RNA processing protein DGCR8 (12) and also interact with the ATP-dependent nucleosome remodeling machinery (9,13).

SUMO belongs to the Ubiquitin like (Ubl) protein family and is conjugated to target proteins on lysine residues. The SUMO protein family is comprised of SUMO1-4, which have molecular weights of ~12KDa. SUMO2, 3 and 4 are almost identical, however, SUMO1 shares only ~50% identity with other SUMO family members. SUMO proteins are translated in a precursor form. SUMO-specific proteases cleave the precursor SUMO protein into a mature form with a di-glycine motif on its C-terminus, which eventually gets conjugated to the lysine residue of target proteins. Mature SUMO is conjugated to target proteins in three steps:

i) Activation where a thioester bond is formed between SUMO and a cysteine residue of Uba2 by a heterodimer containing E1 activation enzymes Aos1 and Uba2; **ii)** Conjugation, where Uba2-SUMO transfers SUMO to Ubc9, the only known SUMO E2 conjugation protein. A thioester bond is formed between the C-terminal GG motif of SUMO and cysteine 93 of Ubc9. Ubc9-SUMO then interacts with and transfers SUMO to protein substrates (14). One consensus site for SUMOylation contains a hydrophobic amino acid (ψ), a lysine for SUMO conjugation (K) and an acidic amino acid (E/D) on its 1st, 2nd or 4th position (ψKXE/D where X is any amino acid); **iii)** Ligation, the final step. Transferring SUMO to target proteins is often stabilized or facilitated by another class of proteins, the SUMO E3 Ligases. Unlike E1 and E2 enzymes, there are many SUMO E3 ligases. SUMOylation can result in disruption of protein-protein interactions, promote protein-protein interactions or result in structural changes. SUMOylation of transcription factors and chromatin remodeling proteins has often been linked to gene repression (15), and in a few instances, to gene activation (16) loss of repression (17).

Although roles for several of the ING proteins have been described in diverse cellular processes (18) few reports exist describing regulation of the INGs by post-translational modifications (PTMs) (19-23). Exceptions to this are for the role of ING1b phosphorylation at two different serine residues; serine 126, which affects protein stability (23) and serine 199, which affects subcellular localization (24). Also, src mediated ING1b phosphorylation affects protein stability and ING1b levels (19), ING2 SUMOylation mediates ING2-Sin3a interaction (21) and ING4 citrullination affects ING4-p53 interactions (22). In this study we find that ING1b is SUMOylated mainly on lysine 193 and that this is catalyzed by the E3 SUMO ligase PIAS4 PIAS4 and E2 SUMO ligase Ubc9 . We also find that ING1b SUMOylation regulates the promoter occupancy and expression of the *ISG15* and *DGCR8* genes.

Materials and Methods

Cell culture and transfection.

Immortalized human osterosarcoma cells (U2OS) and human embroyonic kidney cells (HEK-293) were obtained from the American Type Culture Collection (ATCC). U2OS and HEK-293 cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. PEI (Sigma) and Lipofectamine LTX (Invitrogen) reagents were used to transfect plasmids into HEK293 cells and U2OS cells respectively.

Plasmids.

The ING1b mutants, ING1b K193R, ING1b E195A, ING1b S199D, ING1b S199A were generated with a QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) from pcDNA3.1-ING1b. The primers were: 5'-AGCGCTCCAAGGCCAGGGCGGAGC-3' (sense) and 5'-GCTCCGCCCTGGCCTTGGAGCGCT-3' (antisense) for ING1b K193R; 5'-GGCCAAGGCGGCGCGAGAGGCGT-3' (sense) and 5'-ACGCCTCTCGCGCCGCCTTGGCC-3' (antisense) for ING1b E195A; 5'-GGAGCGAGAGGCGGACCCTGCCGACCTC-3' (sense), 5'-GAGGTCGGCAGGGTCCGCCTCTCGCTCC-3' (antisense) for ING1b S199D and 5'-AGCGAGAGGCGGCCCCTGCCGAC-3' (sense), 5'-GTCGGCAGGGGCCGCCTCTCGCT-3' (antisense) for ING1b S199A. All mutated ING1b constructs were verified by sequencing. HA/SUMO1, HA/UBC9, HA/UBC9CS, FLAG/PIAS1, 2, 3, 4, FLAG/SUMO1, FLAG/ING1b have been described elsewhere (25).

Western-blotting and Immunoprecipitation.

Cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin) or RIPA (20mM Tris-HCl, pH 7.5, 100mM NaCl, 5mM KCl, 1mM EDTA, 0.25%

deoxycholate, 0.25% Nonidet P-40, 0.25% Tween-20) containing EDTA free protease tablets (Roche Diagnostics) and 1mM PMSF was used for protein extraction and immunoprecipitation, respectively. Modified RIPA buffer containing 0.1% SDS and 20mM NEM was used for immunoprecipitation of SUMOylated proteins under denaturing conditions. Antibodies were αING1 (26), αHA (Covance), αFLAG (Sigma), αPIAS4, αSIN3a and αACTIN (SCBT). For affinity purification of HA or FLAG tagged SUMO conjugated proteins, αHA affinity matrix (Roche) and anti-FLAG M2 affinity resin (Sigma) were used. For densitometry analysis of western blot bands, Image J (http://imagej.nih.gov/ij/) software was used and graphs were drawn using Graphpad Prism.

Indirect immunofluorescence.

Transfection of cells was performed with cells plated on glass coverslips. 24 hours after transfection immunofluorescence was performed as reported previously. For immunostaining, an undiluted mixture of ING1 monoclonal antibodies (Cabs) (26) was used as primary antibody and images were visualized using a Leica SP8 immunofloresence microscope.

RNA extraction and real time-PCR analysis.

Total RNA from cells was isolated using RNAeasy kits (Qiagen, USA), and 1 µg of total RNA was transcribed into cDNA using a First-Strand kit (Applied Biosystems, USA). Real-time PCR was carried out with qPCR MasterMix Plus for SYBR Green (Fermentas) using the company's standard manual procedure. The primers used for real-time measurement of PCR were as follows: *GAPDH*, 5'-GTCAGTGGTGGACCTGACCT-3' and 5'-TGAGCTTGACAAAGTGGTCG-3'; *ING1b*, 5'-CAACAACGAGAACCGTGAGA-3' and 5'-GAGACCTGGTTGCACAGACA-3'; *ISG15*, 5'-ACTCATCTTTGCCAGTACAGGAG-3' and 5'-CAGCATCTTCACCGTCAGGTC-3' and *DGCR8* are 5'-TGG-AGT-ATG-CAG-TGC-TCG-ATG-3' and 5'-GGC-TGC-CAA-CAT-ACC-TCG-TA-3'. The expression of each gene was

normalized using *GAPDH* mRNA as an internal control. The relative amounts of each product were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method described in the ABI 7900HT Fast Real-time PCR System (Applied Biosystems, USA). Results represent differences in *ISG15* and *DGCR8* relative to *ING1b* expression.

Chromatin immunoprecipitation.

ChIP was performed using the EpiTect ChIP OneDay Kit (Qiagen, Courtaboeuf, France) following manufacturer's instructions. Briefly, the crosslinking was performed using 1% formaldehyde solution in PBS. Before the immunoprecipitation, 1% of each input fraction was saved and used in blots as a positive control. The supernatant was immunoprecipitated with either anti-ING1 or anti-mouse IgG as a negative control at 4°C for 4 hours. Then, a mixture of protein A/G agarose beads (Santa Cruz Biotechnology) was added and incubated at 4°C for 1 hour. DNA samples were then subjected to Q-PCR, and results were analyzed according to the manufacturer's instructions. The differential occupancy results were calculated by the normalization of the immunoprecipitation differences (ΔΔCt: ΔCt[IP: treated sample]-ΔCt[IP: control sample]). The fold changes in *ISG15* or *DGCR8* promoter occupancy were calculated following the $2^{-\Delta\Delta Ct}$ method. Primer sequences spanning the upstream region of *ISG15* are 5'-AGCATCTCACTGGGGTTTT-3'and 5'-CTGATGAGGGCATAGCATCC-3' and *DGCR8* are 5'-GACTCTCGTCGCTGTCCG-3' and 5'-ACACCTTTCCCGCCTGAAG-3'.

Results

ING1b is modified by SUMO1.

ING1 serves as the targeting component of HDAC complexes and contributes to regulating gene transcription via effects on the histone code. However, the mechanism by which ING1

activation or participation in the complex is regulated is not clear. Regulation of a gene or its product can occur through transcriptional or translational regulation, or by post-translational modifications (PTMs). Indeed, SUMOylation of ING2 was recently reported to increase its occupancy in the Sin3a-HDAC1 complex (21). To test whether ING1b was similarly modified, it was co-expressed with increasing amounts of HA/SUMO1 plasmid in HEK293 cells. As shown in Figure 1A, denaturing immunoprecipitation (IP) with αING1 followed by immunoblotting (IB) with αHA revealed an HA reactive band ~20 kDa higher than unmodified ING1b. To determine if ING1b was also SUMOylated in another cell type, HA/SUMO1 was expressed alone or with ING1b expressing plasmid in U2OS cells. As shown in Figure 1B and supplemental Figure S1A, SUMOylated ING1b migrated at ~55 kDa on 10% SDS-PAGE. This is consistent with reports (27) indicating a shift of 15-20 kDa with the addition of a single SUMO1 moiety and suggests that ING1b is primarily mono-SUMOylated in different cell types. To further investigate if endogenous ING1b was modified by SUMO1, U2OS cell lysates, with or without expression of HA/SUMO1, were subjected to IP to detect endogenous sumoylated ING1b. As shown in Figure 1C, modified endogenous ING1b migrated at ~55 kDa, consistent with the size of mono-SUMOylated ING1b protein. As shown in Figure S1B and S1C, the ING1b antibody recognized both the unmodified and modified forms of ING1b with high specificity. To better understand the mechanism by which ING1b was SUMOylated, lysates from cells overexpressing ING1b, HA/SUMO1 and HA/Ubc9 wild type (HA/Ubc9 WT) were immunoprecipitated with αING1. As shown in Figure 1D and supplemental Figure S2A, Ubc9 enhanced ING1b SUMOylation in a dose-dependent manner. The cysteine residue on the 93rd amino acid position of Ubc9 is known to facilitate SUMO conjugation (28), and thus mutation of this cysteine residue to a serine (Ubc9CS) abrogates its conjugation activity. As shown in Figure 1D, expression of HA/Ubc9CS blocked ING1b SUMOylation, suggesting that

it can act in a dominant negative fashion. In the absence of N-Ethylmaleimide (NEM), a SUMO iso-peptidase inhibitor, the HA-reactive band was lost, confirming that it is indeed a SUMOylated protein. Furthermore, as shown in Figure S2C, we also observed an interaction between flag tagged ING1b and endogenous Ubc9.

SUMOylation is a transient PTM and at steady state, only a very small fraction (1-2%) of proteins are SUMOylated (27). SUMO is cleaved from proteins by SUMO specific iso-peptidases. Of the 6 described major de-SUMOylation enzymes (SENP1,2,3,5,6,7) SENP1 and SENP2 target both SUMO1 and SUMO2, whereas other SENPs prefer SUMO2 and/or SUMO3. SENP1 and SENP2 localize in nuclear pores and are found in the nucleoplasm as nuclear speckles (29). SENP3 and SENP5 localize in the nucleolus and SENP6 and SENP7 primarily localize in the nucleoplasm (29). Given that ING1b is primarily nuclear and is modified by SUMO1, we examined if SENP1 and 2 regulated ING1b SUMOylation. As shown in Figure 1E, overexpression of Flag tagged SENP1 or SENP2 efficiently de-SUMOylated ING1b, further suggesting its role in the ING1b SUMOylation pathway. Together, these data suggest that ING1b is SUMOylated by SUMO1 in an Ubc9-dependent manner and is de-SUMOylated by both SENP1 and SENP2 SUMO-specific iso-peptidases.

ING1b is SUMOylated by the PIAS4 SUMO E3 ligase.

SUMO substrate specificity is regulated by many different SUMO E3 ligases. Although SUMO E3 ligases are dispensable for SUMOylation *in vitro* where the presence of E1 activation enzyme and E2 conjugation enzyme is sufficient for the transfer of SUMO, SUMO E3 ligases fulfill critical roles in many biological pathways (27). The PIAS (Protein Iinhibitor of Activated STAT Protein) family is the most widely characterized group of E3 SUMO ligases, facilitating SUMOylation of a variety of chromatin regulators, transcription factors and tumor

suppressors. These proteins localize primarily to the nucleus and therefore nuclear proteins are believed to be their major substrates. PIAS1 and PIAS4 have been reported to be involved in SUMOylation of proteins involved in the DNA damage response (30). Given that INGs function in response to one or more types of DNA damage (31,32), and in related stress pathways like senescence and apoptosis where PIAS proteins are known to be involved (30,33), we asked if one or more of the PIAS proteins might act as SUMO E3 ligases for ING1b. ING1b was co-expressed with FLAG tagged PIAS1, PIAS2α, PIAS3 and PIAS4, and α-FLAG IPs were performed followed by immunoblotting with αING1. As shown in Figure 2A, only PIAS3 and PIAS4 immunoprecipitated ING1b. Therefore, we next investigated the involvement of PIAS3 and PIAS4 in ING1b SUMOylation. We co-expressed ING1b, HA/SUMO1 and HA/Ubc9 with or without FLAG tagged PIAS3 or PIAS4. HEK293 cell lysates were then subjected to ING1b IP. As shown in Figure 2B, PIAS4 enhanced ING1b SUMOylation. In contrast, PIAS3, which also interacted strongly with ING1b, had a negative effect on ING1b SUMOylation. Consequently, we asked whether PIAS3 acted in a dominant negative manner, competing with PIAS4 and blocking its ability to SUMOylate ING1b. We co-expressed ING1b, HA/SUMO1 and HA/Ubc9 with FLAG/PIAS4, with PIAS3 or with both, in both U20S and in HEK293 cells. As shown in Figure 2C and supplemental Figure S3, PIAS4 enhanced ING1b SUMOylation. However, co-expression of PIAS3 with PIAS4 inhibited PIAS4 mediated ING1b SUMOylation to levels similar to those seen in the absence of exogenous PIAS4. As shown in Figure 2D, a physical interaction occurs between endogenous ING1b and PIAS4, further supporting the idea that PIAS4 functions as a major SUMO1 E3 ligase for ING1b.

Lysine 193 is the major SUMO1 acceptor on ING1b.

SUMO1 is often conjugated to a short consensus sequence consisting of ΨK*XD/*E where Ψ

is a hydrophobic residue and X is any amino acid. The SUMO consensus motifs (ΨK*XD/*E) serve as recognition modules enabling Ubc9 to interact with target proteins. Lysine within this module serves as the SUMO acceptor site and the acidic amino acid residue is important for Ubc9 interaction (14). Bioinformatics analysis using the SUMOplot program (http://www.abgent.com/sumoplot) confirmed the presence of three SUMO consensus motifs within ING1b, however, sequence conservation analysis using the ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) revealed that the motif containing lysine 193 (Figure 3A) was the highest scoring SUMO consensus motif and was highly conserved between human, mouse, rat and other vertebrates (Figure 3B). To test if lysine 193 was the major SUMO acceptor on ING1b, lysine 193 was mutated to arginine, another basic amino acid but one that could not be SUMOlated (ING1b K193R). ING1b and ING1b K193R were co-expressed with HA/SUMO1 in HEK293 cells and were subjected to denaturing ING1 IP. As observed in Figure 3C, SUMO1 was not conjugated onto the ING1b K193R mutant suggesting that lysine 193 was the major ING1b SUMO acceptor site, or perhaps the only one. Lysine 193 on ING1b could be the target of a multitude of different lysine specific PTMs like acetylation, ubiquitination, methylation and other modifications that could interfere with our analysis of SUMOylation. Therefore, we mutated the glutamic acid residue at position 195 to alanine (ING1b E195A), a manipulation that is predicted to only influence SUMOylation (14). This disrupts the sumoylation consensus motif and thus should selectively prevent SUMOylation, but not other modifications on K193. As shown in Figure 3D, consistent with ΨK*XD/*E being the SUMO consensus site and also being important for Ubc9 and target protein interaction, site directed mutagenesis of glutamic acid 195 strongly inhibited, but did not totally abrogate SUMOylation.

ING1b K193 is also a PDSM.

The phosphorylation dependent SUMOylation motif (PDSM) contains a SUMOylation target residue governed by the phosphorylation of a serine residue downstream. The consensus PDSM is of the sequence ΨKXEXXSP where ΨKXD/E is the SUMO consensus motif and the downstream serine is the phospho-acceptor residue (34). ING1b possesses the sequence AK(193)XE(195)REAS(199)P that is very similar to a PDSM and contains the target K193 residue. The ING1b PDSM is well conserved within vertebrates and lies between the NLS and PHD of ING1b as indicated in Figure 4A. The PDSM in ING1b overlaps with its 14-3-3 binding motif and 14-3-3 binding to ING1b is dependent on the phosphorylation status of serine 199 (24). To test whether phosphorylation of ING1b on this site affected its sumoylation, mutagenesis was performed to mutate serine 199 to glutamic acid (ING1 S199D) which should serve as a phosphomimic. As presented in Figure 4B, denaturing αHA IPs of cell lysates expressing ING1b WT or ING1b S199D and HA/SUMO1, showed that the S199D phosphomimic mutant showed a significant increase in ING1b SUMOylation, suggesting that phosphorylation of S199 promotes SUMOylation of K193. However, we saw no difference in ING1b SUMOylation when the serine was mutated to alanine as shown in supplementary figure S4A.

ING1 SUMOylation does not alter its subcellular localization.

SUMOylation has been widely linked to protein relocalization. Attachment of a SUMO moiety can influence translocation or recruitment of proteins to different sub-cellular compartments or to macromolecular protein complexes. Consequently sumoylation could alter the interaction of ING1b with transport machinery proteins such as 14-3-3η (24). To test whether ING1b SUMOylation affected its localization, we transfected cells with ING1b, ING1b K193R or ING1b E195A and performed indirect immunofluorescence. As shown in Figure 5A, ING1b WT and SUMOylation deficient mutants ING1b K193R and ING1b E195A localized similarly

in the nucleus, suggesting that there was no significant role for SUMOylation in subcellular relocalization under unstressed conditions. However, ING1b binds to chromatin and plays a role in apoptosis in response to exogenous stress (7,35,36). To ask if SUMOylation of ING1b affected chromatin binding, we performed experiments with ING1b WT, ING1b K193R and ING1b E195A. We purified a chromatin-enriched fraction (CEF) from cells transfected with ING1b WT or the mutants and probed for chromatin bound ING1b. The E195A or K193R mutants did not appear to bind chromatin markedly differentially compared to ING1b WT as shown in Figure 5B.

SUMOylation of ING1b regulates its recruitment to the ISG15 and DGCR8 promoters.

ING1 affects gene expression by regulating the acetylation status of core histones on the promoters of genes such as *DGCR8*, *ITSN1*, and *mi204a* among others (12,37,38). To test the role of ING1b SUMOylation on its ability to regulate transcription, we performed q-PCR on 12 differents ING1b target genes recently identified in a microarray screen (unpublished data in preparation) and *DGCR8,* a previously identified ING1b target (12). Among the 13 genes examined, we found that ING1b SUMOylation reproducibly affected the expression of only two genes, *ISG15*, which codes for the ubiquitin like protein ISG15 and *DGCR8,* which codes for a protein involved in microRNA processing (39). As shown in Figure 6A, overexpression of wild-type ING1b repressed the expression of *ISG15* & *DGCR8*. Interestingly, SUMOylation-resistant mutant ING1b E195A regulated their transcription differently. ING1b E195A did not repress *ISG15* while it was more effective in *DGCR8* repression. To further understand how ING1b SUMOylation could regulate *ISG15 and DGCR8* transcription, we further analysed ING1b binding on their promoters by chromatin-immunoprecipitations (ChIP). Lysates from cells transfected with either an empty vector, ING1b WT or ING1b E195A were subjected to ChIP. As illustrated in Figure S7, IgG was used as a negative control. Our data showed that

the SUMOylation defective mutant ING1b E195A, bound more avidly compared to the ING1b WT for both of these genes. However, ING1b E195A failed to repress *ISG15* expression, while ING1b E195A repressed *DGCR8* more efficiently. Given that ING1b is a stoichiometric member of Sin3a/HDAC complexes, we tested if SUMOylation influenced its interaction with this complex. As shown in supplemental Figure S5, unlike ING2, ING1b SUMOylation did not appear to affect its interaction with Sin3a. Collectively, our results indicate that SUMOylation of ING1b can have different roles based on which promoter it binds to. Our data are consistent with several reports suggesting a role for SUMOylation in regulating gene expression (15,25,37,40).

Discussion

The roles of the ING protein family in multiple cellular processes are being widely investigated by many groups. However, how ING proteins are themselves regulated has received very little attention. INGs have been classified as type II tumor suppressors because they are often down-regulated or mislocalized, but not frequently mutated in cancers (41). High throughput proteomic analyses have identified several different types of post-translational modifications on all of the INGs (20) and understanding the significance of those modifications could substantially add to our understanding of how members of the ING family affect several biological processes. In this study, we report that SUMOylation of ING1b occurs at K193 and is mediated by the SUMO E2 conjugation enzyme Ubc9 and SUMO E3 ligase PIAS4. Our data also indicate that ING1b is modified by SUMO1. ING1b modified by SUMO1 migrates at 55KDa, which is consistent with other studies reporting an electrophoric shift of ~20 KDa (27) upon conjugation of one SUMO moiety.

ING1b contains at least 6 recognizable domains (8) and although the ING1b SUMO

consensus lysine is the penultimate amino acid within the NLS, its SUMOylation did not significantly affect ING1b subcellular localization. This does not exclude the possibility of SUMO mediated ING1b relocalization under stress conditions. We also identified a novel PDSM within ING1b. The PDSM was first identified as ΨKxExxSP in HSF1, HSF4b, GATA1 and MEF2A and was predicted to occur in many other proteins (34). In this study, we show that the sequence AK(193)AE(195)REAS(199)P in ING1b acts as a PDSM. We previously reported that S199 is phosphorylated and that this affects binding to 14-3-3η, regulating ING1b shuttling from the nucleus to the cytoplasm (24). However, under unstressed conditions ING1b SUMOylation did not modify its subcellular localization. ING1b sumoylation might thus have little effect on its binding to 14-3-3η and consequently no effect on its localization. Interestingly, a PDSM is also conserved in ING2. Serine 201 within the ING2 PDSM is likely to be involved in crosstalk with ING2 SUMOylation at the K197 residue (21) and preliminary experiments suggests that this is also regulated in a similar manner to that of ING1b (data not shown). This may be relevant to the biological functions of ING1b and ING2 since they are both stoichiometric members of HDAC complexes (9).

ING1b interacts with SUMO E2 conjugation enzyme Ubc9 and SUMO E3 ligase PIAS3 & PIAS4. Ubc9 affected ING1b SUMOylation in a dose dependent manner however, among the two interacting PIAS proteins, only PIAS4 mediated ING1b sumoylation. This is of particular interest since a role for PIAS4 was found in the DNA damage response (30). PIAS4 mediates SUMO1 conjugation to a variety of different DNA damage repair proteins unlike PIAS1, which mediates SUMO2/3 conjugation. These observations suggest that PIAS4 may have a selective function during the DNA damage response or other forms of genomic instabilities such as UV-mediated stress response or replication stress, in which ING1b has been implicated by numerous studies. For example, ING1b association with the PCNA

component of DNA repair complexes increases by >10 fold in response to UV-induced DNA damage, and binding occurs through a PCNA-interacting protein (PIP) motif found in ING1b (35). An ability to affect DNA repair in immortalized cells was also seen in an independent study (42). ING1b was also recently reported to affect genomic stability during replication (43) and thus PIAS4 mediated ING1b SUMOylation could be a mechanism for directing its function in response to DNA damage, particularly since PCNA is also known to be SUMOylated at stalled replication forks in response to DNA damage (44).

In this study, we also found that PIAS3 interacts with ING1b. However, PIAS3 overexpression, in contrast to PIAS4 overexpression, decreased ING1b SUMOylation. When PIAS3&4 were co-expressed, PIAS3 inhibited ING1b SUMOylation mediated by PIAS4 suggesting that PIAS3 might act as a dominant negative, perhaps through blocking PIAS4 access to ING1b K193. The PIAS family of SUMO E3 ligases contains a SP-RING finger domain, a zinc finger motif that is closely related to the RING finger of Ubiquitin E3 ligases. PHD finger motifs very closely resemble RING finger motifs and are also characterized by $Cys_3HisCys_4$ which co-ordinate Zn^{2+} and that are required for function (45). KAP1, a well-characterized transcription co-repressor, was reported to act as a SUMO E3 ligase for its own SUMOylation, and this was mediated through its PHD finger (46) suggesting that this could also occur with the ING1b protein. However, experiments with a PHD 7CA mutant where 7 conserved cysteines were mutated to alanine did not have any effect on SUMOylation (data not shown), supporting the idea that ING1b does not auto-SUMOylate and at least one SUMO E3 ligase is required to SUMOylate ING1b. Finally, while the PIAS E3 SUMO ligase family is arguably the best understood, there are many other E3 SUMO ligases known, leaving open the possibility that ING1b is also a substrate of other E3 SUMO ligases.

We also found that ING1b SUMOylation at K193 affects the regulation of *ISG15 and*

DGCR8 transcription. First, we found that ING1b WT repressed *ISG15.* In addition, consistent with a previous study (12), we saw that ING1b WT overexpression mediated *DGCR8* repression. While ING1b E195A SUMOylation-resistant mutant repressed *ISG15* twice as efficiently as WT, ING1b E195A acted differently on *DGCR8* and repressed it more than ING1b WT. Although we see a strong enrichment of ING1b E195A on promoters of both these genes, we do not have a clear indication of how ING1b SUMOylation might affect promoters differently. Although we do not see a difference in their binding to Sin3a/HDAC complexes, we cannot rule out a promoter specific or context dependent interaction of SUMO modified ING1b with mSin3a or other HDAC components such as in case of genomic instabilty, or UV mediated stress response, or programmed cell death where ING1b has been implicated. Our data are consistent with ING1b repressing the expression of significantly more genes that it induces (47) and suggests that SUMOylation may define a subset of genes that are differentially sensitive to regulation by ING1b. Along with the role of SUMOylated ING1b in *DGCR8* repression, we now uncover a new ING1b target gene, *ISG15*. Given that DGCR8 plays a vital role in microRNA biogenesis, ING1b SUMOylation may play an important role in the processing of microRNA and therby regulation of multiple processes. The second gene that was significantly repressed by ING1b SUMOylation was *ISG15.* Interestingly, it is an ubiquitin like modifier protein (48) induced by type 1 interferons (49). *ISG15* is upregulated in a variety of different breast cancer cell lines and primary breast cancer tissues (50). This is particularly interesting because ING1b has been reported to be downregulated in 44% of primary breast cancers and in all the widely used breast cancer cell lines (2). This may underline the role of ING1b in repressing *ISG15* expression and preventing tumorigenesis. It was also reported that ISG15 has antiviral activity towards different types of viruses (51,52). A role for ING1b has not been reported in these cellular

processes but it opens the interesting possibility of the ING epigenetic regulators being involved in remodeling DNA in response to viral infection and the subsequent transient and longer-term immune responses that are elicited.

Supplementary material

Supplementary figures S1-7 along with their material & methods and figure legends can be found at http://carcin.oxfordjournals.org/

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Conflicts of interest

The authors declare no conflicts of interest.

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Figure legends

Figure 1. **ING1b is SUMOylated. A.** ING1b expression construct was co-transfected with increasing concentrations of HA/SUMO1 expression construct in HEK293 cells. Cells were lysed in the presence of NEM and lysates were subjected to denaturing αING1 IP and IB with SUMO1 (αHA) and ING1 (Cab1 and Cab5) to detect SUMOylated and unSUMOylated ING1b. **B.** U20S cell lysates expressing HA/SUMO1 with or without ING1b were subjected to αHA IP and IB with HA and ING1 antibodies. **C.** U2OS cell lysates with or without HA/SUMO1 was subjected to anti-HA purification using HA affinity matrix under denaturing conditions. 2X SDS-Laemmli sample buffer was used to elute the SUMOylated proteins and eluent was subjected to SDS-PAGE electrophoresis. αING1 IBs were performed to detect endogenous SUMOylated ING1b protein followed by αHA to detect purified SUMOylated proteins. αING1 & αHA (SUMO1) and α-actin IB were performed to confirm protein expression and equal loading respectively. Black arrows depicting SM (sumoylated) and UM (unmodified) denotes SUMO modified ING1b and unmodified ING1b respectively. Major SUMOylated endogenous protein species of ~68 and ~85 KDa were visualized in the input lysate while a protein of ~85 KDa was recovered from the HA affinity matrix. The band at ~20 KDa could be free SUMO1.**D.** HEK293 cells expressing HA/Ubc9 WT or HA/Ubc9 CS were immunoprecipitated with αING1 followed by IB with αHA and αING1 to detect SUMOylated ING1b, HA/SUMO1, HA/Ubc9 and modified and unmodified ING1 respectively. The associated graph indicates the relative density of SUMOylated ING1b in cells expressing Ubc9 WT or Ubc9 CS mutant. **E.** HEK293 cells expressing ING1b and HA/SUMO1 were co-expressed with FLAG tagged SENP1 or SENP2 and levels of SUMOylated ING1b were assessed by denaturing immunoprecipitation of ING1b and immunoblotting against αHA-SUMO. Expression of the different transfected constructs was checked with αFLAG (SENP1&2), αHA (SUMO1) and

αING1.

Figure 2. **PIAS4 promotes ING1b SUMOylation. A.** HEK293 cell lysates co-expressing FLAG/PIAS1, 2, 3& 4 and ING1b were co-immunoprecipitated with αFLAG and subjected to ING1 IB. Cell lysates were immunoblotted with αING1, αFLAG (PIAS1-4) and α-actin to confirm equal loading. **B.** FLAG/PIAS3&4, ING1b, HA/Ubc9 and HA/SUMO1 were co-expressed in HEK293 and cell lysates were subjected to denaturing immunoprecipitation with αING1 and immuno-blotted with αHA (SUMO1 & Ubc9). The associated graph indicates the relative density of SUMOylated ING1b in cells expressing FLAG/PIAS3 or FLAG/PIAS4 **C.** U2OS cell lysates co-expressing ING1b, HA/Ubc9, HA/SUMO1 with PIAS4 and PIAS3&4 were directly subjected to SDS-PAGE electrophoresis and αING1 to detect modified and unmodified ING1b. SUMO1 modified ING1b is marked by the arrow. ++ represents a higher exposure of the panel marked +. The associated graph indicates the density of SUMOylated ING1b in cells expression FLAG/PIAS4 alone or PIAS3 and FLAG/PIAS4 together with ING1b, HA/SUMO1 and HA/Ubc9 **D.** U2OS cell lysates were subjected to IgG or αPIAS4 IP followed by αING1 IB. Cell lysates were probed for αING1, αPIAS4 and α-actin to confirm protein expression and equal protein loading.

Figure 3. **Identification of ING1b SUMO acceptor sites**. **A.** SUMOplot software (Abgent) predicts the presence of three consensuses SUMO sites from the primary protein sequence of ING1b and a score is assigned based on the hydrophobicity of the first residue and the presence of an acidic amino acid on the fourth position of the consensus motif. **B.** Sequence analysis revealed that the site with the highest probability containing lysine 193 is completely conserved in other vertebrates. **C.** αING1b denaturing immunoprecipitation was performed with HEK293 cell lysates expressing ING1b wild type (WT) and the ING1 K193R (K193R) point mutant with and without HA/SUMO1. Expression of the transfected constructs and

equal loading were confirmed using αING1 & αHA (SUMO1) and α-actin, respectively. **D.** A reciprocal αHA immunoprecipitation was performed with U2OS cell lysates expressing ING1b WT, K193R & E195A (SUMOylation specific point mutant) and HA/SUMO1 to confirm the presence of a functional SUMO consensus site (AKAE) containing lysine 193 and glutamic acid 195.

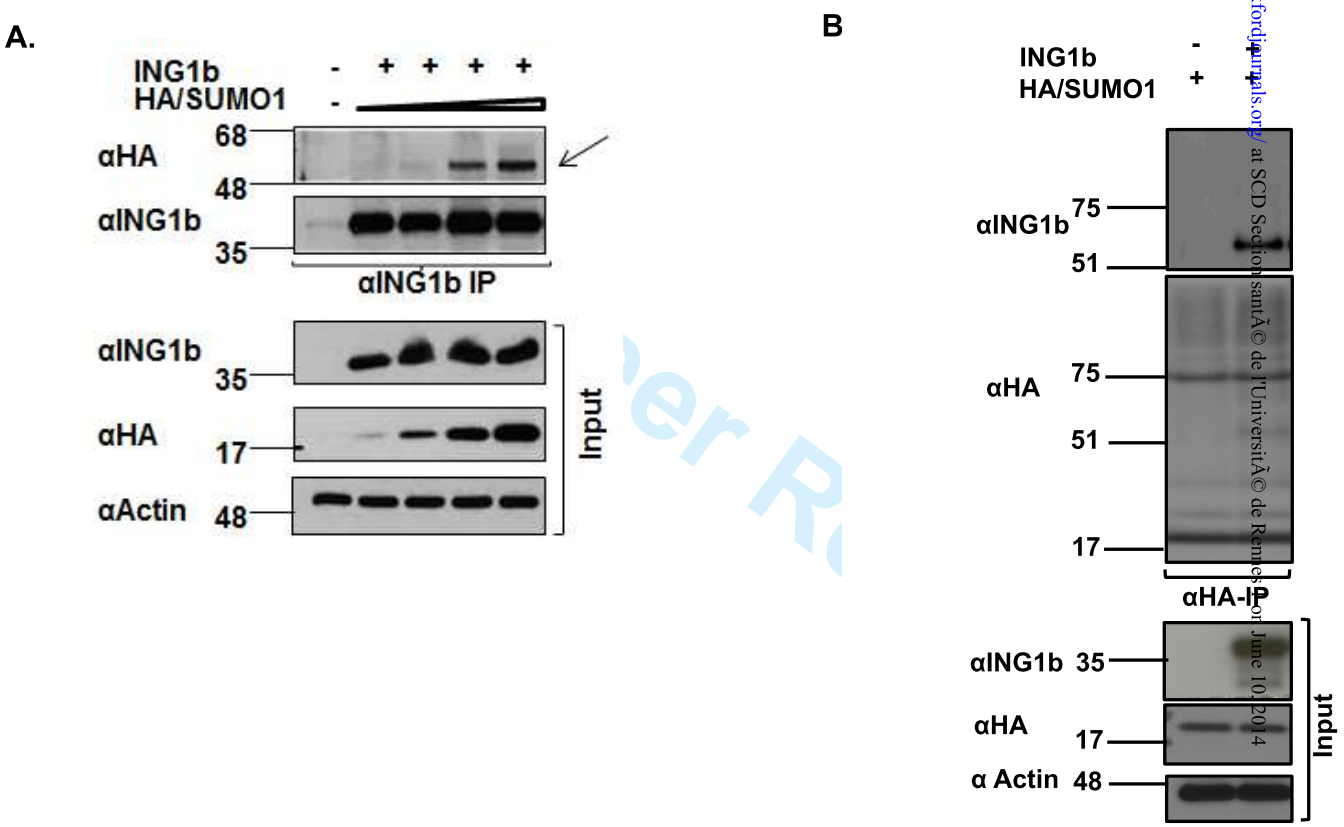
Figure 4. **Identification of a novel PDSM in ING1b. A.** Lysine 193 of the SUMO consensus is the second last amino acid within the NLS, however, the rest of the SUMO consensus and PDSM lies between the NLS and PHD domains of ING1b. **B.** U2OS cell lysates expressing ING1b WT or ING1b S199D mutant and HA/SUMO1 were subjected to αHA IP followed by IB with αING1, αHA (SUMO1) and α-actin for detecting SUMO modified ING1, protein expression and protein loading controls respectively.

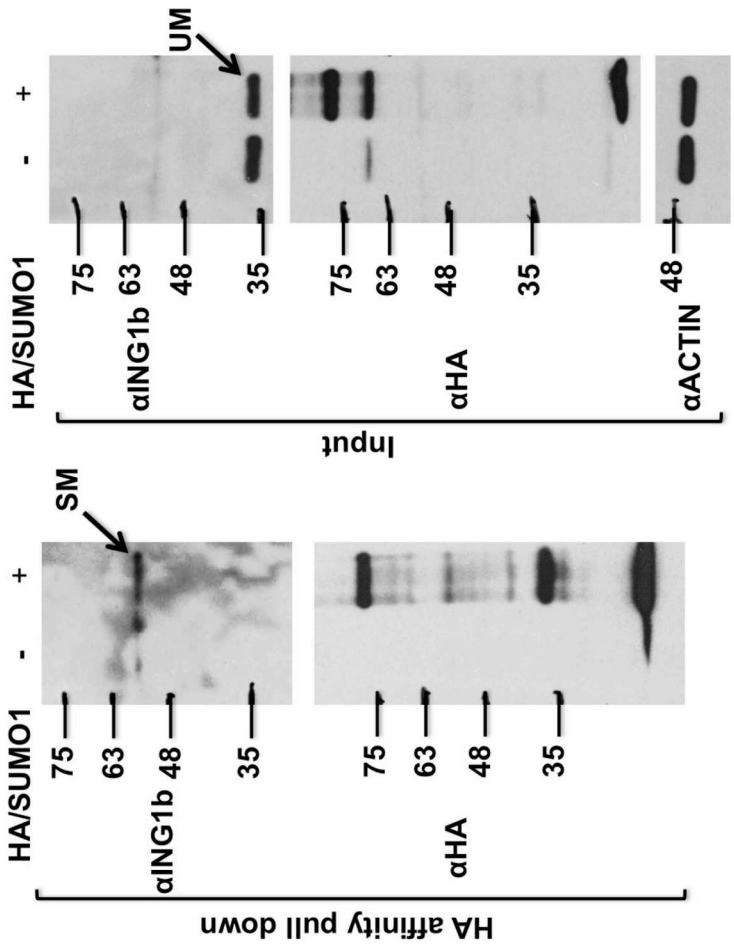
Figure 5. SUMOylation does not alter ING1b localization. A. U2OS cells transfected with control vector or either ING1b or SUMO deficient mutants (K193R or E195A) were stained with αING1 or DAPI to stain ING1b protein and DNA, respectively. Cells expressing ING1b, ING1b K193R and ING1b E195A were imaged with the same exposure time and for cells expressing control vector, exposure time was increased in order to detect endogenous ING1b. **B.** The chromatin enriched-fractions (CEF) or whole cell extracts (WCE) from U2OS cells transfected with empty vector, ING1bWT or SUMO specific mutant (ING1bE195A) were subjected to IB using αING1 to detect chromatin bound ING1b and αH3 for establishing equal loading of CEF. WCE was probed for αING1 and with actin to confirm equal protein expression and loading, respectively.

Figure 6. **ING1b sumoylation affects ING1b binding to, and modulates transcription of *ISG15* and *DGCR8*. A.** *ISG15* and *DGCR8* mRNA expression was quantified by RT-qPCR in cells either expressing empty vector, ING1b WT or ING1b E195A. All the gene expression

levels were normalized to *GAPDH.* Results represent differences in *ISG15* and *DGCR8* relative to *ING1b* expression ± SD. (*p<0.05; **p<0.01; ***p<0.001; NS, not significant). **B.** Panel (i) and (ii) Binding of ING1b on *ISG15* and *DGCR8* promoters was analysed by chromatin immunoprecipitation. Mouse IgG was used as a negative control. Bar graph**s** represent fold changes in ING1b on *ISG15* [panel (i)] and *DGCR8* [panel (ii)] promoter occupancy of cells transfected as in Figure 1A ± SD. (*p<0.05; **p<0.01; ***p<0.001; NS, not significant).

Figure1.





C.

Figure 1.

Figure 1. D.

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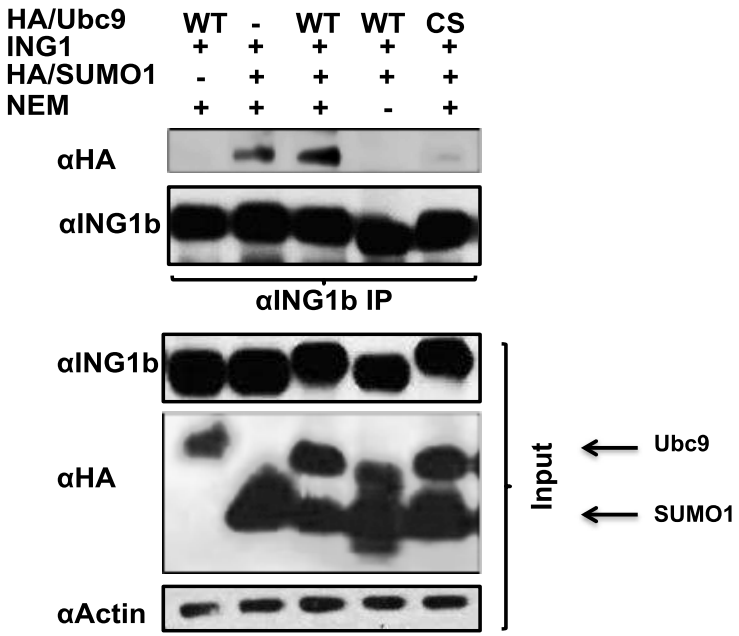

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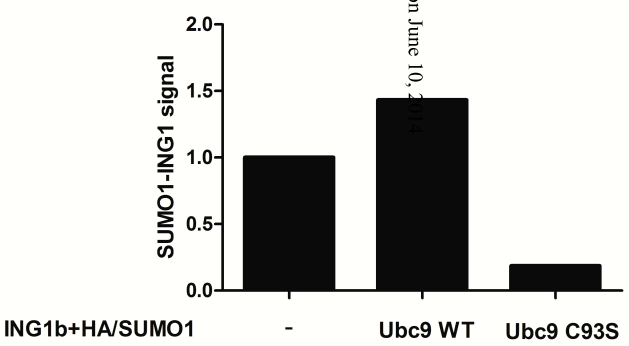

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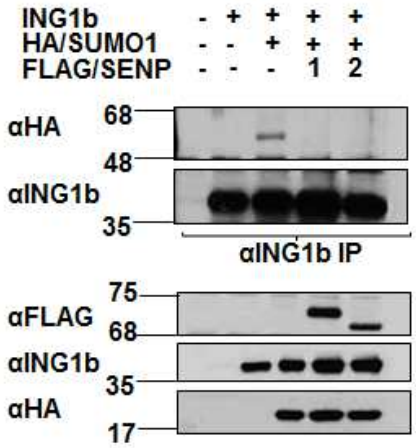
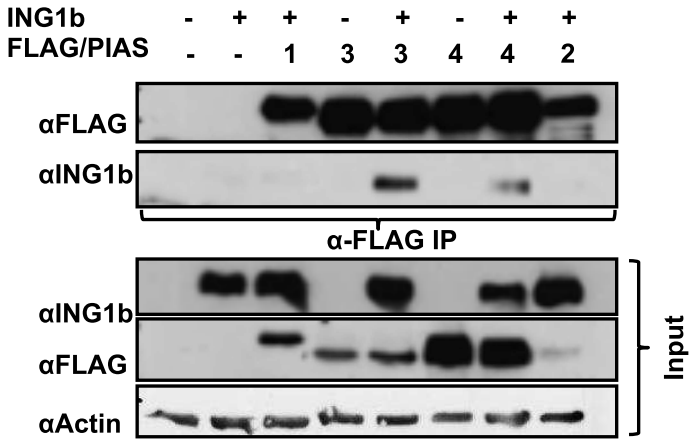


Figure 2.

A.



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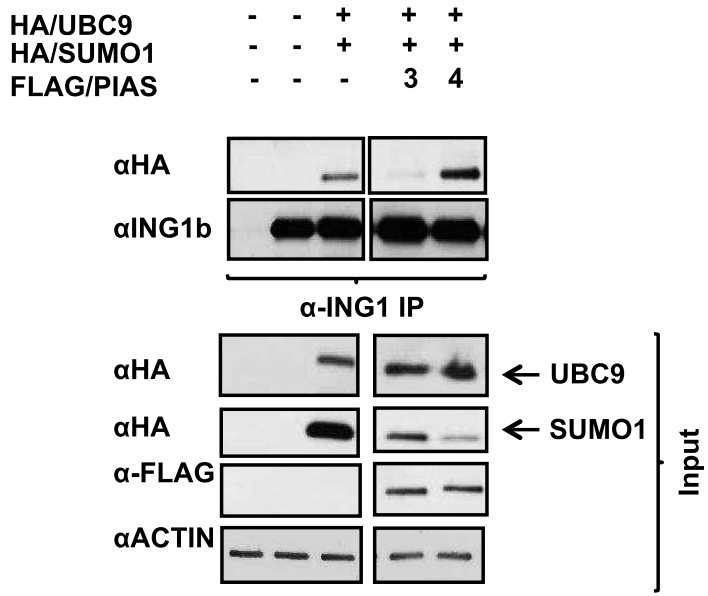

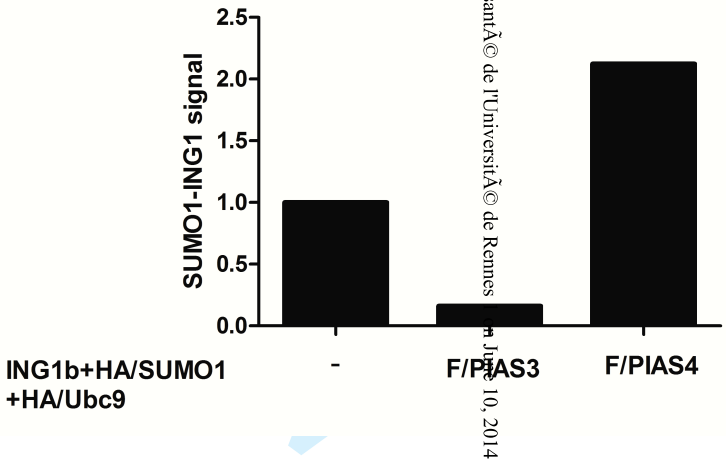

Figure 2.

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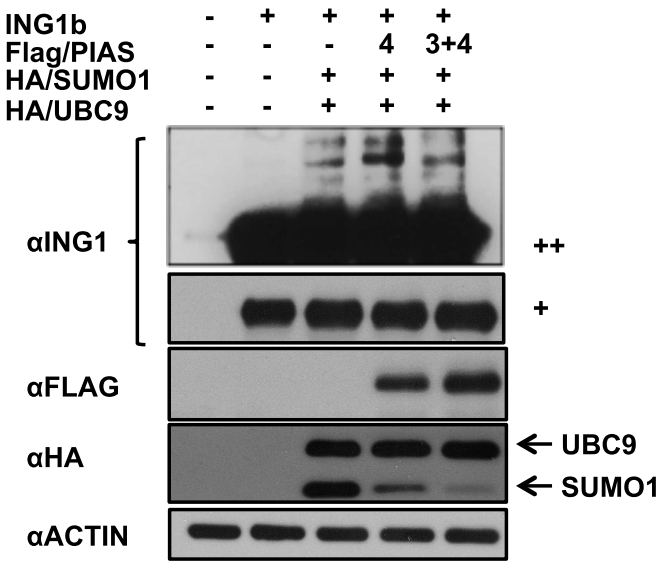

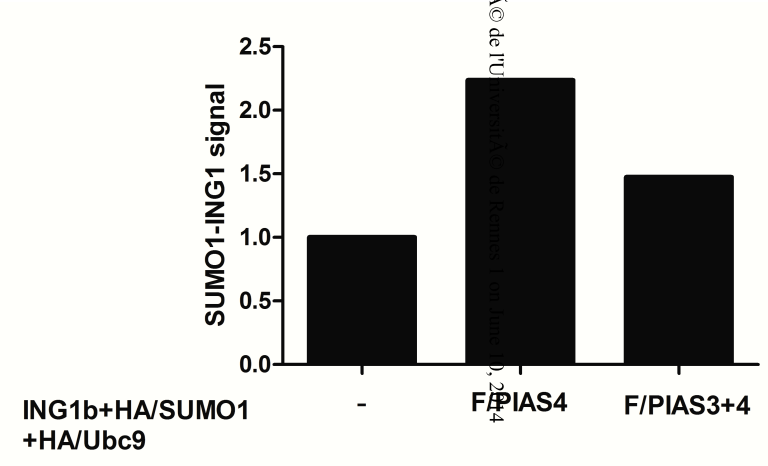

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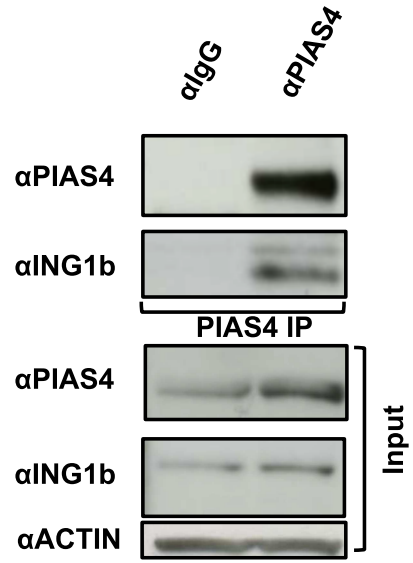


Figure 3.

A.

No.	Position	Group	Score
1.	K193	KKRSK AKAE REASP	0.79
2.	K135	AGADR PKGE AAAQA	0.61
3.	K128	TAGNS GKAG ADRPK	0.50

B.

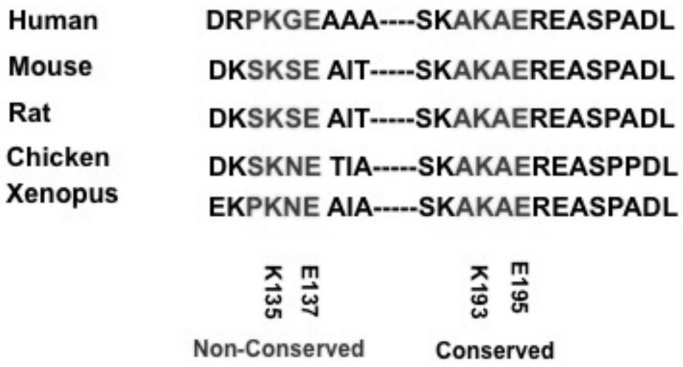
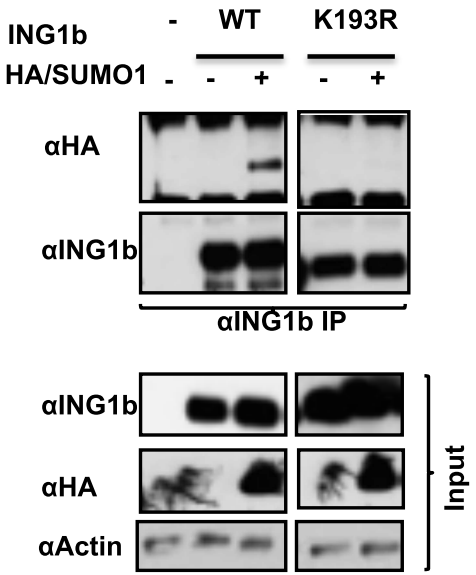


Figure 3.

C.



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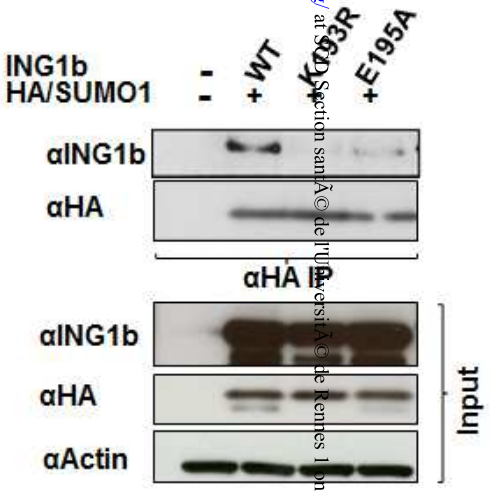
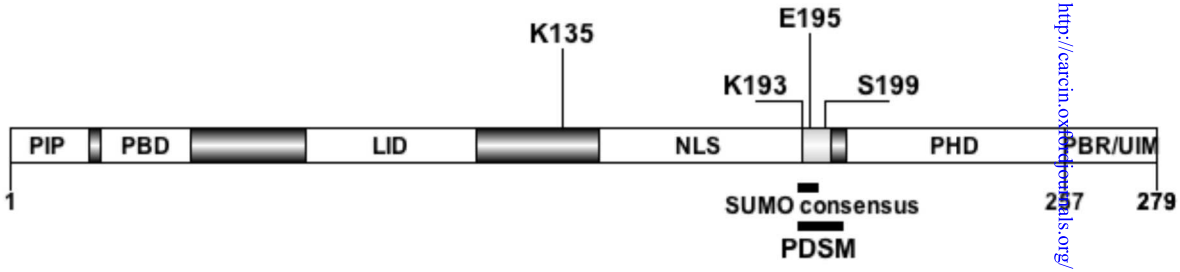


Figure 4

A.



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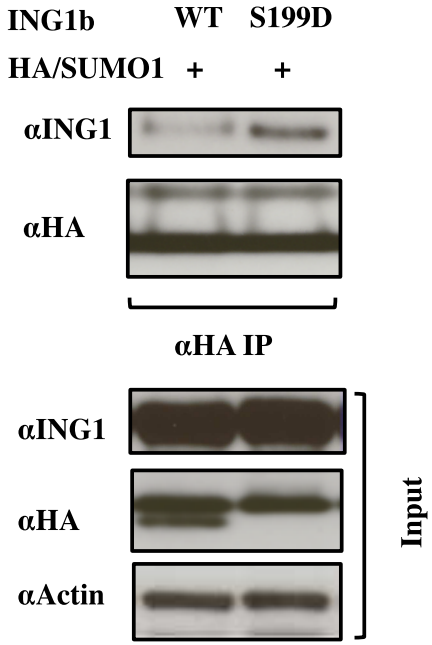
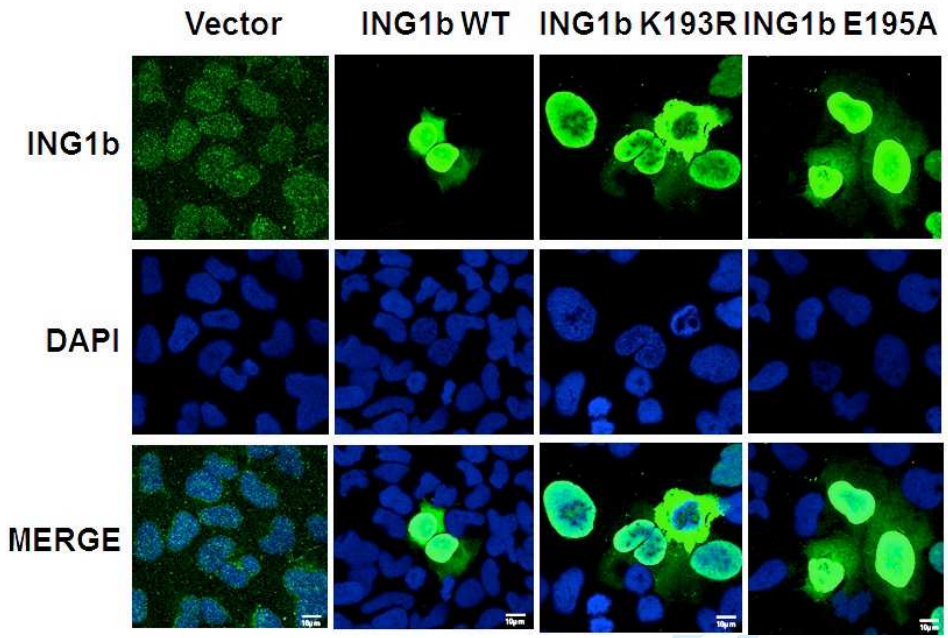


Figure 5.

A.



B.

Figure 6.

A.

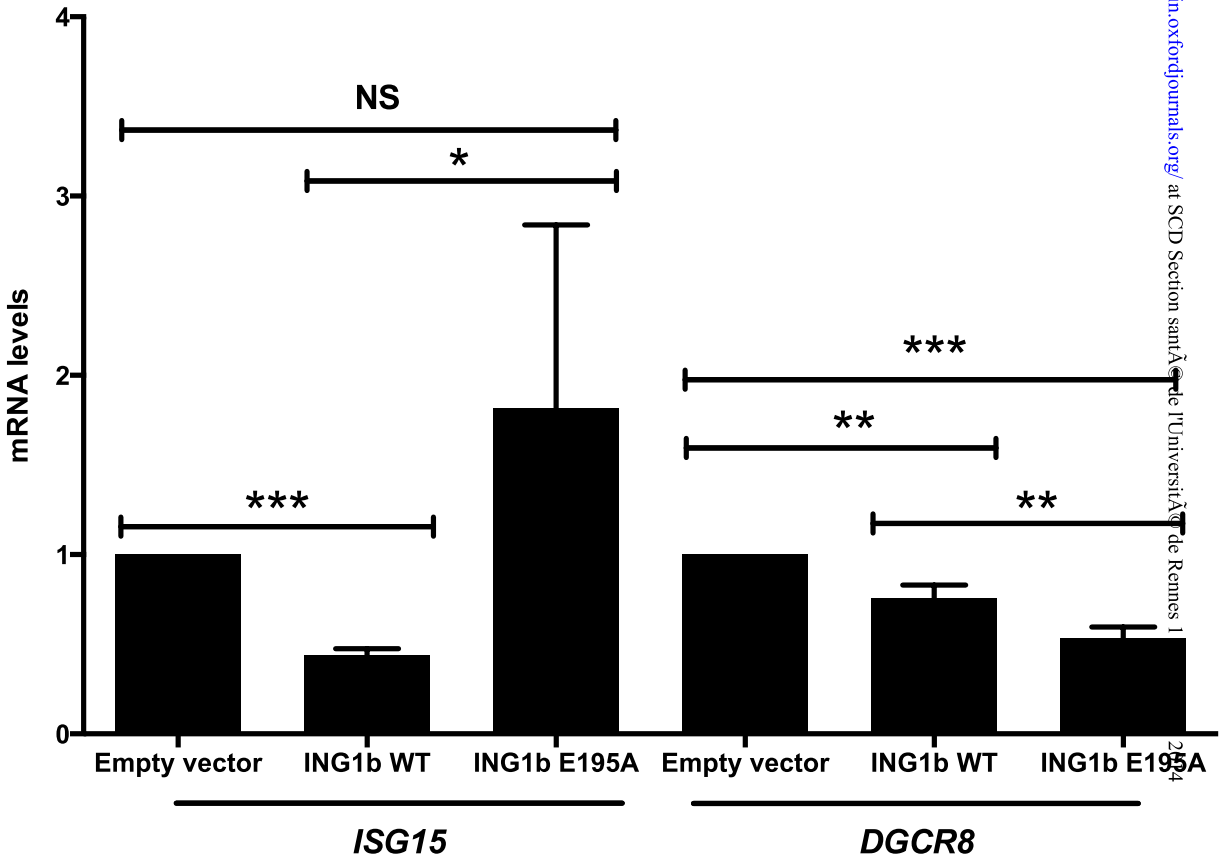
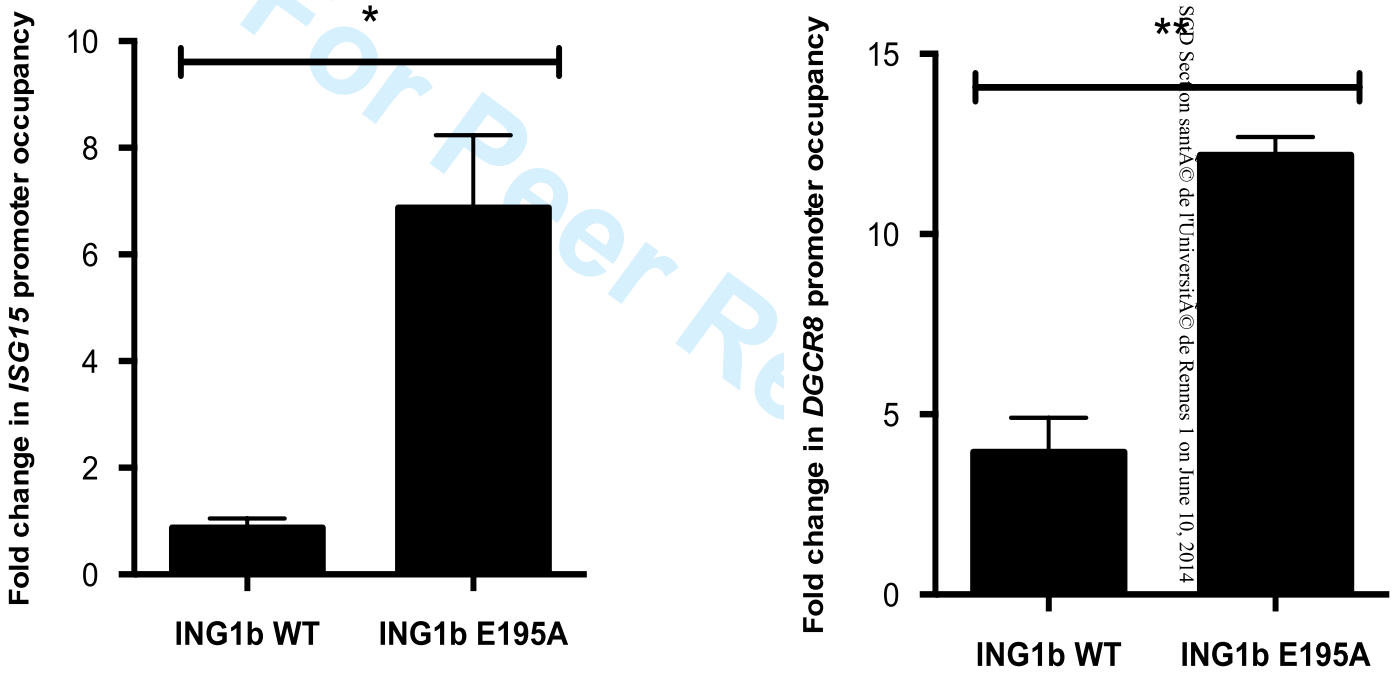


Figure 6

B.



Supplementary:

Material and methods:

Apoptosis and cell cycle analyses.

HEK293 cells were transfected with either empty vector, pcdna/ING1b WT or pcdna/ING1b E195A. After 48 hours apoptosis was analysed on a Gallios cytometer (Beckman Coulter) using PE & active capase-3 apoptosis kit (BD Pharmigen) and cell cycle was analysed using DAPI staining (SIGMA) using Kaluza 1.3 and FlowJo vX softwares.

Figure legends:

Figure S1. A. HEK293 cell lysates co-expressing HA/SUMO and pcdna/ING1b were co-immunoprecipitated with αHA and subjected to ING1b IB. Cell lysates were immunoblotted with αING1b, αHA and α actin to confirm equal loading. **B**. U20S cell lysates expressing either empty vector or ING1b and HA/SUMO1 was subjected to αING1b IP and IB with ING1b and β-actin antibodies. SM : SUMO Modified ING1b ; UM : UnModified ING1b ; Ig : immunoglobulin. **C.** U20S cell lysates transfected with empty vector or pcdna/ING1b were subjected to αING1b IP and IB with ING1b and actin antibodies. ++ or + denotes high or low exposure.

Figure S2 A. HA/Ubc9 dose dependent ING1b sumoylation. U2OS cell lysates coerexpressing Flag/SUMO, HA/Ubc9 and ING1b were subjected to Flag IP followed by αING1b IB. Cell lysates were probed for HA, Flag and β-actin to confirm protein expression and equal protein loading. **B**. U2OS cell lysates overexpressing Flag/ING1b were subjected to Flag IP followed by αUbc9 IB. Lysates were probed for Flag and β-actin to confirm protein expression and equal protein loading.

Figure S3 A. HEK293 cell lysates co-expressing pcdna/ING1b, HA/SUMO, HA/Ubc9, Flag/PIAS3 and Flag/PIAS4 were directly subjected to SDS-PAGE electrophoresis and αING1b to detect modified (SM) and unmodified (UM) ING1b and with αHA, αFlag and α β-actin antibodies. ++ or + denotes high or low exposure.

Figure S4 U2OS cell lysates expressing either empty vector, pcdna/ING1b WT or pcdna/ING1b S199A and Flag/SUMO were subjected to αFlag IP followed by IB with αING1b, αFlag and α β-actin to detect ING1b sumoylation, protein expression and protein loading controls respectively.

Figure S5 U2OS cell lysates expressing either empty vector, ING1b WT or ING1b E195A were subjected to αSin3a IP followed by IB with αING1, αSin3a and α β-actin to detect ING1b interaction with Sin3a, protein expression and protein loading controls respectively.

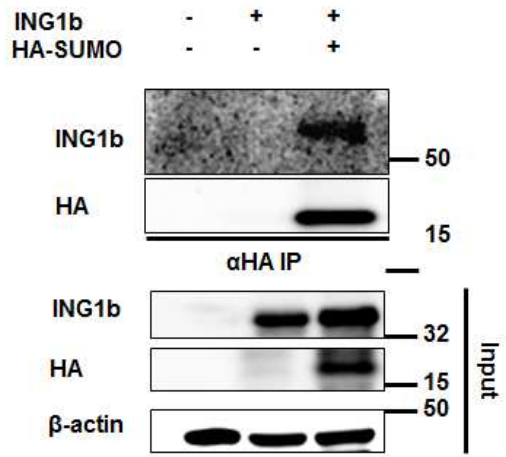
Figure S6 HEK293 cells were transfected with either empty vector, ING1b WT or ING1b E195A and assessed for apoptosis **(A)** and cell cycle **(B)** by flow cytometry 48 hours later. **A.** Apoptosis was evaluated by the use of active caspase-3 staining. Bar graph represents the percentage of positive active caspase 3 cells ± SD. (*P<0.05; **P<0.01; ***P<0.001; NS, not significant). **B.** Cell cycle was analyzed by visualization of DNA content stained with DAPI.

Figure S7 A. Efficiency of immunoprecipitations in ChIP experiments. *ISG*15 **(A)** and *DGCR*8 promoters **(B)** were immunoprecipitated with ING1 or IgG antibodies from U2OS cells expressing empty vector, pcdna/ING1b and pcdna/ING1b E195A ± SD. In **(A)** and **(B)**, ING1b immunoprecipitations were significantly greater than IgG immunoprecipitations following an ING1b WT induced expression. (*p<0.05; **p<0.01; ***p<0.001; NS, not significant).

Supplemental figures with legends:

FigureS1

A.



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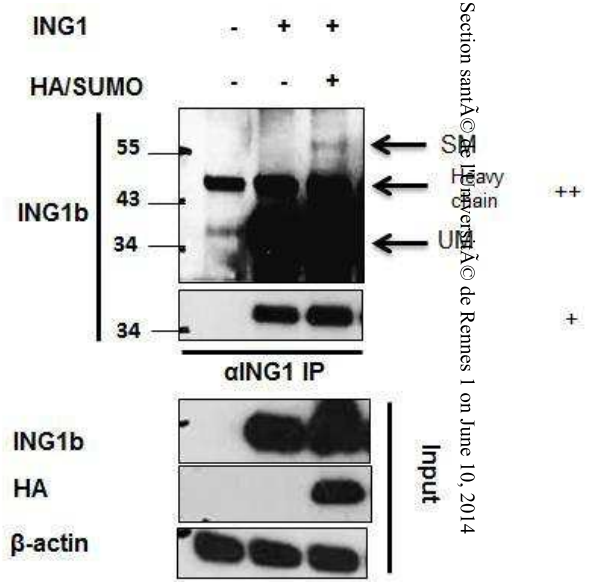

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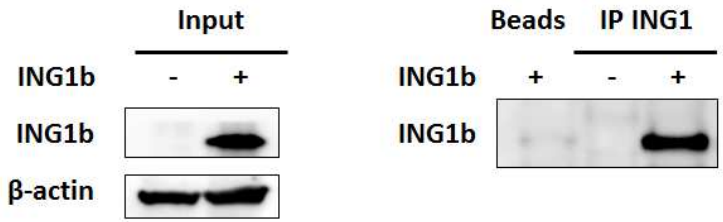
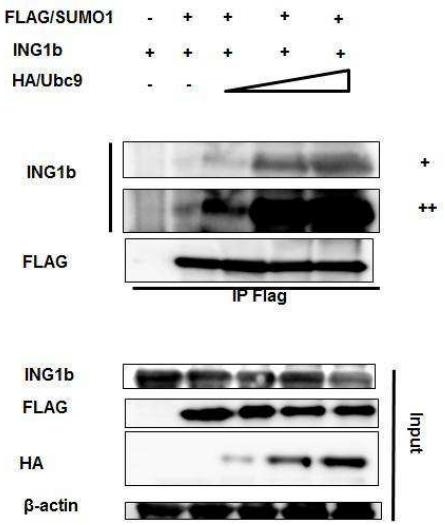


FigureS2

A.



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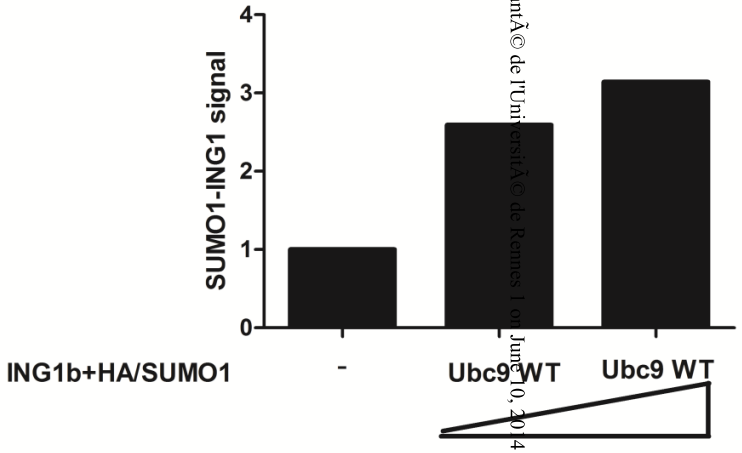

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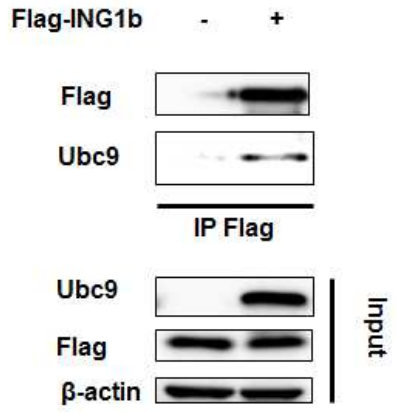

Figure S3

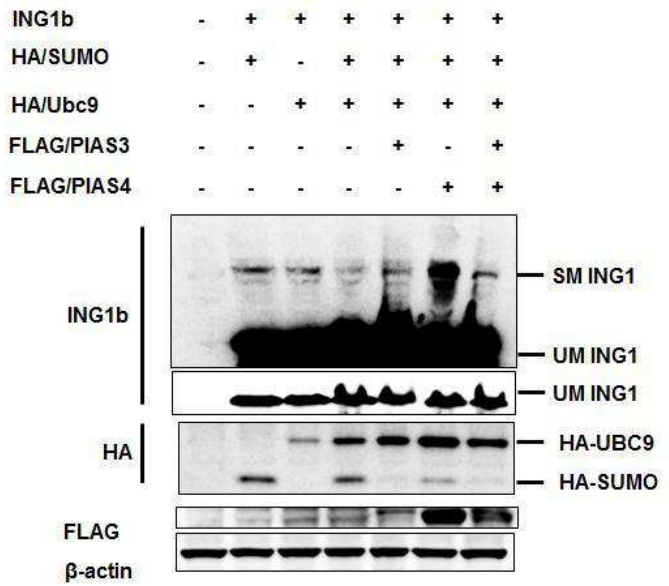

Figure S4

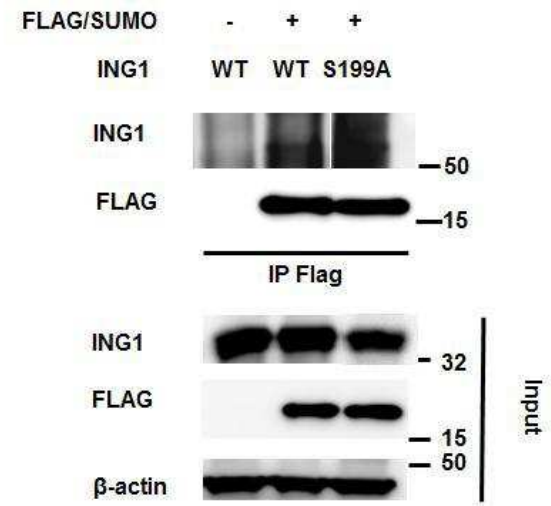

Figure S5

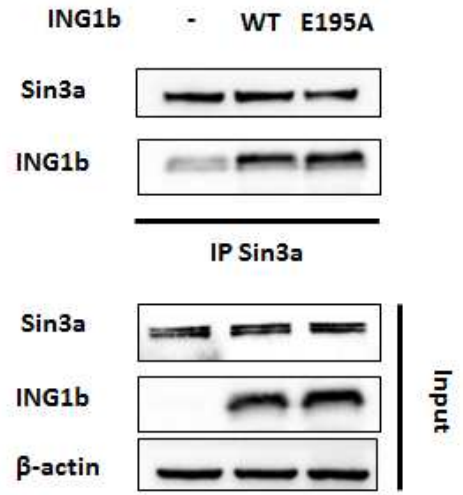

Figure S6

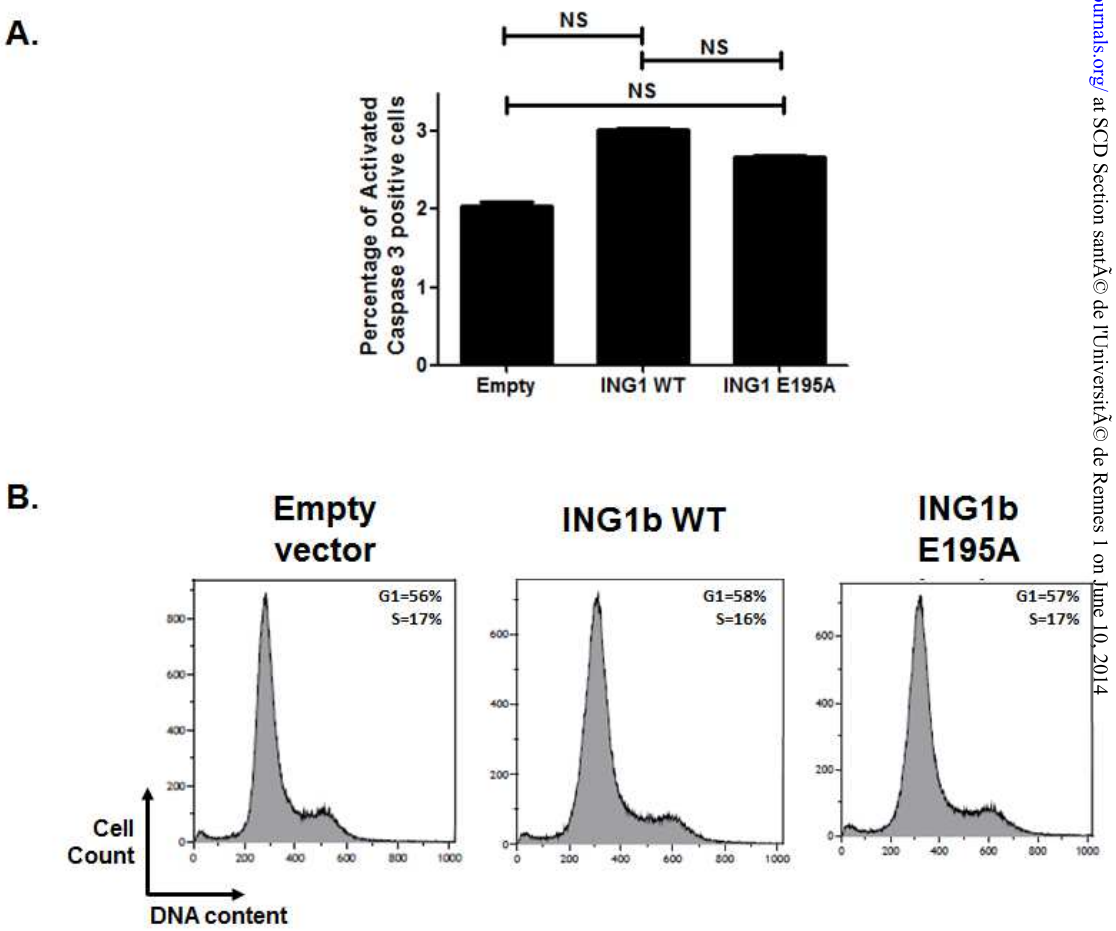

Figure S7