



# Control of nuclear HIPK2 localization and function by a SUMO interaction motif

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## ABSTRACT

The serine/threonine kinase HIPK2 regulates gene expression programs controlling differentiation and cell death. HIPK2 localizes in subnuclear speckles, but the structural components allowing this localization are not understood. A point mutation analysis allowed mapping two nuclear localization signals and a SUMO interaction motif (SIM) that also occurs in HIPK1 and HIPK3. The SIM binds all three major isoforms of SUMO (SUMO-1–3), while only SUMO-1 is capable of covalent conjugation to HIPK2. Deletion or mutation of the SIM prevented SUMO binding and precluded localization of HIPK2 in nuclear speckles, thus causing localization of HIPK2 to the entire cell. Functional inactivation of the SIM prohibited recruitment of HIPK2 to PML nuclear bodies and disrupted colocalization with other proteins such as the polycomb protein Pc2 in nuclear speckles. Interaction of HIPK2 with Pc2 or PML in intact cells was largely dependent on a functional SIM in HIPK2, highlighting the relevance of SUMO/SIM interactions as a molecular glue that serves to enhance protein/protein interaction networks. HIPK2 mutants with an inactive SIM showed changed activities, thus revealing that non-covalent binding of SUMO to the kinase is important for the regulation of its function.

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## 1. Introduction

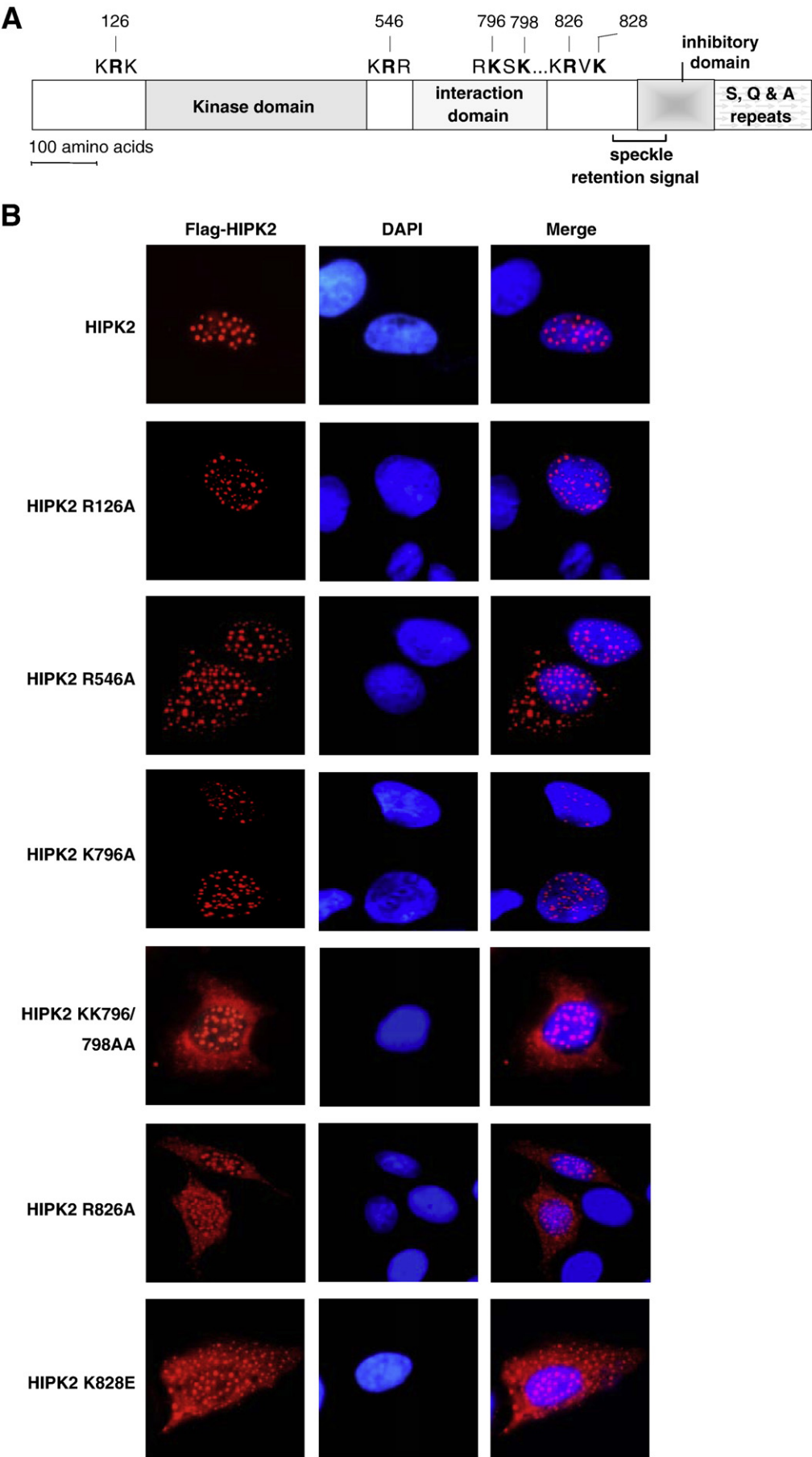
Post-translational modification by attachment of SUMO (small ubiquitin-like modifier) to lysines plays an important role for the regulation of a wide range of cellular functions. The family of human SUMO proteins comprises three well-characterized paralogues, SUMO-1 to SUMO-3, while SUMO-4 has only been proposed based on DNA sequence analysis [1]. Within the group of SUMO proteins, SUMO-2 and SUMO-3 are highly homologous and share 97% sequence identity. The distinct biological functions of SUMO-1 and SUMO-2/3 rely on sequence differences in the N-termini, which allow SUMO-2/3 to generate polymers, while SUMO-1 occurs only in a monomeric form [2,3]. SUMO proteins can be conjugated covalently to target lysines, but they also have the ability to bind to target proteins in a non-covalent manner via so-called SUMO interaction motifs (SIMs). They typically consist of a hydrophobic core, which is often flanked by acidic residues [4,5]. SIMs allow binding to SUMO proteins and thus can act as a docking motif to enhance or specify the interaction with other SUMOylated

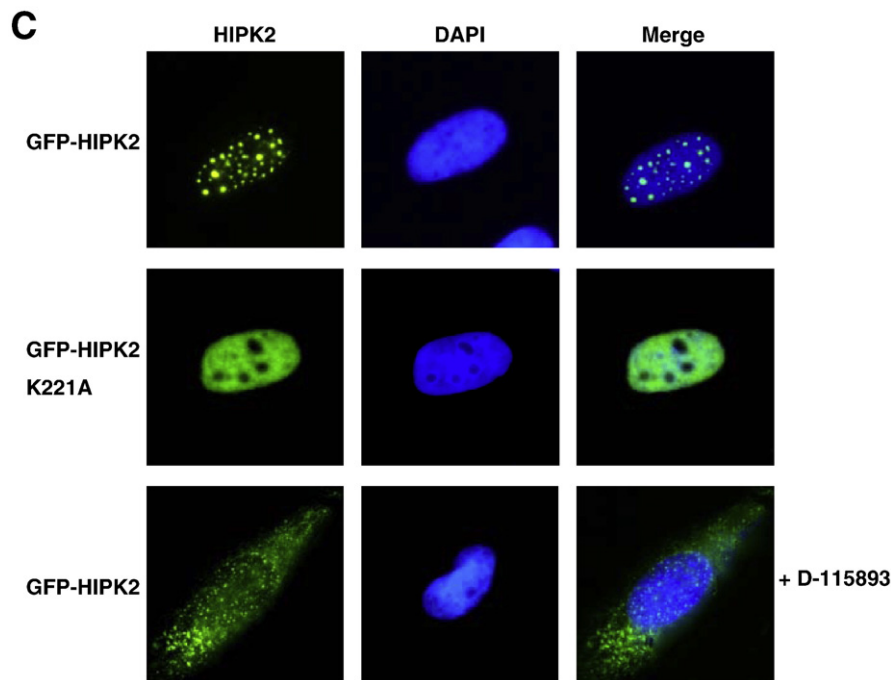
proteins. Intriguingly, mutation of lysines used for covalent attachment of SUMO is scarcely associated with a clear phenotype, while mutation of SIMs often causes drastic changes. This highlights the importance of SUMO/SIM interactions, as illustrated by numerous examples showing their relevance for DNA replication, assembly of nuclear bodies, the replicative stress response, and regulation of thymine DNA glycosylase [1,6].

Among the numerous SUMO substrates is also HIPK2 (homeodomain-interacting protein kinase 2), an evolutionary conserved serine/threonine kinase [7,8]. HIPK2 is activated in response to DNA-damaging or morphogenic signals and accordingly HIPK2-guided gene expression programs trigger differentiation and development or alternatively apoptosis [9]. HIPK2 regulation employs several mechanisms such as control of its stability by the degradative ubiquitination or caspase-mediated cleavage of the autoinhibitory domain, thus generating HIPK2 fragments with higher activity [10,11]. HIPK2 functions as a general regulator of gene expression but also as a kinase that phosphorylates and regulates transcription factors such as p53 and IPF1/PDX (insulin promoter factor-1/pancreatic duodenal homeobox-1) [12–14]. Besides its ability to phosphorylate transcription factors, HIPK2 can also control the abundance of some transcription factors as exemplified by the ability of HIPK2 to repress the expression of hypoxia-inducible factor (HIF) [15]. Under hypoxic conditions, HIPK2-mediated repression of HIF and many of its target genes is relieved by ubiquitin/proteasome-dependent degradation of HIPK2 on a pathway employing the ubiquitin E3 ligase Siah2 (seven-in-absentia) [16]. HIPK2 belongs to a small family of kinases, which includes HIPK1, HIPK2, and HIPK3 and also the more

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**Fig. 1.** Identification of NLS sequences in HIPK2. (A) Schematic representation of HIPK2 domains. The sequence of each putative NLS is given, the mutated amino acids are indicated in bold. (B) U2OS cells were transfected to express Flag-tagged HIPK2 wild-type and point mutated versions thereof as indicated. The intracellular localization of HIPK2 was detected by indirect immunofluorescence, nuclear DNA was stained with DAPI. Representative pictures are displayed. (C) U2OS cells were transfected to express GFP-tagged HIPK2 or a kinase inactive point mutant thereof. Cells expressing wild-type HIPK2 were incubated with the HIPK2 inhibitor D-115893 (10 nM) overnight as shown, and localization of HIPK2 was revealed by the intrinsic fluorescence of GFP. Chromosomal DNA was stained with DAPI.

distantly related protein HIPK4. The C-terminal parts of HIPK1, 2, and 3 show only limited homology, while the kinase domains show >90% identity. This is also reflected by a certain degree of functional redundancy, as mice deficient for the *Hipk1* or *Hipk2* genes are viable whereas double deficient mice die within the first 2 weeks after fertilization [17]. However, most functions are non-redundant, as single HIPK2 knockout mice develop more skin tumors and are subjected to faster disease progression than wild-type mice after two-stage skin carcinogenesis treatment [18].

HIPK2 typically localizes to subnuclear speckles termed HIPK domains or also nuclear deposition sites (NUDES) [19,20]. A minor fraction of the kinase colocalizes with the promyelocytic leukemia (PML) tumor suppressor protein in PML nuclear bodies (PML-NBs). Some cells also show HIPK2 localizing more diffusely in the nucleoplasm and also the cytoplasm [20,21]. HIPK2 is composed of an N-terminal kinase domain, which is preceded by a SUMO attachment site at lysine 25 [22,23]. The SUMO modification of HIPK2 employs the SUMO E3 ligase Pc2 (also known as CBX4), which is a member of the polycomb (PcG) class of proteins [24]. The kinase domain is followed by a region allowing interactions with homeodomain transcription factors and a variety of further proteins. The C-terminal portion of the kinase harbors an autoinhibitory domain between position 935 and 1050, which partially overlaps with a speckle retention signal (SRS, between amino acids 860 and 967) [25]. Previous deletion experiments have shown that HIPK2 variants lacking the SRS fail to localize to HIPK domains, but the molecular mechanisms explaining this effect are not known.

In this study, we have systematically analyzed the sequence elements in HIPK2 mediating its localization (I) in the nucleus and (II) in nuclear bodies. While nuclear localization employs two nuclear localization sequences (NLS), retention in nuclear bodies and recruitment to PML-NBs was critically dependent on a functional SIM contained in the SRS. Functional studies revealed a role of the SIM for protein/protein interactions and HIPK2 activity.

## 2. Materials and methods

### 2.1. Cell culture and transfections

HEK293T cells and U2OS cells were grown in DMEM containing 10% FCS and 1% (w/v) penicillin/streptomycin at 37 °C and 5% CO<sub>2</sub>. The p53-deficient H1299 cells were cultivated in supplemented RPMI medium. Cells were plated out 1 day prior to transfection, which was done using Rotifect (Roth) according to the manufacturer's instructions. DNA amounts in each transfection were kept constant upon addition of empty expression vector.

### 2.2. Antibodies and plasmids

#### 2.2.1. Antibodies, plasmids, and reagents

Antibodies recognizing Flag (M2) and  $\beta$ -tubulin (tub 2.1) (Sigma), anti-HA (3F10) and anti-GFP (7.1 and 13.1) (Roche Applied Science), anti-T7 (Novagen), anti-p53 (DO-1), and anti-PML (PG-M3) (Santa Cruz) were from the indicated companies. The phospho-p53 (ser46) and control IgG antibodies were from Cell Signaling Technology, and anti-phospho-PML [26] and the anti-phospho-Siah2 [16] antibodies have been described. Expression vectors for Flag-HIPK2, Flag-HIPK2 K221A, GFP-HIPK2, p53, GFP-SUMO-1, PML-IV [13], epitope-tagged Pc2 [27], HA-Siah2 [16], IPF1/PDX [14], and GFP-Hp66 $\beta$  [28] were published previously. Also the reporter plasmids p53-Luc [13] and 5x P1-Luc [14] were described. The vectors encoding GFP-SUMO-2 and -3 are kind gifts from Hans Will (Heinrich Pette Institut Hamburg), vectors for GST-SUMO fusion proteins were from Ivan Dikic (Frankfurt), and Flag-SUMO-1-GG/AA was obtained from Stefan Müller (Martinsried). The various point mutants were generated using QuickChange® site-directed Mutagenesis Kit (Stratagene), and deletion mutants were generated by PCR cloning. The authenticity of the constructs was verified by DNA sequencing. Details on the cloning strategies can be obtained from the authors upon request. The HIPK2

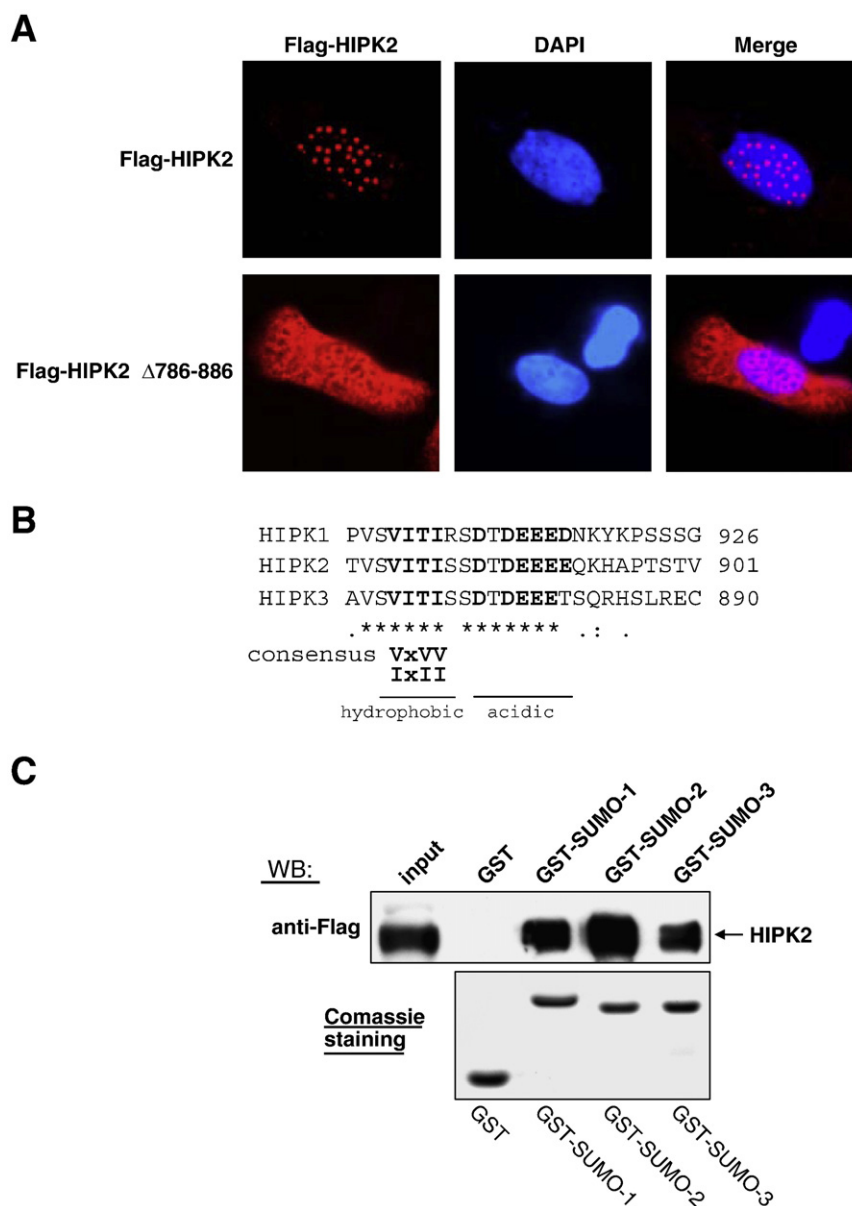
inhibitor D-115893 was a kind gift from Æterna Zentaris (Frankfurt, Germany).

### 2.3. Cell lysis protocols and luciferase reporter assays

Soluble fractions were obtained after lysis of cells in NP-40 buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl-fluoride, 10 mM NaF, 0.5 mM sodium orthovanadate, leupeptine (10 µg/ml), aprotinin (10 µg/ml), 1% (v/v) NP-40, and 10% (v/v) glycerol). After centrifugation of lysates at 4 °C, the supernatant was either mixed with SDS sample buffer and further analyzed by immunoblotting or used for determination of luciferase activity in a Berthold LB 9507 luminometer. Protein SUMOylation was tested after directly lysing an aliquot of the cells in 1× SDS sample buffer and sonification.

### 2.4. Coimmunoprecipitation and Western blotting

For coimmunoprecipitation experiments, cells were washed in phosphate-buffered saline (PBS) and incubated in the presence of 0.5 mM of the membrane-permeable crosslinker dimethyl-3-3'-dithiobispropionimidate 2-HCl (Pierce) as previously described [24]. Cells were lysed in IP buffer (50 mM Hepes pH 7.5, 50 mM NaCl, 1% (v/v) Triton X-100, 2 mM EDTA, 10 mM sodium fluoride, 0.5 mM sodium orthovanadate, leupeptine (10 µg/ml), aprotinin (10 µg/ml), and 1 mM PMSF), followed by a sonication step. Cell debris was removed by centrifugation, and extracts were precleared with A/G sepharose. The supernatants were mixed with 2 µg of precipitating antibodies together with 25 µl of protein A/G sepharose. Tubes were rotated for 2 h on a spinning wheel at 4 °C. The immunoprecipitates were washed 5× with IP buffer and eluted by boiling in 1× SDS sample buffer. Equal amounts of protein were separated by SDS-PAGE, followed by semidry blotting to



**Fig. 2.** Identification of a functional SIM in HIPK2. (A) The indicated HIPK2 constructs were expressed in U2OS cells and analyzed for HIPK2 localization by indirect immunofluorescence. (B) The sequences corresponding to the putative SIM in all HIPK forms and the positions of the amino acids are indicated. Identical amino acids are marked by stars, homologous, and related amino acids are indicated. The lower part shows the hydrophobic SIM consensus sequence, x stands for any amino acid. The hydrophobic core and the block of acidic amino acids are highlighted by writing in bold. (C) HIPK2 binds non-covalently to GST-SUMO-1-3 *in vitro*. Total cell extract from cells transfected to express Flag-HIPK2 was tested for interaction with bacterially produced GST and GST-SUMO-1-3 proteins by pull-down experiments as shown. The upper part shows a Western blot (WB) displaying the input material and the eluates, the lower part shows the Coomassie-stained GST fusion proteins used for this experiment.

a polyvinylidene difluoride membrane (Millipore). After blocking of the membrane with milk powder, primary antibodies were added. Appropriate secondary antibodies coupled to horseradish peroxidase were detected by enhanced chemiluminescence.

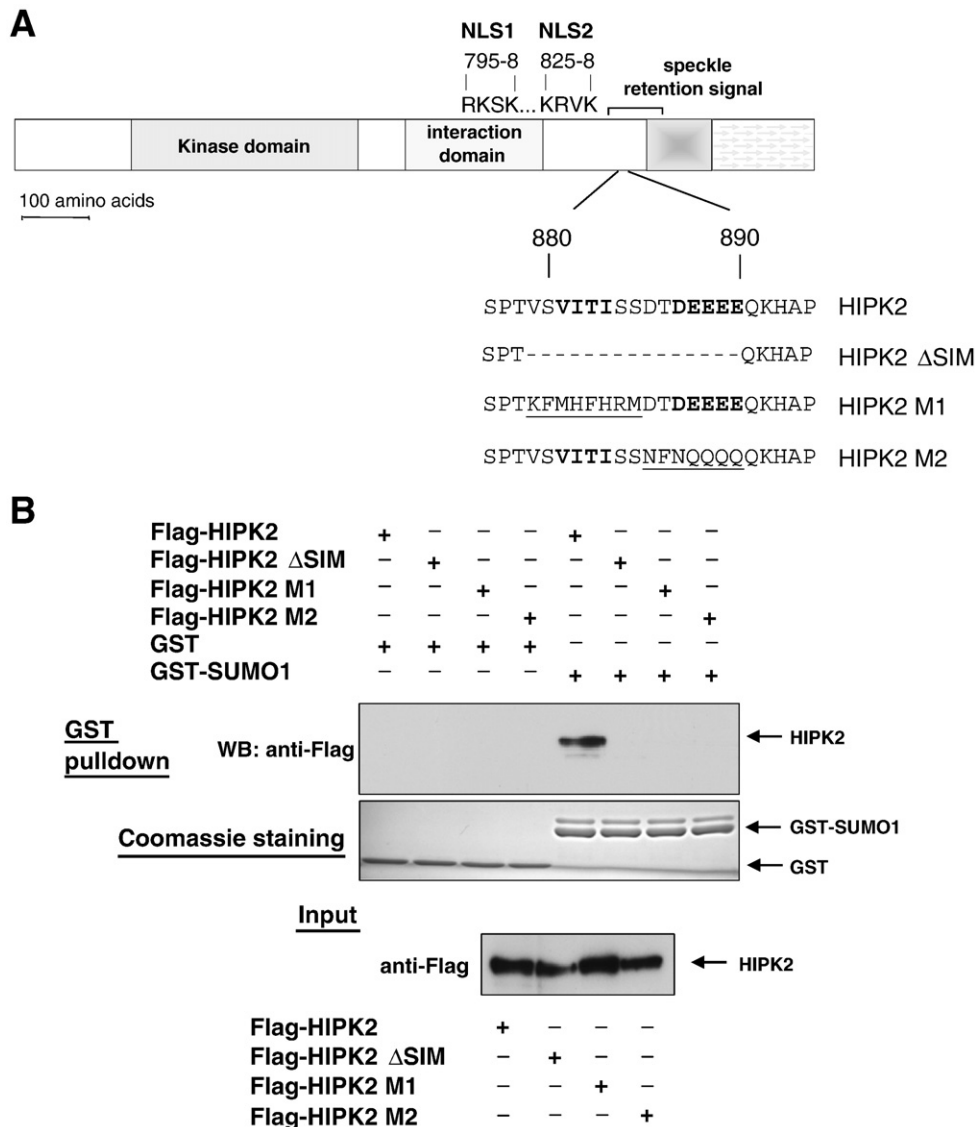
## 2.5. GST pull-down assays

Recombinant GST fusion proteins were produced in *Escherichia coli* BL21 and purified on GSH-coupled beads by standard methods. The eluates were dialyzed against PBS buffer and frozen in aliquots. HIPK2 and its derivatives were produced by expression in transiently transfected HEK293T cells, followed by cell lysis. Ten percent of the lysate was used for the input control, while the remaining extract was divided to be used for incubation with 4 µg of GST or the GST fusion proteins, respectively. Binding occurred at 4 °C for 3 h and GSH-coupled sepharose beads were added for another hour. After extensive washing in PBS, bound proteins were eluted with 1× SDS sample buffer and subsequent boiling for 5 min. Eluates were further analyzed by denaturing SDS-PAGE and

Western blotting, while the recombinant GST proteins were controlled by SDS-PAGE and Coomassie staining.

## 2.6. Immunofluorescence

Cells were grown on coverslips and transfected with indicated vectors. The next day, cells were washed once with PBS and fixed for 5 min at −20 °C in methanol/acetone (1:1). Dried cells were blocked for 30 min at room temperature in phosphate-buffered saline containing 10% (v/v) goat serum. Cells were incubated for 1 h with primary antibodies at room temperature, washed extensively with PBS, and incubated for another 45 min with appropriate fluorochrome-conjugated secondary antibodies. Chromosomal DNA was visualized by DAPI staining. Stained cells were mounted on glass slides and examined using an inverted Nikon Eclipse 2000E microscope. Dying or mitotic cells and also cells expressing aberrantly high levels of the proteins were not analyzed. All immunofluorescence data are representative for >80% of interphase cells.



**Fig. 3.** Characterization of a functional SIM in HIPK2. (A) Schematic display of the wild-type HIPK2 protein and the mutants either lacking a SIM or changed in the underlined amino acids. (B) Lysates of HEK293T cells transfected to express the indicated HIPK2 proteins were tested for binding to GST or GST-SUMO-1 by pull-down experiments. The upper part shows the Western blot revealing the bound HIPK2 protein and the Coomassie-stained input control. The lower part shows the HIPK2 variants as detected by immunoblotting. (C) Localization of the indicated HIPK2 variants was analyzed in U2OS cells by immunofluorescence, nuclear DNA was stained with DAPI.



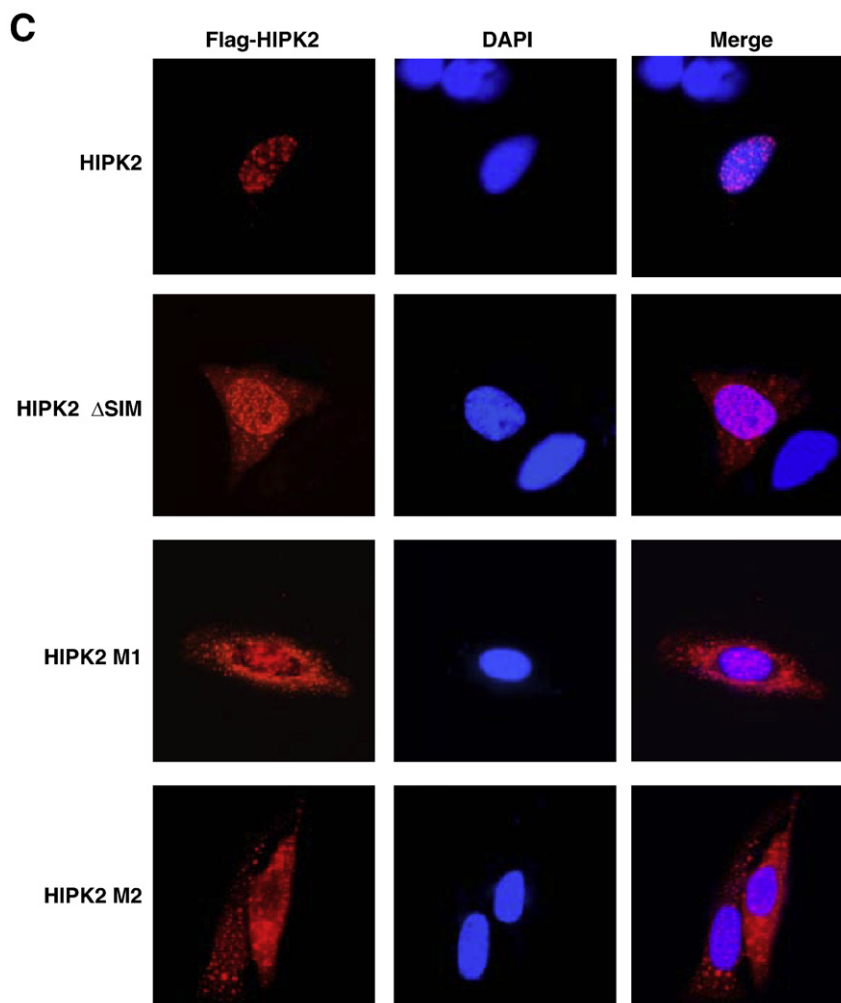


Fig. 3 (continued).

### 3. Results

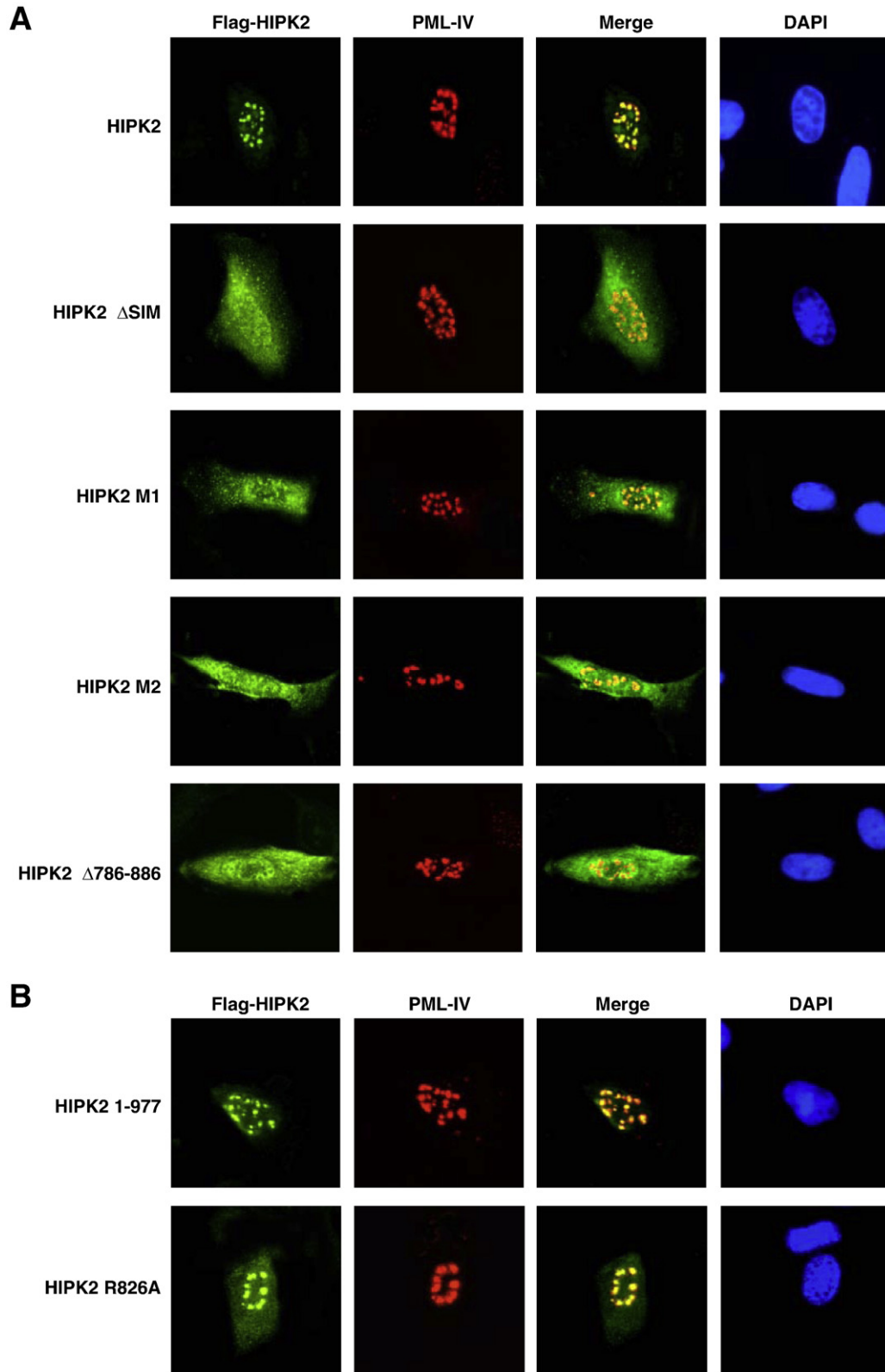
#### 3.1. Mapping of the NLS in HIPK2

All results on the structural requirements dictating intracellular localization of HIPK2 are based on the use of deletion mutants lacking significant parts of the protein [25]. These mutants provide valuable information but, on the other hand, do not allow the identification of the involved short signal sequences and may also affect the overall structure or conformation of the kinase. In order to identify the NLS sequence(s) responsible for the mainly nuclear localization of HIPK2, the amino acid sequence was analyzed for the occurrence of clusters containing basic amino acids typical for NLS sequences [29]. We identified 4 such clusters, as schematically shown in Fig. 1A. Their possible contribution for nuclear localization of HIPK2 was tested upon mutation of basic amino acids contained in these clusters to alanine and analysis of HIPK2 localization by immunofluorescence (Fig. 1B). Mutation of R126 had no influence on HIPK2 localization to nuclear HIPK domains, while mutation of R546 still allowed the occurrence of HIPK2 in the nucleus but slightly increased the percentage of cells with cytosolic HIPK2. Changing lysine 796 alone to alanine caused no alteration of HIPK2 localization, while additional mutation of lysine 798 resulted in the localization of HIPK2 within the entire cell, thus identifying this sequence as an NLS. In addition, mutation of either R826 to alanine or K828 to a negatively charged amino acid resulted in prominent loss of nuclear localization, thus

revealing this region as a second NLS. In summary, these experiments allowed to identify two NLS for HIPK2 consisting of the sequences 795-RKSK-798 (NLS1) and 825-KRVK-828 (NLS2). Early work on HIPK2 showed a mainly nucleoplasmic localization of the HIPK2 kinase inactive point mutant HIPK2 K221A [8]. Also other HIPK2 mutants with absent kinase activity localize to the nucleoplasm (Supplementary Fig. 1), thus revealing the general importance of the kinase function for localization to nuclear bodies. These results also raise the question whether the inactivation of the kinase function of the wild-type kinase already existing in nuclear speckles can be affected by a small molecule kinase inhibitor. To address this question, we took advantage from the availability of the HIPK2 inhibitor D-115893, which inhibits HIPK2 *in vitro* (data not shown) and HIPK2-dependent functions such as p53 serine 46 phosphorylation (Supplementary Fig. 2). The addition of nanomolar amounts of D-115893 resulted in a drastic loss of nuclear HIPK2 localization (Fig. 1C). These results collectively show that ongoing kinase activity together with a bipartite NLS is required for the occurrence of HIPK2 in the nucleus.

#### 3.2. Identification and functional characterization of a SIM in HIPK2

We observed that a HIPK2 mutant lacking the region between 786 and 886 lost its ability to localize to speckles (Fig. 2A), suggesting the relevance of the deleted region for proper localization. Reinspection of the sequence allowed to identify the sequence 880-VITI-883, which



**Fig. 4.** The HIPK2 SIM is required for uptake into PML-NBs and binding to PML-IV. (A) U2OS cells were transfected to express the PML isoform PML-IV along with different HIPK2 forms as shown. The proteins were detected with Cy3 and Cy5-conjugated secondary antibodies, the merge reveals areas of overlapping localization in yellow. (B) The experiment was done as in (A) with the exception that different HIPK2 constructs were used. (C) PML-IV and the indicated forms of Flag-tagged HIPK2 were coexpressed in HEK293T cells as shown. Interacting proteins were crosslinked for 30 min to preserve protein/protein interactions occurring in intact cells. After removal of the crosslinker and quenching, cells were lysed and subjected to immunoprecipitation (IP) using anti-Flag antibodies. The precipitates and input controls were analyzed by immunoblotting. (D) Cells were transfected to express PML-IV along with HIPK2 wild-type or mutants thereof at the indicated combinations. After 36 h, cells were harvested and cell extracts were analyzed by immunoblotting for the occurrence and phosphorylation of the indicated proteins and for tubulin as a loading control.

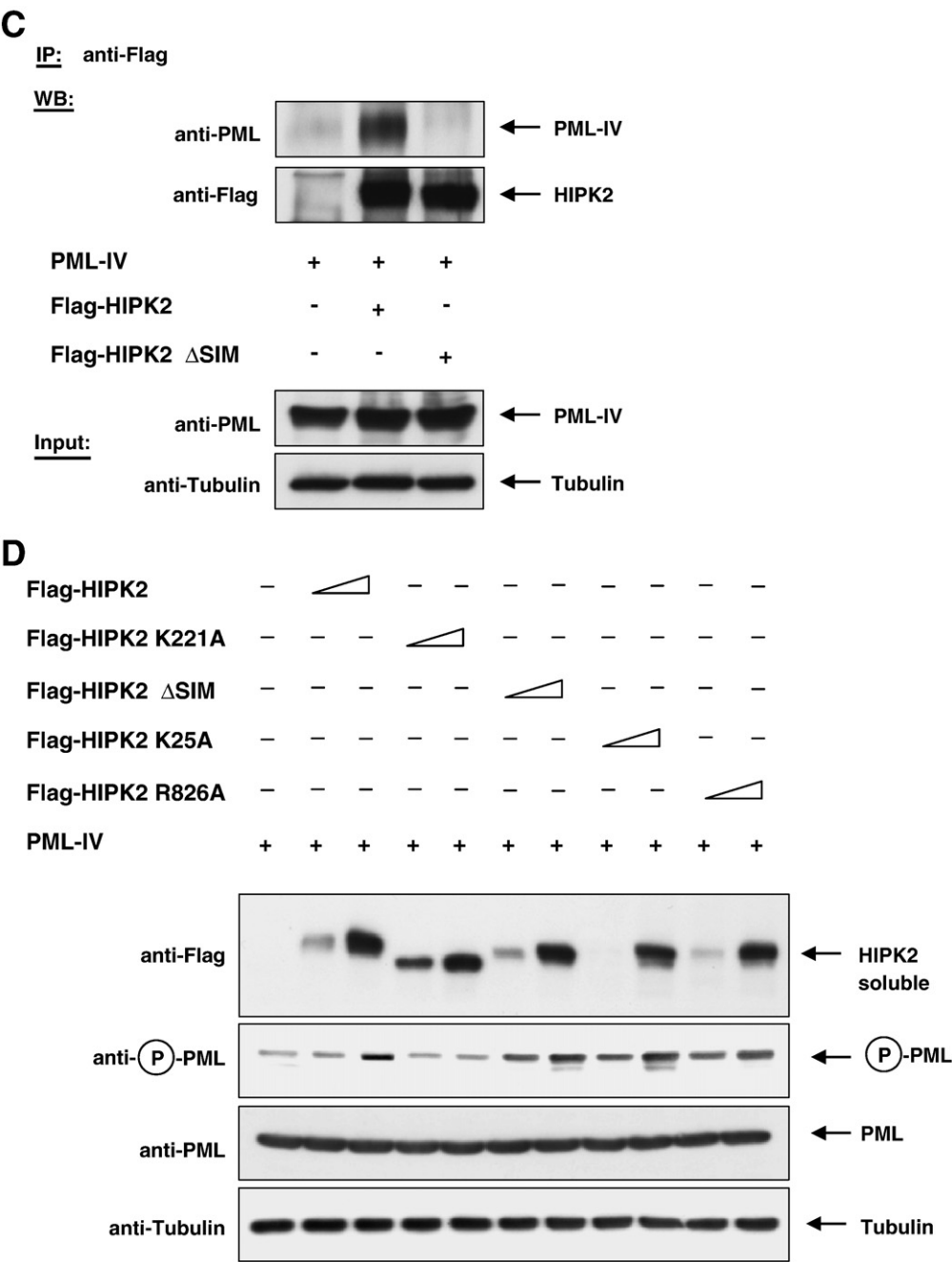


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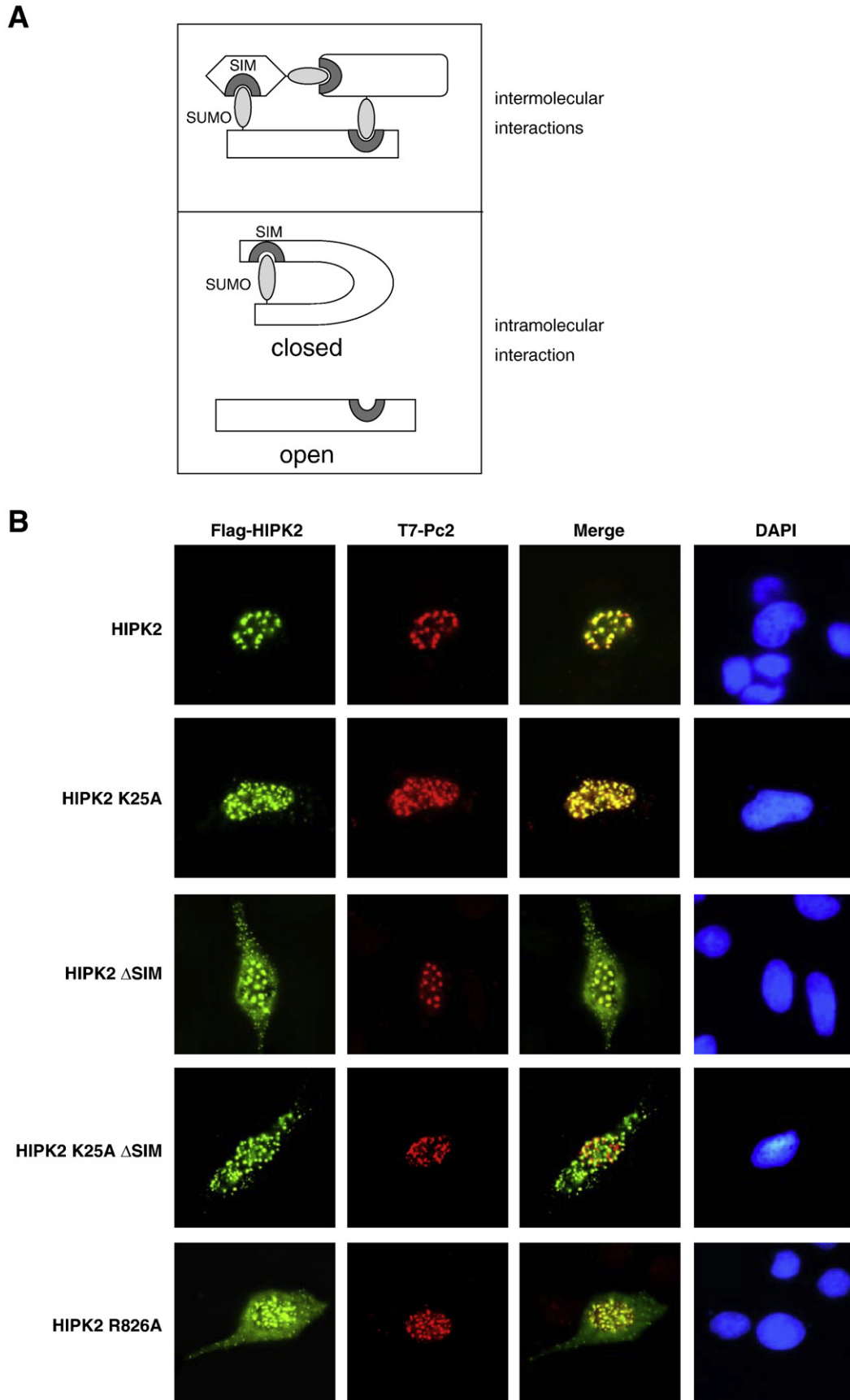
corresponds to the reported SIM consensus sequence V/I-x-V/I-V/I [6]. As this sequence is flanked by a patch of 6 acidic amino acids (Fig. 2B), this region is a high-probability candidate for a SIM. To test binding of HIPK2 to the three SUMO isoforms, pull-down assays were performed. Bacterially expressed and purified GST-SUMO-1, GST-SUMO-2, and GST-SUMO-3 efficiently captured HIPK2 (Fig. 2C), showing that all major SUMO isoforms have the ability to interact with this kinase. To investigate the relevance of the putative SIM for SUMO binding, HIPK2 mutants were produced that either lack the SIM or were mutated in order to target either the hydrophobic patch (HIPK2 M1) or the block of acidic amino acids (HIPK2 M2), as schematically displayed in Fig. 3A. GST pull-down experiments revealed that deletion or mutation of the SIM completely destroyed the ability of HIPK2 to interact with SUMO (Fig. 3B), thus identifying

the sequence between 880 and 890 as a functional SIM. The HIPK2 mutants either lacking the SIM or containing mutated versions of the hydrophobic or the acidic patch had lost the ability to localize in nuclear domains and were found throughout the cell (Fig. 3C).

3.3. The HIPK2 SIM is required for recruitment to PML-NBs

In unstressed cells, only a minor fraction of HIPK2 colocalizes with PML-NBs, while DNA damage increases the colocalization of both proteins and allows for HIPK2-mediated phosphorylation of PML at serines 8 and 38. The PML protein occurs in differentially spliced forms exerting different functions, as, for example, only the splice variant PML-IV promotes senescence in human diploid fibroblasts [30]. PML-IV localizes in nuclear speckles (Supplementary Fig. 3) and





**Fig. 5.** SUMO/SIM interactions contribute to Pc2 binding of HIPK2. (A) Schematic model summarizing the potential contribution of SUMO/SIM association for the formation of intermolecular (upper) and intramolecular (lower) interactions. (B) T7-tagged Pc2 and the indicated Flag-tagged HIPK2 variants were coexpressed in U2OS cells and analyzed for intracellular localization by immunofluorescence. (C) T7-tagged Pc2 and HIPK2 wild-type and mutant proteins were coexpressed in HEK293T cells and analyzed by crosslinking and coimmunoprecipitation as shown. (D) Cells were transfected to express HIPK2 along with GFP-SUMO-1 or a SUMO-1 variant defective for covalent attachment to a target lysine (SUMO-1 GG/AA). Intracellular localization of the proteins was analyzed by immunofluorescence.

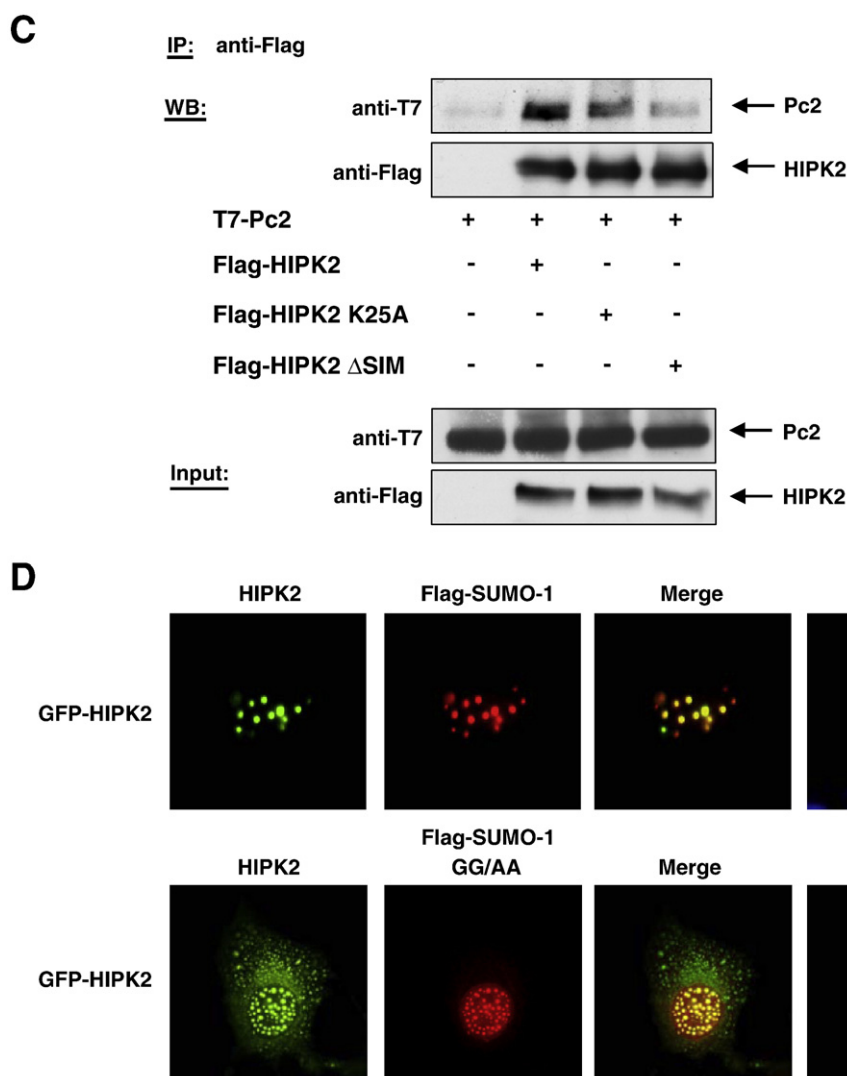


Fig. 5 (continued).

has the ability to recruit HIPK2 to PML-NBs [26], raising the question whether the SIM in HIPK2 is required for efficient uptake of the kinase in PML-NBs. To address this question, HIPK2 or mutant versions of the kinase were coexpressed along with PML-IV. While the wild-type kinase was efficiently recruited into PML-NBs, all mutants either lacking the entire SIM or mutated in the hydrophobic or acidic patch were not enriched in PML-NBs (Fig. 4A). Using a similar experimental approach, we also tested PML-NB recruitment of a HIPK2 fragment that is generated during caspase-dependent cleavage of HIPK2 (HIPK2 1-977) or a HIPK2 variant with a mutated NLS as exemplified by HIPK2 R826A with a defect NLS2. Both mutants could be largely recruited to PML-NBs, although a small but significant fraction of HIPK2 R826A stayed in the cytosol (Fig. 4B). The impact of the SIM on interaction between HIPK2 and PML was directly addressed by coimmunoprecipitation experiments. These experiments require crosslinking of the interacting proteins in intact cells, as SUMO modification is quickly lost during standard lysis conditions [1]. HEK293T cells were transfected to express PML-IV along with HIPK2 or HIPK2 ΔSIM. Cells were treated with a membrane-permeable crosslinker to allow covalent coupling of proteins occurring in very close proximity, followed by a coimmunoprecipitation experiment. While the wild-type form of HIPK2 was found in association with PML-IV, the deletion of the HIPK2 SIM largely precluded HIPK2/PML-IV binding (Fig. 4C).

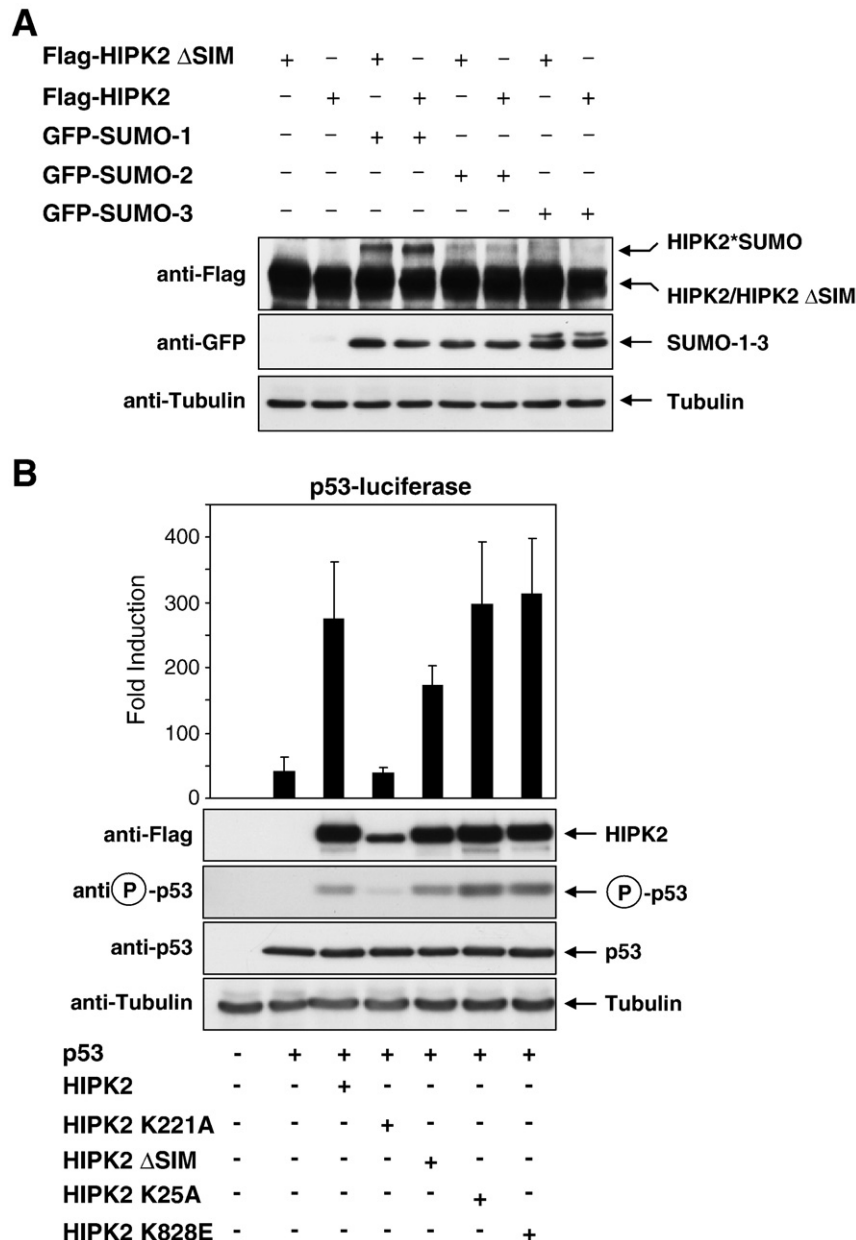
### 3.4. Pc2 binding to HIPK2 largely depends on a functional SIM

It was then interesting to test the importance of NLS and SIM sequences for the ability of HIPK2 to phosphorylate the PML protein. To address this question, PML-IV was coexpressed with HIPK2 and various mutants defect in SUMO binding or nuclear localization, followed by analysis of PML-IV phosphorylation using a phospho-specific antibody (Fig. 4D). The wild-type kinase induced PML phosphorylation, while the NLS mutant HIPK2 R826A showed an impaired PML phosphorylation. The HIPK2 ΔSIM mutant and the HIPK2 K25A mutant harboring a mutation in the covalent SUMO attachment site showed an intact PML phosphorylation despite their reduced colocalization with PML.

SUMO/SIM interactions play an important role for the control of protein/protein interaction affinities [31]. These interactions can occur in an intermolecular and/or intramolecular fashion as schematically displayed in Fig. 5A. The contribution of the SUMO/SIM system in HIPK2 for intermolecular binding was investigated for the Pc2 protein, as it also binds covalently and non-covalently to SUMO [32,33] and serves as a SUMO E3 ligase for HIPK2 [24]. The colocalization between both proteins was studied by immunofluorescence after coexpression of Pc2 with either wild-type HIPK2 or various mutants. While the non-SUMOylatable mutant HIPK2 K25A still colocalized with Pc2, deletion of the HIPK2 SIM or mutation of

NLS2 resulted in a strongly reduced colocalization (Fig. 5B), showing that the SIM of HIPK2 is of central relevance for colocalization with Pc2. The role of the HIPK2 SIM for association with Pc2 was investigated by crosslinking and coimmunoprecipitation experiments in cells transfected to express T7-tagged Pc2 along with HIPK2, HIPK2 K25A, or HIPK2  $\Delta$ SIM. These experiments revealed that Pc2 binding of the non-SUMOylatable HIPK2 K25A mutant was only slightly reduced, while deletion of the SIM strongly impaired the interaction with Pc2 (Fig. 5C). These data highlight the relevance of the SIM for protein/protein interactions, while covalent attachment of SUMO to its target lysine seems to be of minor relevance. Accordingly, coexpression of

HIPK2 K25A with a Pc2 variant mutated in the main SUMOylation site (Pc2 K492R) still allowed large colocalization between both proteins (Supplementary Fig. 4). The model shown in Fig. 5A implies that overexpression of a SUMO variant defect for the attachment to its target lysine should be able to compete with the endogenous SUMO for SIM binding and thus disrupt a fraction of SUMO/SIM interactions. We tested this hypothesis experimentally and measured HIPK2 localization in the presence of overexpressed wild-type SUMO-1 and a point mutant changed in the two C-terminal glycines needed for coupling to the target lysine. While the wild-type HIPK2 localized to nuclear bodies and showed colocalization with SUMO-1, the SUMO-1



**Fig. 6.** The SIM is not required for the SUMOylation of HIPK2 but controls its activity. (A) HEK293T cells were transfected to express Flag-HIPK2 or HIPK2  $\Delta$ SIM together with GFP-tagged SUMO1–3 as shown. Equal amounts of protein contained in cell lysates were analyzed by immunoblotting for the occurrence of a slower migrating HIPK2 form corresponding to the SUMOylated form. (B) H1299 cells were transfected with a p53-dependent reporter gene and expression vectors for p53, HIPK2 and HIPK2 variants as shown. After 36 h, cells were lysed and either analyzed for protein expression and p53 serine 46 phosphorylation (lower) or for luciferase activity (upper). Error bars show the standard deviations from four independent experiments. (C) HEK293T cells were transfected with a reporter gene under the control of 5 binding sites for IPF/PDX together with expression vectors for IPF/PDX and the indicated HIPK2 variants. Induction of transcription and protein expression were measured as in (B). (D) Cells were transfected with expression vectors encoding Flag-tagged Siah2 with the indicated HIPK2 mutants in the presence of MG-132, cell lysates were prepared and separated by extended SDS-PAGE in order to facilitate the detection of upshifted bands corresponding to phosphorylated Siah2. Phosphorylation of Siah2 was additionally revealed by phosphospecific antibodies, and comparable expression of the HIPK2 mutants was ensured using Flag antibodies. The positions of phosphorylated and hyperphosphorylated Siah2 are indicated. (E) U2OS cells were transfected to express GFP-tagged p66 $\beta$  along with the indicated HIPK2 variants. Indirect immunofluorescence was used to reveal the intracellular localization of both proteins.

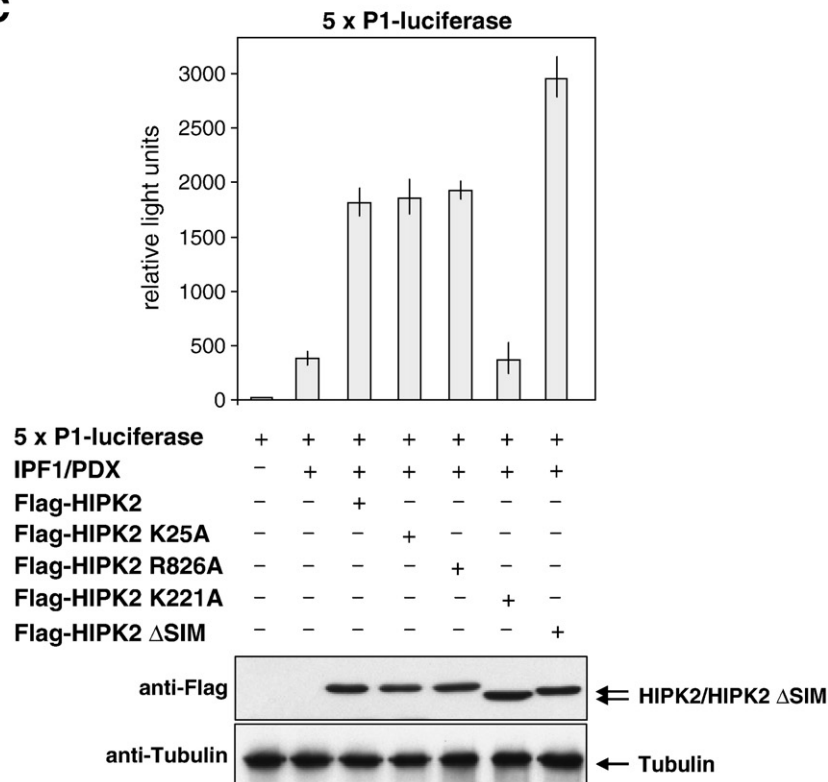
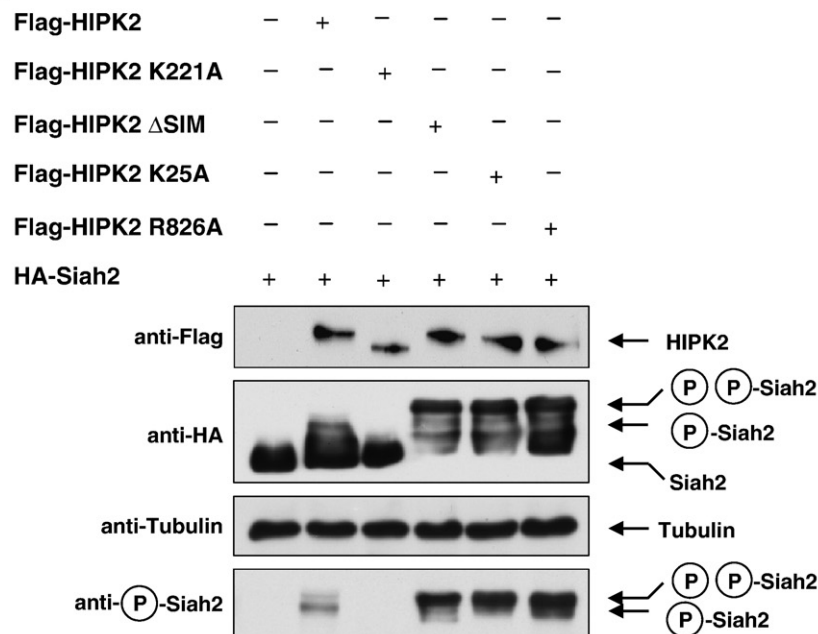
**C****D**

Fig. 6 (continued).

GG/AA mutant caused mislocalization of a substantial fraction of HIPK2 which also occurred in the cytosol (Fig. 5D).

### 3.5. A functional role of SUMO/SIM interactions for HIPK2 activity

SUMO/SIM interactions control several parameters including SUMO E3 ligase activity of Pc2 and also selection of the adequate SUMO paralogue. For example, non-covalent interactions between

SUMO-2/3 and the Bloom syndrome protein are required for preferential modification by SUMO-2/3 [34]. To test whether the SIM in HIPK2 has any influence on the SUMOylation efficiency and SUMO isoform selection, HIPK2 or HIPK2 ΔSIM were expressed alone or with GFP-tagged versions of all three major SUMO isoforms. Coexpression of HIPK2 with GFP-tagged SUMO-1 resulted in the occurrence of a slower migrating band corresponding to the expected molecular weight of the SUMOylated kinase, while SUMO-2 and

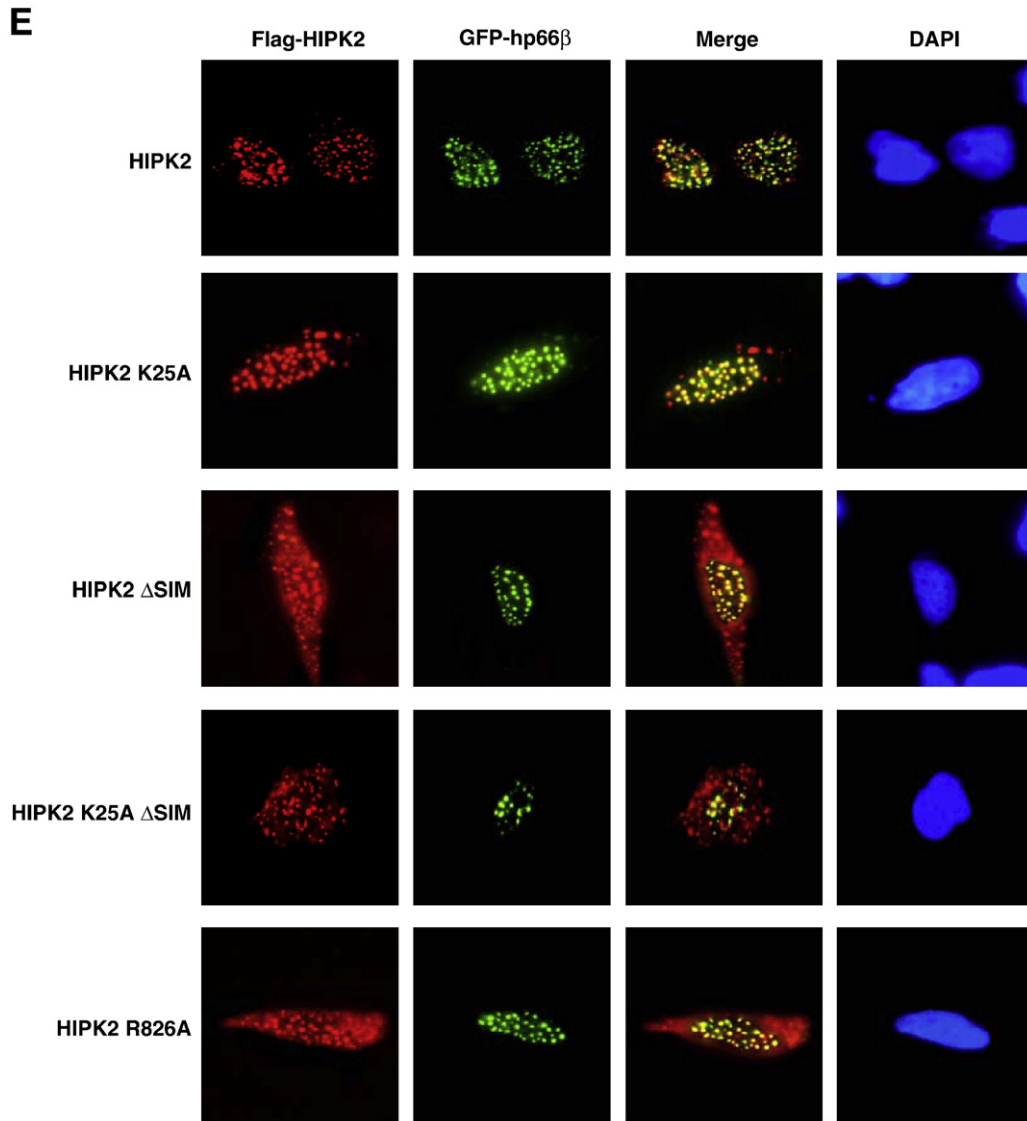


Fig. 6 (continued).

SUMO-3 were not conjugated to HIPK2 (Fig. 6A). Deletion of the SIM remained without impact on the HIPK2 modification by SUMO-1, indicating that a functional SIM is not required for this process.

As HIPK2 regulates the activity of several transcription factors, it was interesting to compare the HIPK2 mutants for their ability to regulate gene expression. HIPK2 and variants thereof were compared for their ability to phosphorylate p53 and to trigger its transcriptional activity. To address this question, p53-deficient H1299 cells were transfected with a p53-dependent reporter gene and expression vectors for p53, HIPK2 and its variants. While p53 serine 46 phosphorylation was roughly comparable between HIPK2 and its mutants, deletion of the SIM significantly impaired the ability of HIPK2 to trigger p53-dependent transcription (Fig. 6B). To investigate whether HIPK2 ΔSIM also shows altered activities towards other transcription factors, cells were transfected with a IPF1/PDX-dependent reporter construct along with an expression vector for IPF1/PDX and various HIPK2 expression constructs. Analysis of luciferase activity showed the importance of HIPK2 kinase function for its ability to trigger IPF1/PDX activity (Fig. 6C). While the activity of HIPK2 K25A was similar to that of the wild-type kinase, deletion of the SIM allowed for an augmented activity. These results suggest that the SIM can be either important for HIPK2 function (as is the case for p53) or even play an inhibitory role, which is in line with the

reported role of SUMO in transcriptional repressor complexes [35]. Is there any role of intramolecular SUMO/SIM interactions that may determine the activity of HIPK2? To explore this possibility, HIPK2 mutants were compared for their ability to phosphorylate a cytosolic substrate protein. The best known cytosolic HIPK2 substrate is Siah2, which gets phosphorylated by HIPK2 at many sites including threonine 26, serine 28, and serine 68 [16]. Lysates from cells transfected to coexpress Siah2 and various HIPK2 mutants were analyzed for Siah2 phosphorylation in two ways: extended gel electrophoresis was performed to detect phosphorylated and thus upshifted Siah2 bands as described [16] and in parallel serine 28 phosphorylation was detected using a phospho-specific antibody. Mutation of lysine 25 or deletion of the SIM resulted in a strongly elevated kinase function of HIPK2 (Fig. 6D), a result that is compatible with the idea that intramolecular SUMO/SIM interactions renders HIPK2 more active.

#### 4. Discussion

Using a point mutation strategy, this study identifies two NLS sequences for HIPK2 at position 795 to 798 (NLS1) and 825 to 828 (NLS2) and also a SIM between 880 and 890.



This is consistent with a seminal study showing the importance of the region between 629 and 859 for nuclear localization of this kinase [25]. The same paper also showed that the SRS can be separated from other regions in HIPK2 that still allow for nuclear localization. The SIM is entirely contained in the SRS region (860–967) identified in this previous study, thus providing evidence that the speckle retaining function of this region is attributable to a functional SIM. Mutation of only one amino acid in NLS2 is sufficient to interfere with nuclear localization, while the inactivation of NLS1 required the mutation at two residues, which might be an indication that NLS2 is of major importance. While most HIPK2 occurs in nuclear domains, some cells contain significant amounts of the kinase in the cytosol. A possible molecular mechanism responsible for differential localization may employ high-mobility group protein A1, as increased expression of this protein leads to increased cytosolic localization of HIPK2 [21]. It would thus be interesting to know whether the reported interaction between HIPK2 and high-mobility group A1 affects the accessibility of the HIPK2 NLS regions. Mutation of each individual NLS results in the cytosolic localization of HIPK2 without excluding it from the nucleus where a significant fraction of the kinase is still detectable in HIPK domains. This residual localization may well be due to SUMO/SIM interactions that mediate trapping of HIPK2 in nuclear speckles upon binding to other nuclear proteins such as Pc2 or PML. Mutation of the HIPK2 SIM did not only reduce binding to Pc2 and PML but also prohibited recruitment of the kinase to PML-NBs, which also depend on a functional SIM in the PML protein [36].

Given broad spectrum of SUMOylated proteins, it is reasonable to assume that the HIPK2 SIM will allow for assembly with even more SUMOylated proteins. Along this line, we found the relevance of the SIM for coupling to p66 $\beta$  (Fig. 6E), a potent transcriptional repressor that interacts with the methyl-CpG-binding domain (MBD) proteins MBD2 and MBD3 [28]. These observations suggest that SUMO/SIM interactions contribute to the stable assembly of nuclear bodies hosting macromolecular multi-protein complexes. These aggregates are characterized by macromolecular crowding and individual proteins are integrated into multi-protein networks. In such a situation with an extremely slow diffusion, the deconjugation of SUMO from an individual protein would still retain it in the protein complex due to SUMO/SIM meshworks with neighboring proteins. This concept also integrates the finding of normal speckled localization of SUMOylation-deficient HIPK2 and Pc2 point mutants (Supplementary Fig. 4), while inactivation of the HIPK2 SIM causes delocalization from HIPK domains. Depending on the cellular context, the HIPK2 SIM may serve different functions. On one hand, it can lead to transcriptional repression, as the SUMO/SIM system is known to recruit repressor complexes and histone deacetylases [35]. On the other hand, it allows cross-coupling and binding to other proteins such as Pc2 and PML, thus enabling protein/protein interactions and signal transmission. An intriguing possibility is the potential occurrence of intramolecular SUMO/SIM interactions, which is supported by the observation of enhanced Siah2 phosphorylation by HIPK2 K25A.

While SUMO/SIM interactions are an important glue that enhances protein/protein affinities, they do not allow to explain the specificity of protein/protein interactions, which rely on direct contacts between the proteins. Regulation of protein/protein affinities by the SUMO/SIM systems is thus a versatile tool to control the strength of protein/protein interaction networks. This raises the need to regulate SUMOylation, which can be achieved by several ways. We have previously shown that activation of HIPK2 by genotoxic stress allows for HIPK2-mediated phosphorylation of Pc2 at threonine 495, which in turn enhances its SUMO E3 ligase activity towards HIPK2 [24]. This mechanism may also explain that loss of HIPK2 kinase function results in nucleoplasmic localization of HIPK2, as a kinase inactive HIPK2 point mutant is not efficiently SUMOylated [24]. But also the deconjugation of SUMO can be a regulated process. Previous studies showed that deconjugation of SUMO from HIPK2 can be mediated by the isopeptidases SENP1 (sentrin-specific protease 1) and the SENP2 splice form SuPr-1

[22,37]. The regulation of these enzymes is not well explored, but one clue that may allow their differential activity is their distinct intracellular localization. SENP1 shuttles between the cytosol and the nucleus, while SuPr-1 localizes to PML-NBs. Another potential layer of regulation comes from experiments describing the regulation of SUMO/SIM affinities by phosphorylation. As exemplified by PIAS1 and PML, phosphorylation of amino acids contained in the SIM adds negative charge and thus increases binding affinities. Thus it would be interesting to study potential phosphorylation of serines and the threonine contained in the HIPK2 SIM.

## 5. Conclusions

This study allowed to map two independent nuclear localization signals in HIPK2 and to reveal the importance of a C-terminal SIM for its localization to nuclear speckles and its uptake in PML-NBs. These data also show that SUMO/SIM interactions function as a molecular adhesive that serves to enhance protein/protein interaction networks and to control various HIPK2 functions. Thus the regulation of SUMO/SIM interactions, as it occurs by HIPK2-dependent control of Pc2 activity, allows to regulate localization and activity of this tumor suppressor kinase.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamcr.2010.11.022.

## References

- [1] K.A. Wilkinson, J.M. Henley, Mechanisms, regulation and consequences of protein SUMOylation, *Biochem. J.* 428 (2010) 133–145.
- [2] M.C. Geoffroy, R.T. Hay, An additional role for SUMO in ubiquitin-mediated proteolysis, *Nat. Rev. Mol. Cell Biol.* 10 (2009) 564–568.
- [3] D. Walker, Sumoylation: wrestling with filaments, *Nat. Rev. Mol. Cell Biol.* 11 (2010) 3.
- [4] C.M. Hecker, M. Rabiller, K. Haglund, P. Bayer, I. Dikic, Specification of SUMO1- and SUMO2-interacting motifs, *J. Biol. Chem.* 281 (2006) 16117–16127.
- [5] J. Song, L.K. Durrin, T.A. Wilkinson, T.G. Kroniris, Y. Chen, Identification of a SUMO-binding motif that recognizes SUMO-modified proteins, *Proc. Natl. Acad. Sci. USA* 101 (2004) 14373–14378.
- [6] O. Kerscher, SUMO junction-what's your function? New insights through SUMO-interacting motifs, *EMBO Rep* 8 (2007) 550–555.
- [7] M.A. Calzado, F. Renner, A. Roscic, M.L. Schmitz, HIPK2: a versatile switchboard regulating the transcription machinery and cell death, *Cell Cycle* 6 (2007) 139–143.
- [8] Y.H. Kim, C.Y. Choi, S.J. Lee, M.A. Conti, Y. Kim, Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors, *J. Biol. Chem.* 273 (1998) 25875–25879.
- [9] C. Rinaldo, A. Prodosmo, F. Siepi, S. Soddu, HIPK2: a multitasking partner for transcription factors in DNA damage response and development, *Biochem. Cell Biol.* 85 (2007) 411–418.
- [10] Y. Rui, Z. Xu, S. Lin, Q. Li, H. Rui, W. Luo, H.M. Zhou, P.Y. Cheung, Z. Wu, Z. Ye, P. Li, J. Han, S.C. Lin, Axin stimulates p53 functions by activation of HIPK2 kinase through multimeric complex formation, *EMBO J.* 23 (2004) 4583–4594.

- [11] E. Gresko, A. Roscic, S. Ritterhoff, A. Vichalkovski, G. del Sal, M.L. Schmitz, Autoregulatory control of the p53 response by caspase-mediated processing of HIPK2, *EMBO J.* 25 (2006) 1883–1894.
- [12] G. D'Orazi, B. Cecchinelli, T. Bruno, I. Manni, Y. Higashimoto, S. Saito, M. Gostissa, S. Coen, A. Marchetti, G. Del Sal, G. Piaggio, M. Fanciulli, E. Appella, S. Soddu, Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis, *Nat. Cell Biol.* 4 (2002) 11–19.
- [13] T.G. Hofmann, A. Moller, H. Sirma, H. Zentgraf, Y. Taya, W. Droge, H. Will, M.L. Schmitz, Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2, *Nat. Cell Biol.* 4 (2002) 1–10.
- [14] M.J. Boucher, M. Simoneau, H. Edlund, The homeodomain-interacting protein kinase 2 regulates insulin promoter factor-1/pancreatic duodenal homeobox-1 transcriptional activity, *Endocrinology* 150 (2009) 87–97.
- [15] L. Nardinocchi, R. Puca, A. Sacchi, G. Rechavi, D. Givol, G. D'Orazi, Targeting hypoxia in cancer cells by restoring homeodomain interacting protein-kinase 2 and p53 activity and suppressing HIF-1 $\alpha$ , *PLoS ONE* 4 (2009) e6819.
- [16] M.A. Calzado, L. de la Vega, A. Moller, D.D. Bowtell, M.L. Schmitz, An inducible autoregulatory loop between HIPK2 and Siah2 at the apex of the hypoxic response, *Nat. Cell Biol.* 11 (2009) 85–91.
- [17] K. Isono, K. Nemoto, Y. Li, Y. Takada, R. Suzuki, M. Katsuki, A. Nakagawara, H. Koseki, Overlapping roles for homeodomain-interacting protein kinases hipk1 and hipk2 in the mediation of cell growth in response to morphogenetic and genotoxic signals, *Mol. Cell. Biol.* 26 (2006) 2758–2771.
- [18] G. Wei, S. Ku, G.K. Ma, S. Saito, A.A. Tang, J. Zhang, J.H. Mao, E. Appella, A. Balmain, E.J. Huang, HIPK2 represses beta-catenin-mediated transcription, epidermal stem cell expansion, and skin tumorigenesis, *Proc. Natl Acad. Sci. USA* 104 (2007) 13040–13045.
- [19] A. Moller, H. Sirma, T.G. Hofmann, S. Rueffer, E. Klimczak, W. Droge, H. Will, M.L. Schmitz, PML is required for homeodomain-interacting protein kinase 2 (HIPK2)-mediated p53 phosphorylation and cell cycle arrest but is dispensable for the formation of HIPK domains, *Cancer Res.* 63 (2003) 4310–4314.
- [20] C. Rinaldo, F. Siepi, A. Prodosmo, S. Soddu, HIPKs: Jack of all trades in basic nuclear activities, *Biochim. Biophys. Acta* 1783 (2008) 2124–2129.
- [21] G.M. Pierantoni, C. Rinaldo, M. Mottolese, A. Di Benedetto, F. Esposito, S. Soddu, A. Fusco, High-mobility group A1 inhibits p53 by cytoplasmic relocation of its proapoptotic activator HIPK2, *J. Clin. Invest.* 117 (2007) 693–702.
- [22] T.G. Hofmann, E. Jaffray, N. Stollberg, R.T. Hay, H. Will, Regulation of homeodomain-interacting protein kinase 2 (HIPK2) effector function through dynamic small ubiquitin-related modifier-1 (SUMO-1) modification, *J. Biol. Chem.* 280 (2005) 29224–29232.
- [23] E. Gresko, A. Moller, A. Roscic, M.L. Schmitz, Covalent modification of human homeodomain interacting protein kinase 2 by SUMO-1 at lysine 25 affects its stability, *Biochem. Biophys. Res. Commun.* 329 (2005) 1293–1299.
- [24] A. Roscic, A. Moller, M.A. Calzado, F. Renner, V.C. Wimmer, E. Gresko, K.S. Ludi, M.L. Schmitz, Phosphorylation-dependent control of Pc2 SUMO E3 ligase activity by its substrate protein HIPK2, *Mol. Cell* 24 (2006) 77–89.
- [25] Y.H. Kim, C.Y. Choi, Y. Kim, Covalent modification of the homeodomain-interacting protein kinase 2 (HIPK2) by the ubiquitin-like protein SUMO-1, *Proc. Natl Acad. Sci. USA* 96 (1999) 12350–12355.
- [26] E. Gresko, S. Ritterhoff, J. Sevilla-Perez, A. Roscic, K. Frobius, I. Kotevic, A. Vichalkovski, D. Hess, B.A. Hemmings, M.L. Schmitz, PML tumor suppressor is regulated by HIPK2-mediated phosphorylation in response to DNA damage, *Oncogene* 28 (2009) 698–708.
- [27] M.H. Kagey, T.A. Melhuish, S.E. Powers, D. Wotton, Multiple activities contribute to Pc2 E3 function, *EMBO J.* 24 (2005) 108–119.
- [28] M. Brackertz, J. Boeke, R. Zhang, R. Renkawitz, Two highly related p66 proteins comprise a new family of potent transcriptional repressors interacting with MBD2 and MBD3, *J. Biol. Chem.* 277 (2002) 40958–40966.
- [29] D. Kalderon, B.L. Roberts, W.D. Richardson, A.E. Smith, A short amino acid sequence able to specify nuclear location, *Cell* 39 (1984) 499–509.
- [30] O. Bischof, O. Kirsh, M. Pearson, K. Itahana, P.G. Pelicci, A. Dejean, Deconstructing PML-induced premature senescence, *EMBO J.* 21 (2002) 3358–3369.
- [31] B.T. Seet, I. Dikic, M.M. Zhou, T. Pawson, Reading protein modifications with interaction domains, *Nat. Rev. Mol. Cell Biol.* 7 (2006) 473–483.
- [32] S.H. Yang, A.D. Sharrocks, The SUMO E3 ligase activity of Pc2 is coordinated through a SUMO interaction motif, *Mol. Cell. Biol.* 30 (2010) 2193–2205.
- [33] J.C. Merrill, T.A. Melhuish, M.H. Kagey, S.H. Yang, A.D. Sharrocks, D. Wotton, A role for non-covalent SUMO interaction motifs in Pc2/CBX4 E3 activity, *PLoS ONE* 5 (2010) e8794.
- [34] J. Zhu, S. Zhu, C.M. Guzzo, N.A. Ellis, K.S. Sung, C.Y. Choi, M.J. Matunis, Small ubiquitin-related modifier (SUMO) binding determines substrate recognition and paralogue-selective SUMO modification, *J. Biol. Chem.* 283 (2008) 29405–29415.
- [35] S.H. Yang, A.D. Sharrocks, SUMO promotes HDAC-mediated transcriptional repression, *Mol. Cell* 13 (2004) 611–617.
- [36] T.H. Shen, H.K. Lin, P.P. Scaglioni, T.M. Yung, P.P. Pandolfi, The mechanisms of PML-nuclear body formation, *Mol. Cell* 24 (2006) 331–339.
- [37] Y.H. Kim, K.S. Sung, S.J. Lee, Y.O. Kim, C.Y. Choi, Y. Kim, Desumoylation of homeodomain-interacting protein kinase 2 (HIPK2) through the cytoplasmic-nuclear shuttling of the SUMO-specific protease SENP1, *FEBS Lett.* 579 (2005) 6272–6278.