A Novel SUMO1-specific Interacting Motif in Dipeptidyl Peptidase 9 (DPP9) That Is Important for Enzymatic Regulation

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Background: Interactions of SUMO isoforms/paralogs involve a groove on SUMO1–3 and a SIM on the downstream effector.

Results: A novel motif in DPP9 binds to a loop on SUMO1, leading to allosteric activation of DPP9.

Conclusion: The SUMO1-loop is an additional surface for noncovalent interactions, allowing discrimination between SUMO1–3.

Significance: Learning how SUMO isoforms/paralogs are recognized advances our understanding on events downstream of sumoylation.

Sumoylation affects many cellular processes by regulating the interactions of modified targets with downstream effectors. Here we identified the cytosolic dipeptidyl peptidase 9 (DPP9) as a SUMO1 interacting protein. Surprisingly, DPP9 binds to SUMO1 independent of the well known SUMO interacting motif, but instead interacts with a loop involving Glu67 of SUMO1. Intriguingly, DPP9 selectively associates with SUMO1 and not SUMO2, due to a more positive charge in the SUMO1-loop. We mapped the SUMO-binding site of DPP9 to an extended arm structure, predicted to directly flank the substrate entry site. Importantly, whereas mutants in the SUMO1-binding arm are less active compared with wild-type DPP9, SUMO1 stimulates DPP9 activity. Consistent with this, silencing of SUMO1 leads to a reduced cytosolic prolyl-peptidase activity. Taken together, these results suggest that SUMO1, or more likely, a sumoylated protein, acts as an allosteric regulator of DPP9.

SUMOs are small proteins that act as post-translational protein modifiers (1–3). Modification of proteins by SUMO (sumoylation) affects many cellular pathways including cell cycle progression, chromatin structure, DNA repair, transcription, and trafficking (e.g. reviewed in Refs. 4–7). Humans express three functional SUMO paralogs: SUMO1–3, which are conjugated to their targets in a reversible manner. SUMO2 and SUMO3 are highly homologous (97% identity) but share only 50% identity with SUMO1. The three paralogs appear to serve overlapping but also distinct functions, because some proteins are modified preferably by one of the SUMO paralogs (8–11).

Sumoylation regulates the molecular interactions of the modified proteins leading to changes in the localization, activity, solubility, or even stability of respective target proteins. Consequently, novel interactions depend on the presence of downstream effector proteins that contain motifs for noncovalent binding to SUMO (3, 12). A single SUMO-interacting motif (SIM),3 is currently known, which is characterized by a cluster of hydrophobic residues (13–17). Some SUMO-SIM interactions are regulated by phosphorylation of serine residues located in close proximity to the hydrophobic core (18, 19). NMR and crystal structures reveal that SIMs bind to a SIM-interacting groove (SIG) formed between the α-helix and β-sheet of all three SUMO paralogs (14, 15, 17, 20, 21). How SUMO-interacting proteins differentiate between the SUMO paralogs is only partially understood. In some cases negative charges flanking the SIM, lead to a preferable interaction of the SIMs with SUMO1 over SUMO2/3 (17, 19).

To get more insights into downstream events of sumoylation, it is important to understand how proteins interact specifically with one SUMO isoform. Here we screened for proteins showing preferable interaction with either SUMO1 or SUMO2. In this screen, we identified two prolyl peptidases of the S9B/DP-PIV family, dipeptidyl peptidase 8 (DPP8) and dipeptidyl peptidase 9 (DPP9) as specific interactors of SUMO1.

Peptidases of the S9B/DPPIV family are unique in their ability to cleave off N-terminal dipeptides from substrates that have a proline residue at the second position (Xaa-Pro). Four active

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3 The abbreviations used are: SIM, SUMO-interacting motif; SIG, SIM-interacting groove; AMC, 7-amino-4-methylcoumarin; SUBA, SUMO-binding arm; DPP, dipeptidyl peptidase; EIL, E67-interacting loop.
members of this family are known, two of which are cell surface peptidases: dipeptidyl peptidase IV (DPPIV) and the fibroblast activation protein α (22). DPPIV inhibitors are used for treatment of diabetes type II, because they stabilize the incretin hormones glucagon-like peptide and the glucose-dependent insulinotropic polypeptide, which are important for glucose homeostasis (23).

The two cytosolic members of the DPPIV family are DPP8 and DPP9, which share approximately 60% identity (24, 25). Both enzymes are expressed in multiple tissues, predominantly in lymphocytes and epithelial cells of many organs (26–30). In vitro, DPP8 and DPP9 are similar in their biochemical properties, including enzyme kinetics and substrate specificity (31, 32).

The physiological roles of DPP8 and DPP9 are only emerging. siRNA-mediated down-regulation of both peptidases in several cell lines reveals that DPP9 but not DPP8, is rate-limiting for degradation of most cytosolic proline containing peptides (31). Changes in the expression levels of DPP8 and DPP9 are critical for survival and proliferation of different cell lines, such as human hepatoma and embryonic kidney cells as well as cells originating from the Ewing sarcoma family of tumors (33, 34). Overexpression of both peptidases was reported to impair cell adhesion and migration (35).

Recently, we showed that several tumor-related antigens, which contain a proline residue in position 2, are processed in vitro by DPP8 and DPP9. Among these antigens was RU1 (34–42). Down-regulation of DPP9 by siRNA treatment increased the presentation of the RU1 (34–42) antigen on MHC class I alleles to RU1-specific cytotoxic T cells; thus identifying this antigen as the first endogenous substrate of DPP9, and linking DPP9 to the antigen presentation pathway (31). Currently, little is known about regulation of DPP9, e.g., by binding partners.

Importantly, here we show that DPP9 and SUMO1 interact in a SIM-independent manner, and define a novel surface on SUMO1 for noncovalent interactions with downstream effectors. Moreover, we show that SUMO1 binds to an arm motif in DPP9, located in proximity to the substrate entrance site. Furthermore, we find that the SUMO-binding arm is important for DPP9 activity, and that DPP9 enzymatic activity is increased in the presence of SUMO1. Finally, down-regulation of SUMO1 by siRNA oligos leads to reduced prolyl peptidase activity in cell extracts. Taken together these results show that noncovalent interaction of DPP9 with SUMO1 leads to activation of the peptidase.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HEK293T and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum.

**Antibodies**—Goat anti-Uba2 were previously described (36). Rabbit anti-PIAS2 antibodies and mouse GST antibodies were purchased from Sigma and Santa Cruz Biotechnology, respectively. Mouse anti-GMP1 antibodies were obtained from the Developmental Studies Hybridoma Bank. Rabbit anti-DPP9 antibodies were purchased from Abcam. For ELISA we produced goat anti-DPP9 specific antibodies by injecting a goat with 500 μg of full-length DPP9 on days 0, 40, and 70. Goat anti-DPP9 antibodies were affinity purified from the serum.

**Plasmids**—SUMO1 and SUMO2 were cloned into pET11 (37, 38). DPP8 and DPP9 were subcloned into a pFASTBacHT plasmid (Invitrogen), baculoviruses were generated according to the instructions of the Bac to Bac Baculovirus expression system, using Sf9 cells. DPP9 was cloned into pcDNA3.1 vector for expression of N-terminal HA- or FLAG-tagged DPP9 (using the BamHI and NotI restriction sites). Single point mutations in SUMO1, SUMO2, or DPP9 were generated using primers for site-directed mutagenesis.

**Peptides**—Peptide sequences are included in supplemental “Materials and Methods”. All peptides (>80% purity) were purchased from Genescript. Protein purification methods are described in supplemental “Materials and Methods”.

**SUMO Pull-down Screen**—SUMO proteins were coupled to CNBr-Sepharose (Sigma) at a concentration of 1 mg of protein/ml of beads. To identify SUMO-interacting proteins, 25 g of frozen HeLa cell pellets were resuspended in 50 ml of TB (20 mM Hepes/KOH, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM EGTA), supplemented with protease inhibitors (1 μg/ml of leupeptin, pepstatin, and aprotinin, and 2 mM DTT) and homogenized by douncing. Cell lysates were centrifuged at 10,000 × g for 15 min, and again at 100,000 × g for 1 h. 10 ml of supernatant was incubated at 4 °C with 200 μl of SUMO1/SUMO2 or control beads that were inactivated with ethanolamine. After 2 h of incubation, beads were washed and eluted in TB supplemented with protease inhibitors and increasing NaCl concentrations (0.25, 0.5, or 1 M). Eluted proteins were precipitated with methanol/chloroform, resuspended in LDS sample buffer, separated on a 4–12% NuPAGE gradient gel (Invitrogen), and stained with colloidal Coomassie Brilliant Blue. In-gel trypptic digestion, desalting, and LC-MS/MS analysis were performed as reported previously (39). All pkl files corresponding to one sample were merged into a single mascot generic data file and searched against the human IPI protein database (version 3.26) using Mascot with Mascot Server (version 2.2). The following settings were used: digestion with trypsin allowing one miss cleavage, carbamylation of cysteine as fixed modification, oxidation of methionine as variable, 150 ppm MS mass accuracy, and 0.3 Da for fragmentation masses.

**SUMO Pull Downs with Recombinant Proteins**—500 ng of recombinant proteins were incubated for 1 h at 4 °C with bead-immobilized SUMO1, SUMO2, or ovalbumin in TB supplemented with 0.5% Tween 20 and 0.2 mg/ml of ovalbumin. After 1 h, beads were washed in TB containing 0.5% Tween 20 and protease inhibitors, and eluted with sample buffer. For peptide competition assays: recombinant DPP9 (500 ng) was incubated with peptides from the SUMO1 peptide library (0.1 mg/ml) for 1 h prior to incubation with the SUMO-immobilized beads.

**ELISA**—A 96-well plate (Immuno 96 MicroWell™ Solid Plates, Nunc) was coated with 600 ng of purified recombinant proteins per well, overnight at 4 °C. Next, wells were blocked with ELISA buffer (TB supplemented with 3% BSA) for 1 h at room temperature. After blocking, recombinant interacting proteins were added to the wells. Following 2 h of incubation at 25 °C, wells were washed four times. Interacting proteins were...
detected using goat anti-DPP9 or mouse anti-GMP (SUMO1) antibodies. After incubation with secondary antibodies, wells were washed with ELISA buffer and ddH2O. Reactions were developed with 3,3',5,5'-tetramethylbenzidine-substrate reagent set from BD Biosciences (OptEIA substrate). Absorbance was measured at 420 nm on an Applikan microplate fluorimeter (Thermo Scientific) and Skanit software. Experiments were performed at least three times, in triplicates. For competition assays: SUMO1 was incubated with the DPP9-arm or a control peptide for 30 min prior to incubation with immobilized DPP9 or immobilized ovalbumin.

Immunoprecipitations—HEK293T cells were transfected with pcDNA3 vectors using the calcium-phosphate precipitation method. 48 h later, cells were harvested and dounced in ice-cold TB supplemented with 0.2% Tween 20. The homogenate was centrifuged at 100,000 × g at 4 °C for 20 min. Supernatants were pre-cleared for 30 min with protein G-coupled beads, followed by immunoprecipitation for 2 h at 4 °C with mouse anti-FLAG beads (SIGMA). After extensive washing with TB containing 0.2% Tween 20, bind proteins were eluted with 0.5 mg/ml of FLAG peptide in the same buffer.

Size Exclusion Chromatography—200 nm purified DPP9 wild-type or V285A mutant in TB buffer were loaded on an analytical Superdex 200 column (General electric) for size exclusion chromatography.

Kinetics Assays—Unless otherwise stated: purified recombinant DPP9 (25 nm) was incubated with varying concentrations of GP-AMC, in TB supplemented with 0.2% Tween 20, 0.2 mg/ml of BSA, 1 mM dithiothreitol. For activation assays: DPP9 was incubated with 2.5 μM SUMO proteins, RanGAP or RanGAP modified with SUMO1 for 2 h at 4 °C prior to the assay. Fluorescence was measured using the Applikan microplate fluorimeter (Thermo Scientific) with 380-nm (excitation) and 480-nm (emission) filters and Skanit software, and analyzed using Prism software. Each experiment was performed at least three times, in replicates of four.

Prolyl Peptidase Activity in SUMO1-silenced Cells—siRNA oligos against SUMO1 (supplementary) were synthesized by Invitrogen. HeLa cells that were 30% confluent were transfected with 120 pmol of siRNA using Oligofectamine reagent (Invitrogen) in antibiotic-free medium. 48 h later cells were retransfected with SUMO1 siRNA and harvested after a total of 72 h. Nontargeting-siRNA was used as control. 72 h after transfection, cells were washed in PBS, harvested, and lysed in TB supplemented with 1 mM DTT and 400 nM HA-SUMO-2 Vinyl Sulfone (Biomol). Equal amounts of cell lysates from each silenced sample (5 μg protein) were tested for hydrolysis of 0.5 mM GP-AMC.

RESULTS

Identification of a Novel Interaction between SUMO1 and the Cytosolic Prolyl Peptidases DPP8 and DPP9—We identified DPP8 and DPP9 in a screen for proteins interacting preferentially with either SUMO1 or SUMO2 (Fig. 1A). Using Western blot analysis we verified that both peptidases interacted with SUMO1. For control we blotted for the cytosolic prolyl endopeptidase (POP), which did not interact with the SUMO beads (Fig. 1B). In addition, we tested for the binding of Uba2, which was previously shown to contain a SIM (14). Importantly, whereas Uba2 interacts with both SUMO1 and SUMO2, both DPP8 and DPP9 showed a strong preference toward SUMO1 binding (Fig. 1B).

Next, following the incubation of cell lysates with immobilized SUMO beads, the SUMO-bound fractions were eluted and assayed for prolyl-peptidase activity by measuring the release of AMC from the model substrate Gly-Pro-AMC (GP-AMC). Prolyl-peptidase activity was recovered specifically in fractions eluted from the SUMO1, but not on SUMO2 or ovalbumin beads (bead-bound fraction, Fig. 1C). We then ana-
alyzed the remaining cytosolic fractions that did not bind to the SUMO or control beads (Depleted lysates) for GP-AMC cleavage. As shown in Fig. 1C, depletion of lysates with SUMO1 resulted in a significant decrease (about 40%) of AMC release in the unbound fraction, this decrease was not observed in lysates incubated with SUMO2. These observations suggest that a considerable proportion of endogenous DPP8 and DPP9 can interact with SUMO1, and that this fraction is enzymatically active.

To test whether these interactions are direct, we expressed DPP8 and DPP9 in insect cells (Fig. 1D). Purified recombinant DPP8 and DPP9 were then analyzed for binding to bead-immobilized SUMO1 or SUMO2. In full agreement with the endogenous proteins, recombinant DPP8 and DPP9 interact specifically with SUMO1, but not with SUMO2. In contrast, recombinant Uba2 interacted with both SUMO proteins (Fig. 1E). As an additional approach, we performed ELISA, in which increasing concentrations of SUMO1 were incubated with immobilized recombinant DPP8 or DPP9. We found that SUMO1 interacted directly with DPP8 and DPP9 in a concentration-dependent manner, whereas only background binding was detected in control wells that were coated with ovalbumin (Fig. 1F). Taken together, our results show that DPP8 and DPP9 are novel and direct binding partners specifically of SUMO1.

DPP9 Interacts with a Novel Interaction Surface on SUMO1: E67-interacting Loop (EIL)—To study the preferential association of DPP8 and DPP9 with SUMO1 but not SUMO2, we aimed to identify the surfaces on SUMO1 involved in these interactions. First we analyzed the interaction of DPP9 with SUMO1 mutants in residues that were previously shown via NMR and crystal structures to interact with SIM containing proteins (14, 15, 17, 20). We constructed the following mutants: SUMO1K37AV38A and SUMO1V38AK39A and analyzed their capacity to pull-down DPP8 and DPP9 from cell lysates. For control, we analyzed the binding of known SIM-containing proteins, Uba2 and PIAS2. Neither of these interacted with SUMO1K37AV38A and SUMO1V38AK39A. In striking contrast, both endogenous and recombinant DPP8 and DPP9 still interacted with these SUMO1-SIM mutants (Fig. 2, A and B). Furthermore, in pull down experiments with immobilized SUMO1, we showed that recombinant Uba2 and DPP9 can simultaneously interact with SUMO1 (supplementary Fig. 1). These results show that the interaction of both DPP8 and DPP9 with SUMO1 involves a surface different from the conventional SIG of SUMO1.

To identify the surface of SUMO1 that is important for its interactions with DPP9, we analyzed a library of short 15-mer peptides that covered the complete SUMO1 sequence. In total 12 peptides were tested, each peptide contained a 7-amino acid overlap with the carboxyl terminus of the previous peptide. The peptides were mixed with DPP9 prior to incubation with immobilized SUMO1 beads. If a peptide covers the surface of SUMO1 that associates with DPP9, it may bind to DPP9 and consequently block the SUMO1-DPP9 interaction. Most peptides did not efficiently compete with the DPP9-SUMO1 interaction (Fig. 2C). Also a peptide that includes Val38 and Lys39, corresponding to the β-sheet of the SIG (peptide 5, labeled SIG), did not affect binding of DPP9 to SUMO1. In contrast, preincubation of DPP9 with a peptide covering amino acids 61–75 of SUMO1 (SLRLFEGQRIADNH, peptide 9) strongly reduced the binding of DPP9 to the SUMO1 beads (Fig. 2C). Next we tested whether SUMO1-peptide 9 can displace SUMO1 from the respective DPP8-SUMO1 or DPP9-SUMO1
complexes. For this, recombinant DPP8 or DPP9 were first incubated with bead immobilized SUMO1, to allow complex formation. SUMO1-peptides 5 or 9 were then added and the release of bound DPP8 or DPP9 from the SUMO beads was analyzed by SDS-PAGE (Fig. 2D). Elution was possible with SUMO1-peptide 9 but not with the control peptide that corresponds to the SIG (peptide 5).

Next, single amino acids in SUMO1 between phenylalanine 64 and histidine 75 (corresponding to SUMO1 peptide 9) were mutated to alanines. SUMO1 mutants were immobilized on wells of 96-well plates and their interaction with DPP9 was analyzed in ELISA, and compared with their interaction with wild-type SUMO1 (Fig. 2E). In this assay, SUMO1 mutations covering the sequence between phenylalanine 66 to glutamine 69 as well as a triple mutation SUMO1R63A, R70A, H75A resulted in reduced binding of DPP9. Replacement of glutamic acid 67 of SUMO1 with alanine (S1E67A) led to the most drastic decrease in the SUMO1-DPP9 interaction (Fig. 2E). Finally, we immobilized SUMO1, SUMO1E67A, and SUMO1K37AV38A on beads and tested for interaction with DPP9 and the SIM-containing proteins Uba2 and GST-PIAS3. As shown in Fig. 2F, Uba2 and PIAS3 interacted with SUMO1E67A but not with SUMO1K37AV38A. On the other hand, DPP9 interacted with the SUMO1 SIG mutant but not with SUMO1E67A. These results verify that the sequence covering Phe66-His75, and specifically glutamic acid in position 67 with SUMO1E67A. These results verify that the sequence covering the sequence between phenylalanine 66 to glutamine 69 as well as a triple mutation SUMO1R63A, R70A, H75A resulted in reduced binding of DPP9. Replacement of glutamic acid 67 of SUMO1 with alanine (S1E67A) led to the most drastic decrease in the SUMO1-DPP9 interaction (Fig. 2E). Finally, we immobilized SUMO1, SUMO1E67A, and SUMO1K37AV38A on beads and tested for interaction with DPP9 and the SIM-containing proteins Uba2 and GST-PIAS3. As shown in Fig. 2F, Uba2 and PIAS3 interacted with SUMO1E67A but not with SUMO1K37AV38A. On the other hand, DPP9 interacted with the SUMO1 SIG mutant but not with SUMO1E67A. These results verify that the sequence covering Phe66-His75, and specifically glutamic acid in position 67 of SUMO1 is important for the DPP9-SUMO1 interaction, but not the SIG (Fig. 2F). We termed the sequence covering Phe66-His75 as the E67-interacting loop (EIL) to differentiate this sequence from the well characterized SIG that mediates binding to the SIM. The EIL in SUMO1 is located to the loop that connects the third and fourth β-sheets of SUMO1. The EIL is spatially separated and distinct from the SIG motif, on the “opposite” side of the SIG (Fig. 2G).

Gain of Binding by Point Mutations in SUMO2—To get more insight into the preferential interaction of DPP9 with SUMO1, we analyzed the homology between the SUMO paralogs in the newly identified EIL, and flanking regions. Sequence alignment shows that glutamic acid 67 in SUMO1 is replaced by aspartic acid in SUMO2; because both amino acids are negatively charged, we assumed that this could not explain the strong preference in binding of DPP9 to SUMO1 (Fig. 3A and supplemental Fig. 2). The alignment highlighted four amino acids that are conserved in SUMO1 and SUMO2. We mutated single amino acids in SUMO2 to the corresponding ones in SUMO1 and tested their interaction with recombinant DPP9.

Strikingly, mutation of a single amino acid in SUMO2, aspartic acid 71 to the corresponding histidine residue in SUMO1, resulted in a full gain of binding to DPP9 (Fig. 3, B and C). In contrast to DPP9, Uba2 interacted with all the SUMO2 variants to the same extent. Taken together, these results show that the negative charge in SUMO2 by aspartic acid 71 prevents interaction with DPP9.

SUMO1 Binds to an Arm-like Structure in DPP9—In parallel, we aimed to identify the surface in DPP9 that interacts with SUMO1. For this we constructed several DPP9 mutants, concentrating first on amino acids in the propeller domain of DPP9, assuming that it is important for protein-protein interaction with DPP9, assuming that it is important for protein-protein interactions. Mutants were expressed and purified from HEK293T cells and tested for binding to immobilized SUMO1 beads. Using this approach, we identified a cluster of hydrophobic amino acids in DPP9 that are essential for binding to SUMO1. Replacement of valine 285, isoleucine 288, or valine 290 by alanines resulted in a strong loss of interaction with SUMO1 (Fig. 4A). Strikingly, the single point mutation DPP9-V285A completely abolished the DPP9-SUMO1 association. Mutation in neighboring residues, such as in valine 287, did not affect the binding. To better understand where the SUMO1-binding surface is localized, we turned to a published homology model of DPP9, based on solved structures of other members of the DPPIV family: DPPIV, DPPX, and fibroblast activation protein α (40). A similar model was published by (41). Homology models were successfully used for analyzing and designing inhibitors with increased specificity for DPP8 and DPP9 over DPPIV (42). Crystal structures of members of the DPPIV family show that they all form homodimers, where each monomer is build of two domains, a barrel-like α/β hydrolase and an eight-blade β propeller. An extended arm-like structure projects from blade 4 of the propeller, and is located next to a side opening in DPPIV, which is formed between the hydrolase and the propeller (43–49).

Both published DPP9 homology models predict that valine 285, isoleucine 288, and valine 290 locate to the extended arm of DPP9 (40, 41) (Fig. 4, B and C). We therefore asked whether a peptide corresponding to the extended arm of DPP9...
SUMO1, and define this surface as the SUMO-binding arm (SUBA). We tested if SUMO1 binds to DPP9 by co-exprressing HA-tagged SUMO1 with DPP9 in HEK293T cells. SUMO1 was immunoprecipitated using anti-HA antibodies. The SUMO1/peptide mixture was then added to immobilized DPP9, and interacting SUMO1 was quantified using SUMO1-specific antibodies. Control wells were coated with ovalbumin instead of DPP9, and SUMO1 binding reflects background binding.

(285VEIHVPSALEERKTDSYR305) would inhibit the SUMO1-DPP9 interaction. We tested this hypothesis by incubating SUMO1 with the DPP9-arm peptide prior to incubation with immobilized DPP9. As shown in Fig. 4D, the DPP9-arm peptide competed with DPP9 for binding to SUMO1 in a concentration-dependent manner. Binding of SUMO1 to DPP9 was unaffected in the presence of the same concentrations of a control peptide, or a shorter peptide corresponding to only part of the arm (Fig. 4D and data not shown). Taken together, we conclude that the arm of DPP9 mediates its interaction with SUMO1, and define this surface as the SUMO-binding arm (SUBA) of DPP9.

The SUMO-binding Arm of DPP9 Regulates Enzymatic Activity—The DPPIV arm structure is localized to the dimer interface of DPPIV. Recently, Tang et al. (50) showed that DPPIV mutants, deleted of the arm motif fail to dimerize and are less active. However, they found that the arm of DPP9 was not essential for dimerization, because DPP9 construct lacking a large part of the predicted arm (amino acids corresponding to Ile288-Tyr305) is correctly folded and forms dimers. Interestingly, the deletion mutant was less active compared to the wild-type enzyme (50). The reason for the reduced activity is currently not understood.

The deletion mutant of DPP9 reported by Tang et al. (50) only partially covers the SUBA, because it does not include valine 285, which shows the most dramatic loss in interaction with SUMO1 if replaced by an alanine. Therefore, we tested both the activity of the DPP9V285A mutant, and its ability to form dimers as the wild-type enzyme.

To test whether the DPP9V285A mutant forms dimers, we co-expressed HA- and FLAG-tagged DPP9V285A in HEK293T cells. We then tested whether HA-tagged DPP9V285A co-purifies with FLAG-tagged DPP9V285A in co-immunoprecipitation assays. Wild-type HA or FLAG-tagged DPP9 was transfected in control cells. As shown in Fig. 5A, both wild-type and the DPP9V285A mutant form dimers, which are stable after extensive washing conditions (Fig. 5A). We then compared the activity of recombinant DPP9 wild-type and the V285A mutant. Strikingly, we found that the V285A mutant shows about 60% loss in activity compared with the wild-type enzyme.

These results suggest that the reduced interaction of DPP9V285A with SUMO1, and the reduced activity of the enzyme, is not due to unfolding or lack of dimerization of the DPP9 mutant. Thus, we conclude that the SUMO1-binding arm of DPP9 overlaps with a surface important for its enzymatic activity.

Next, we asked whether SUMO1 influences the enzymatic activity of DPP9. We tested this question by measuring GP-AMC hydrolysis by DPP9 in the presence of recombinant SUMO1. For control, activity was compared with samples containing DPP9 alone. As shown in Fig. 6A, the hydrolysis rate of GP-AMC by DPP9 was higher in the presence of SUMO1 in comparison to control reactions (Fig. 6A). In contrast, the presence of SUMO2, or SUMO2R61L did not affect the enzymatic
activity of DPP9 (Fig. 6B). Activation was also not observed in the presence of SUMO1 mutated in a single amino acid: SUMO1E67A, which cannot bind to DPP9 (Fig. 6C). On the other hand, DPP9 activity was increased in the presence of SUMO2D71H, which can interact with DPP9 (Fig. 6D). Importantly, in contrast to wild-type DPP9, the SUBA DPP9V285A mutant was not activated by SUMO1 (Fig. 6E). We tested also whether in its conjugated form, SUMO1 can activate DPP9. In this assay we analyzed RanGAP as a model protein, because RanGAP alone does not affect DPP9 activity (Fig. 6F and supplemental Fig. 3). As shown in Fig. 6F, when incubated with sumoylated RanGAP, DPP9 activity is clearly increased. Activation was not observed when RanGAP was modified with SUMO2 (not shown).

Taken together, these results show that DPP9 activity can be stimulated by either free or conjugated SUMO1. Activation correlates with the ability of SUMO1 and DPP9 to interact, strongly suggesting that it depends on direct association between the two proteins.

Next, we investigated whether SUMO1 is important for DPP9 activity in cells. Previously, we tested for prolyl peptidase activity in HeLa cell lysates treated with siRNA oligonucleotides against DPP8 and DPP9. We found that down-regulation of DPP9 but not DPP8, strongly reduces the capacity of the lysates to degrade proline containing peptides, including the artificial substrate GP-AMC (31). If SUMO1 indeed regulates the activity of DPP9 in cells, then down-regulation of SUMO1 should result in reduced DPP9 activity, as measured by the capacity to cleave GP-AMC. To test this, we transfected HeLa cells with two different siRNA oligos designed to target SUMO-1, and tested for cleavage of GP-AMC in these lysates. Fig. 6G shows the effective down-regulation of SUMO1 in these cells compared with control cells transfected with noncoding siRNA. Shown in the Western blot is the sumoylated form of RanGAP1, which is the most prominent SUMO-1-modified protein (Fig. 6F). Importantly, cells that were knocked-down for SUMO-1 showed reduced hydrolysis of GP-AMC compared with control cells. Taken together, these results suggest that SUMO1 acts as a positive regulator of DPP9, and that this activation is physiologically relevant in the cell.

**DISCUSSION**

**EIL, a Novel Surface for Noncovalent Interactions of SUMO1**—Downstream events of sumoylation are in most cases due to changes in interactions of the modified proteins. Because some substrates are sumoylated preferentially by one SUMO paralog (e.g. Refs. 37 and 51–54) it is presumed that SUMO receptors can distinguish between the different SUMO proteins. Here we report the identification of DPP8 and DPP9 as such novel SUMO1-specific receptors: they bind in a noncovalent manner preferentially to SUMO1.
Surprisingly, the DPP8 and DPP9 interaction with SUMO1 does not involve the groove of SUMO1, which interacts with the SIM. Instead, we identified a novel interaction surface on SUMO1, between amino acids Phe^{66} and His^{73}, which we term EIL. The EIL is located to the third and fourth β-sheets of SUMO1 and the connecting loop. Strikingly, a single point mutation in glutamic acid 67 in the connecting loop completely abolishes the interaction between DPP9 and SUMO1.

Previously, crystal structures of the SUMO-conjugating enzyme Ubc9 in complex with SUMO showed that noncovalent interaction between Ubc9 and SUMO also includes the surface covering the EIL (55–58). However, in contrast to DPP9, Ubc9 does not differentiate between the SUMO paralogs. Using a mutagenesis approach we find that mutation of a single amino acid in SUMO2, aspartic acid 71, to the corresponding histidine residue in SUMO1 leads to a full gain of binding between SUMO1 and DPP9. These results show that the negative charge in the SUMO2-EIL prevents association with DPP9, and explains the preferential interaction of DPP9 with SUMO1. Our results are in agreement with a recent publication showing that the SUMO-isopeptidases SENP6 and SENP7 interact noncovalently with a surface overlapping the EIL, and do not deconjugate substrates modified by SUMO2 mutated in D71H and N68A (59). These results demonstrate the importance of charge difference in the EIL motif of the SUMO proteins for paralog-specific interactions.

Importantly, the EIL is located on the opposite side of the SIG, on a loop connecting the third and fourth β strands. In comparison, the surface involved in most noncovalent interactions of ubiquitin includes the Ile^{44} hydrophobic patch (located to the third β strand). In the case of the UIM and CUE domains, for example, this interaction is further stabilized by amino acids on the connecting loop of ubiquitin: Ala^{46} and Gly^{47} (Refs. 60 and 61, and reviewed in Ref. 62) (supplemental Fig. 4). These observations show that although the loop of ubiquitin and SUMO differ in their surface charge due to a different amino acid sequence, in both proteins, the loop structure is important for noncovalent interactions, pointing to an evolutionary conservation of this surface for noncovalent interactions of ubiquitin and ubiquitin-like proteins.

Taken together these results show that the EIL functions as a second and independent surface of SUMO for noncovalent interactions, not only with enzymes of the sumoylation pathway, but also for interactions with downstream effectors. It still remains to be shown whether DPP9 is the only downstream effector that interacts with SUMO1 via the EIL, or whether it is shared by additional SUMO1-receptor proteins.

**Allosteric Regulation of DPP9 by SUMO1**—Here we identified the SUBA in DPP9. Interestingly, the SUBA overlaps with a region important for DPP9 activity, because the SUBA mutant is less active compared with the wild-type enzyme. In line with this, free or conjugated SUMO1 stimulates DPP9 activity. Activation is dependent on direct association between DPP9 and SUMO1, because SUMO1E67A and SUMO2 do not activate DPP9, whereas, SUMO2D71H does stimulate enzymatic activity. Finally, SUMO1 does not activate DPP9V285A, mutated in the SUBA.

DPP9 homology models predict that the SUBA is located next to a side opening, formed between the hydrolase and the propeller of DPP9. A crystal structure of DPPIV with a decapeptide suggests that substrates enter the hydrolase domain of DPPIV via this side opening, and not through the propeller funnel (46). The predicted proximity of the SUBA to the substrate entrance cavity raises the intriguing possibility that SUMO1 may activate DPP9 by regulating the access of substrates into the hydrolase domain, or stabilizing a more “active” state of the peptidase (Fig. 7), suggesting that SUMO1 acts as an allosteric regulator of the peptidase.

Furthermore, using siRNA oligonucleotides we found that down-regulation of SUMO1 results in a slower cleavage of GPAMC compared control cells. These findings are important because they show that the levels of endogenous SUMO1 affect the activity of endogenous DPP9 in HeLa cell extracts, and suggest that the activation of DPP9 by SUMO-1 is physiologically relevant. Because SUMO1 is usually found in cells in a conjugated form, it is more likely that an unknown protein regulates DPP9 activity upon sumoylation. Such a scenario would give more specificity to the regulation of DPP9, because it would involve both the SUBA and also other surfaces of DPP9. Sumoylation would initiate the interaction.

How SUMO1 activates DPP9 in cells is currently unclear. One possibility is that sumoylation of a substrate protein would be the cue to target this protein for processing by DPP9, and for stimulating the activity of the peptidase (protein X, Fig. 7, scenario A (marked by arrow)). In an alternative model, DPP9 is regulated by sumoylation of a specific DPP9-interacting protein (protein X, Fig. 7, scenario B (marked by arrow)). In this case, protein X would interact only weakly with DPP9. Sumoylation of protein X leads to a stronger association with DPP9, and activation of the peptidase. In both cases SUMO-specific isopeptidases would terminate the activation.
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