PDSM, a motif for phosphorylation-dependent SUMO modification

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SUMO (small ubiquitin-like modifier) modification regulates many cellular processes, including transcription. Although sumoylation often occurs on specific lysines within the consensus tetrapeptide ΨKxE, other modifications, such as phosphorylation, may regulate the sumoylation of a substrate. We have discovered PDSM (phosphorylation-dependent sumoylation motif), composed of a SUMO consensus site and an adjacent proline-directed phosphorylation site (ΨKxExxSP). The highly conserved motif regulates phosphorylation-dependent sumoylation of multiple substrates, such as heat-shock factors (HSFs), GATA-1, and myocyte enhancer factor 2. In fact, the majority of the PDSM-containing proteins are transcriptional regulators. Within the HSF family, PDSM is conserved between two functionally distinct members, HSF1 and HSF4b, whose transactivation capacities are repressed through the phosphorylation-dependent sumoylation. As the first recurrent sumoylation determinant beyond the consensus tetrapeptide, the PDSM provides a valuable tool in predicting new SUMO substrates.

heat-shock factor | heat-shock protein | transcription

SUMO (small ubiquitin-like modifier) is covalently linked by an isopeptide bond to lysine residues in their substrates (1). SUMO conjugation often requires a consensus sequence ΨKxE (Ψ, large hydrophobic residue; x, any amino acid) around the target lysine (2). Because not all available SUMO consensus sites are modified, other factors must also affect the target site specificity. For example, phosphorylation of the substrate has been shown to regulate the sumoylation both positively and negatively (3–5). However, apart from the ΨKxE tetrapeptide, no common regulatory motifs involved in SUMO conjugation are known (1). The SUMO family consists of four members, SUMO-1, -2, -3, and -4, of which SUMO-1 is best studied; SUMO-4 has been so far detected only at the RNA level (1, 6). SUMO-1 and SUMO-2/3 have, at least partially, distinct substrates and regulation. For example, the general pattern of conjugates and the subnuclear distribution are different between SUMO-1 and SUMO-2/3, and the amount of conjugated SUMO-2/3, but not SUMO-1, is increased upon protein-damaging stresses (7, 8). Beyond this, the functional differences between the SUMO paralogs are largely unknown.

The mammalian HSF family is composed of three members, HSF1, HSF2, and HSF4. HSF1 is the mammalian counterpart of the single invertebrate HSF and is indispensable for the heat-shock response (9). Upon stress, HSF1 is activated by trimerization-induced DNA binding and hyperphosphorylation (10, 11). Moreover, HSF1 is sumoylated on a conserved lysine, lysine-298 (4, 12). We have demonstrated that HSF1 sumoylation depends on phosphorylation of an adjacent site, serine-303 (4). Sumoylation was originally suggested to induce HSF1 DNA binding and to be needed for HSF1-mediated transcription (12). This hypothesis was, however, questioned by our previous study, showing that HSF1 could be fully activated also when the sumoylation was prevented (4). Therefore, the biological consequences of HSF1 sumoylation require further clarification. HSF4 differs functionally from HSF1 (10). HSF4 is constitutively trimeric and exists in two alternatively spliced isoforms; HSF4b displays transactivation capacity, and HSF4a does not (13, 14). Recently, HSF4 was shown to be critical for lens development (15, 16). In lens fiber cells, HSF4b is needed for proper expression of γ-crystallins and small heat-shock proteins (Hsps), whereas in lens epithelial cells HSF4b seems to repress expression of fibroblast growth factors FGF-1, FGF-4, and FGF-7 (16). Thus, HSF4b may function as both a transcriptional activator and repressor in a context-dependent manner. Because the post-translational regulation of HSF4 activity is unexplored, the reasons behind this specificity are not known.

Here we describe a recurrent motif, ΨKxExxSP, involved in phosphorylation-dependent sumoylation of numerous mutually unrelated transcriptional regulators, such as erythroid transcription factor GATA-1 and myocyte-specific enhancer factor 2A (MEF2A), as well as two functionally distinct members of the heat-shock factor family, HSF1 and HSF4b. The bipartite motif, named PDSM (phosphorylation-dependent sumoylation motif), comprises a SUMO consensus site and a proline-directed phosphorylation site, separated by two amino acids. We also demonstrate that this motif can be used to predict novel SUMO substrates. Our functional analyses revealed that sumoylation of the PDSM efficiently represses transactivation capacity of both HSF1 and HSF4b.

Materials and Methods

Plasmid Constructs. For plasmid construction, see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Cell Culture and Transient Transfection. Cell culture and transfections were done as described in ref. 4 (see *Supporting Materials and Methods*).

Immunoprecipitation and Western Blotting. *In vivo* immunoprecipitation experiments were performed as described in ref. 4 (see *Supporting Materials and Methods*). For standard immunoblots, cleared cell lysates (25–35 μg of protein) in lysis buffer were resolved on SDS/PAGE, transferred to nitrocellulose, and blotted with anti-Myc (Sigma), anti-FLAG (Sigma), anti-HSF1 (17),

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Abbreviations: SUMO, small ubiquitin-like modifier; HSF, heat-shock factor; PDSM, phosphorylation-dependent sumoylation motif; MEF, myocyte-specific enhancer factor; ERR, estrogen-related receptor; SENP, SUMO/sentrin-specific protease.

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anti-HSF4 (14), or anti-Hsc70 (SPA-815; StressGen Biotechnologies, Victoria, Canada) antibodies.

EMSA. Whole-cell extracts (15 μg) were incubated with a ^{32}P-labeled oligonucleotide representing the proximal heat-shock element of the human *hsp70* promoter (18) or the GAL4-binding site (19). Samples were resolved on 4% native PAGE and visualized by autoradiography as described in ref. 18.

***In Vivo* ^{32}P-Labeling and Phosphopeptide Mapping.** Plasmids encoding HSF4b WT or S299A, GATA-1 WT or S142A, and MEF2A WT or S408A were transfected into Cos-7 cells 24 h before the labeling. The labeling and subsequent analyses were performed as described in ref. 20 with slight modifications (see *Supporting Materials and Methods*).

***In Vitro* Sumoylation.** Reticulocyte-lysate translated GATA-1 was incubated at 30°C for 2 h in 20 mM Hepes (pH 7.4), 110 mM potassium acetate, 2 mM magnesium acetate, and 5 mM $MgCl_2$ together with 0.1 μg of SAE1/2, 0.4 μg of Ubc9, 0.1 μg of PIASy, and 1 μg of SUMO-1 (21). Samples were resolved by SDS/PAGE and visualized by fluorography. For more details see *Supporting Materials and Methods*.

Luciferase Reporter Assays. Transiently transfected cells were snap-frozen and lysed in passive lysis buffer (Promega) according to the manufacturer's instructions. The cell lysates were cleared by centrifugation (15,000 $\times$ *g* for 2 min), and the firefly luciferase activity produced by the pG5luc reporter plasmid (Promega) was measured by a Luminoskan Luminometer (Labsystems) by using luciferase assay reagent (Promega) as a substrate. The luciferase activity was normalized by using Rous sarcoma virus (RSV) promoter-driven β-galactosidase as an internal control by incubating cell lysates in 100 mM phosphate buffer (pH 7.0) with 0.670 mg/ml *o*-nitrophenyl β-D-galactoside/1.0 mM $MgCl_2$/45 mM 2-mercaptoethanol at 37°C and measuring the absorbance at 420 nm.

Quantitative Real-Time RT-PCR. RNA was isolated by using the RNeasy kit (Qiagen, Valencia, CA). For each sample, 1 μg of RNA was treated with RQ1 DNase (Promega) and reverse-transcribed by using Moloney murine leukemia virus RNase H (−) (Promega). *hsp70* and *gapdh* primers and probes (Cybergene, Huddinge, Sweden) were used at final concentrations of 300 nM and 200 nM, respectively. Final concentrations for *hsp27* were 1,000 nM (primers) and 110 nM (probe). For primers, probes, and PCR conditions, see *Supporting Materials and Methods*. ABsolute QPCR ROX Mix (Advanced Biotechnologies, Columbia, MD) was used to prepare the reaction mixes. Relative quantities of *hsp70* and *hsp27* mRNAs were normalized against *gapdh*, and the fold induction from mock-transfected control sample was calculated. All reactions were made in duplicates in two separate runs with samples derived from four biological repeats.

Results

Many SUMO Substrates Contain a ΨKxExxSP Motif. We showed earlier that sumoylation of HSF1 depends on phosphorylation (4). The proximity of the HSF1 SUMO target site (K298) to the regulatory proline-directed serine (SP) site (S303) led us hypothesize that a similar mechanism could be involved in sumoylation of other proteins. Therefore, we searched the Swiss-Prot protein database for human proteins containing the motif ΨKxExxSP and qualified only those proteins in which this motif was conserved between the human and mouse orthologs. As shown in Table 1, 48 human proteins met these criteria. Strikingly, 71% of the proteins with the motif are involved in transcriptional regulation. Moreover, within many protein families, including

Table 1. Many mutually unrelated transcriptional regulators contain a conserved ΨKxExxSP motif

Protein	Accession	Motif (aa)
Transcriptional regulators		
ATF6-β	Q99941	87–94; 198–205
Bcl-2-associated transcription factor 1	Q9NYF8	490–497
BTB/POZ domain-containing protein 4	Q86UZ6	228–235
c-Myb	P10242	526–533
ETS translocation variant 1 (ER81)	P50549	88–95
ETS translocation variant 4 (E1A-F)	P43268	162–169
ETS translocation variant 5 (ERM)	P41161	88–95
Forkhead box protein C2 (FKHL14)	Q99958	213–220
GATA-1	P15976	136–143
H3 K4-specific MLL3	Q8NEZ4	2822–2829
HSF1	Q00613	297–304
HSF4b	Q9ULV5	293–300
Jumonji/ARID domain-containing protein 1C	P41229	281–288
MEF2A	Q02078	402–409
MEF2C	Q06413	390–397
MEF2D	Q14814	438–445
Nuclear receptor coactivator 2 (NCoA-2)	Q15596	730–737
Nuclear receptor corepressor 1 (N-CoR1)	O75376	1105–1112
NFAT5	O94916	555–562
PPAR-gamma	P37231	106–113
Smad nuclear interacting protein 1 (SNIP-1)	Q8TAD8	29–36
Nuclear receptor ERRalpha	P11474	110–117
Nuclear receptor ERRbeta	O95718	16–23
Nuclear receptor ERRgamma	P62508	39–46
Sterol regulatory element binding protein-2	Q12772	463–470
Transcription factor MEL1	Q9HAZ2	751–758
Transcription factor SOX-3	P41225	374–381
Transcription factor SOX-8	P57073	338–345
Transcription factor SOX-9	P48436	397–404
Thyroid hormone receptor beta-1	P10828	49–56
Transcription cofactor vestigial-like protein 2	Q8N8G2	63–70
Zinc finger protein 295	Q9ULJ3	429–436
Zinc finger protein ZFPM1 (FOG-1)	Q8IX07	498–505
Zinc finger protein ZFPM2 (FOG-2)	Q8WW38	470–477
Others		
ATR-interacting protein	Q8WXE1	233–240
Collagen alpha 1 (II) chain precursor	P02458	238–245
Collagen alpha 1 (III) chain precursor	P02461	859–866
Ecotropic virus integration 1 site protein	Q03112	532–539
Macrophage receptor MARCO	Q9UEW3	374–381
Metabotropic glutamate receptor 4 precursor	Q14833	579–586
Metabotropic glutamate receptor 7 precursor	Q14831	582–589
Metabotropic glutamate receptor 8 precursor	O00222	575–582
Neurofilament triplet H protein	P12036	Several
PH domain-containing protein family A 5	Q9HAU0	647–654
Ras GTPase-activating-like protein IQGAP3	Q86VI3	48–55
Rho GTPase-activating protein 7	Q96QB1	802–809
RNA-binding protein 10	P98175	534–541
Zinc finger protein 106 homolog (Zfp-106)	Q9H2Y7	1199–1206

Human proteins containing a [I, L, V]-K-X-E-X-X-S-P motif were obtained from the Swiss-Prot protein database using the ScanProsite tool.

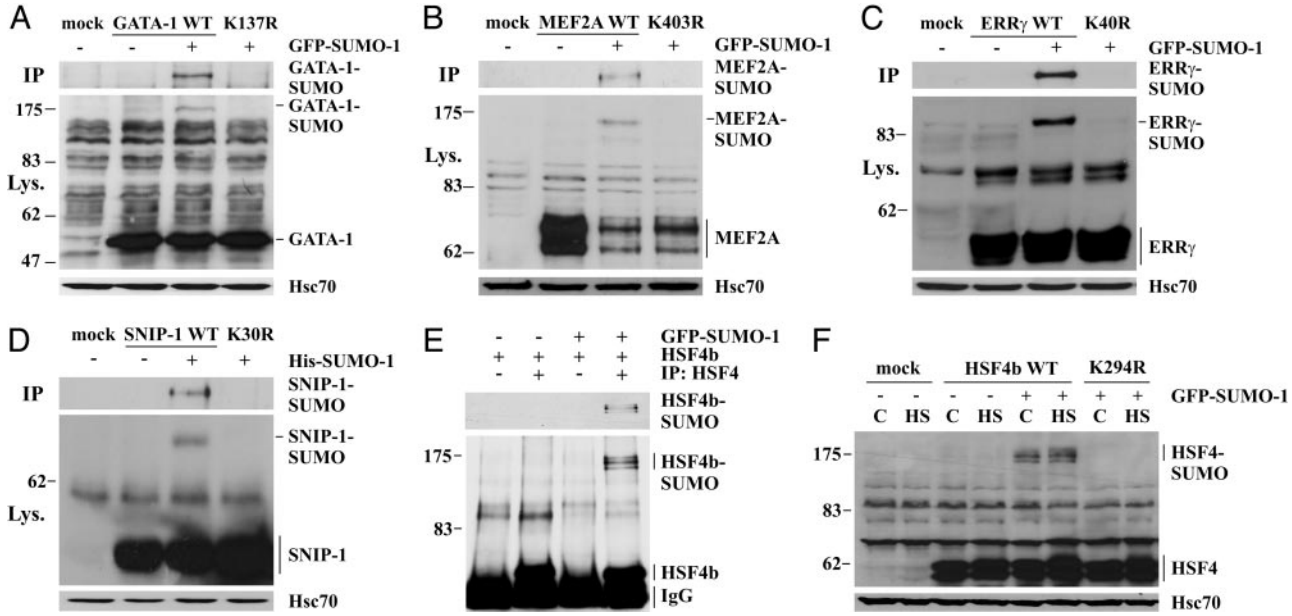

Fig. 1. Several proteins containing the ΨKxExxSP motif are sumoylated. (*A–D*) Cos-7 cells were transfected with empty plasmid (mock) or GATA-1-MycHis (WT or K137R), MEF2A-MycHis (WT or K403R), FLAG-ERRγ (WT or K40R), SNIP-1-MycHis (WT or K30R), together with GFP-SUMO-1, or His-SUMO-1 as indicated. After immunoprecipitation (IP) with antibodies against the epitope tag (Myc or FLAG), sumoylated species were detected by Western blotting with SUMO-1-specific (*A*, *C*, and *D*) or GFP-specific (*B*) antibodies. Sumoylated species are also seen in the input samples (Lys.). (*E*) Cos-7 cells were transfected with HSF4b and GFP-SUMO-1 as indicated. After immunoprecipitation with anti-HSF4 antibodies, HSF4b sumoylation was detected by Western blotting with SUMO-1- or HSF4-specific antibodies. (*F*) Cos-7 cells were transfected with an empty plasmid (mock), WT HSF4b, K294R, and GFP-SUMO-1 as indicated. Cells were exposed to heat shock (HS; 15 min at 42°C) or left untreated (C). HSF4b sumoylation was detected by Western blotting with anti-HSF4 antibodies. Hsc70 is shown for equal loading.

the estrogen-related receptor (ERR) nuclear receptors, ETS translocation variants, MEF2, SOX, and HSFs, there are several motif-containing members. In addition to HSF1, at least GATA-1, MEF2s, c-Myb, nuclear receptor coactivator 2 (glucocorticoid receptor interacting protein 1), peroxisome proliferator-activated receptor γ, SOX-3, and sterol regulatory element-binding protein 2 have been identified as SUMO substrates (21–27).

In agreement with the earlier studies, we observed SUMO modification of GATA-1 and MEF2A, and site-directed mutagenesis verified that SUMO is specifically targeted to the lysine within the conserved motif (Fig. 1 *A* and *B*) (21, 26). To test whether the motif can predict sumoylation of previously unknown substrates, we analyzed sumoylation of three mutually unrelated proteins containing the ΨKxExxSP motif: Smad nuclear interacting protein 1 (SNIP-1), nuclear receptor ERRγ, and HSF4b. All three proteins were prominently sumoylated when coexpressed with SUMO-1 in Cos-7 cells (Fig. 1 *C–F*). Furthermore, all three proteins were specifically modified on the conserved motif, as demonstrated by lack of sumoylation when the target lysine was mutated to arginine. It should be noted that the HSF4a isoform lacks the conserved motif and is not sumoylated (see Fig. 4, which is published as supporting information on the PNAS web site). Taken together, our data demonstrate that the ΨKxExxSP motif can be used to predict novel SUMO target proteins.

Phosphorylation-Dependent Sumoylation of the PDSM. We wanted to investigate whether phosphorylation dependency is a general feature for sumoylation of the motif-containing proteins. Because posttranslational modifications of HSF4b have remained unexplored, we performed *in vivo* ^{32}P-labeling to examine whether HSF4b is phosphorylated. Autoradiography of HSF4 immunoprecipitates showed a strong ^{32}P-labeled band that was not present in the mock-transfected samples, indicating that HSF4b is a phosphoprotein (Fig. 2*A*). We also noticed a modest induction (1.7-fold) of HSF4b phosphorylation by heat shock (Fig. 2*A*). Phosphoamino acid analysis revealed that, similarly to HSF1, HSF4b was phosphorylated on serine residues (Fig. 2*B*). Although the alanine mutant of S299, within the conserved motif of HSF4b, was phosphorylated, the phosphate incorporation was consistently lower when compared with the WT (Fig. 2*A*). Five prominent phosphopeptides were detected in the phosphopeptide map of WT HSF4b (Fig. 2*C*), suggesting that HSF4b is phosphorylated on several sites. However, one of the spots was absent from the map of S299A (Fig. 2*C*), which demonstrates *in vivo* phosphorylation of HSF4b on S299. Next, we addressed the hypothesis that HSF4b sumoylation displays a phosphorylation dependency similar to that of HSF1. Indeed, the S299A mutation strongly inhibited HSF4b sumoylation (Fig. 2*D*), showing that HSF4b sumoylation was phosphorylation-dependent. The finding that the S299A mutation also inhibited SUMO-2 modification of HSF4b (Fig. 2*E*) indicates that the motif does not convey SUMO isoform-specific conjugation. In contrast to SUMO-1, SUMO-2 modification was induced by heat stress, which is likely because of the overall increase in SUMO-2 modification upon heat stress (7).

To assess whether the phosphorylation-dependent sumoylation reaches beyond the HSF family, we analyzed GATA-1 and MEF2, which have previously been shown to be phosphorylated on the motif (28, 29). In agreement with the previous reports, our phosphopeptide mapping analyses verified that both GATA-1 and MEF2A are prominently phosphorylated on the serine residues within the motif (Fig. 2*F*). Similarly to HSFs, sumoylation of GATA-1 and MEF2A was efficiently prevented by mutating the phosphoacceptor sites to alanine (Fig. 2 *G* and *H*). Morever, acidic substitution of the phosphoacceptor sites partially (GATA-1; Fig. 2*G*) or completely (MEF2A; Fig. 2*H*) rescued the sumoylation, supporting the idea that sumoylation of the motif requires prior phosphorylation.

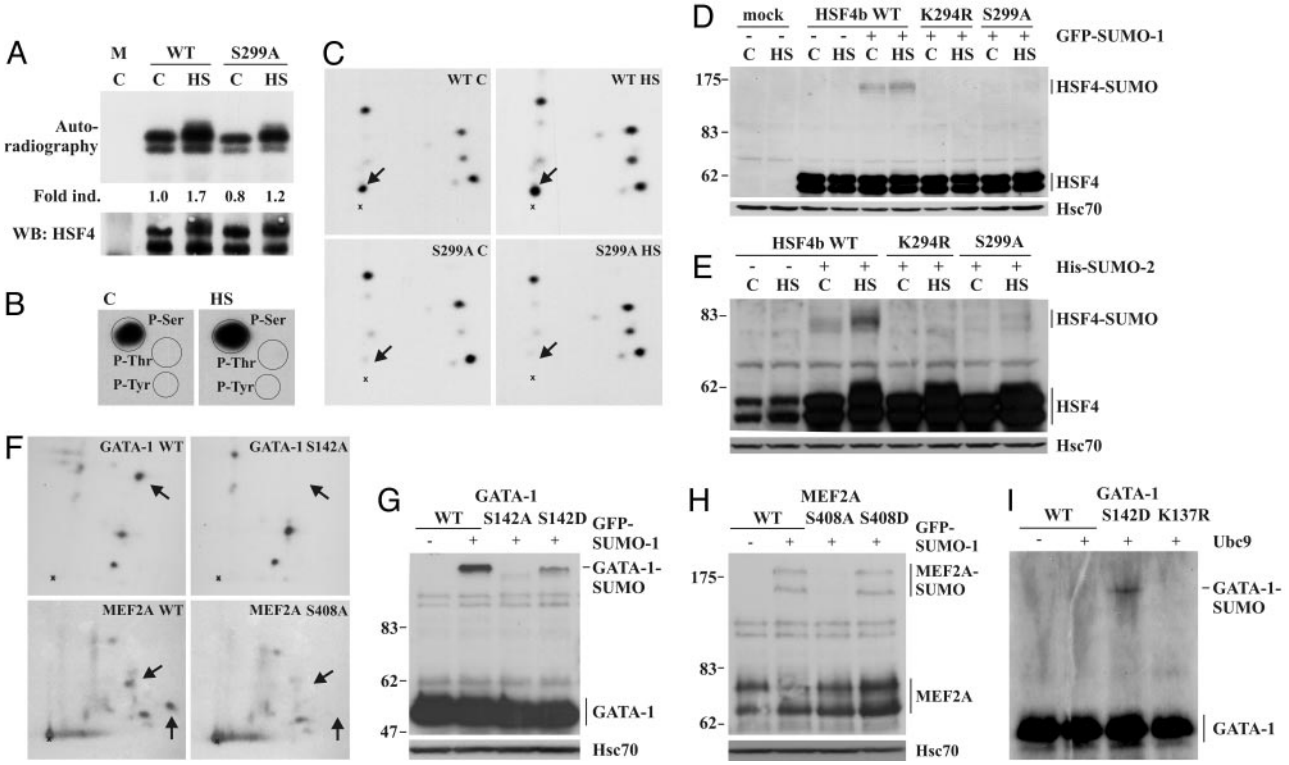

Fig. 2. Phosphorylation-dependent sumoylation of the PDSM. (*A*) Cos-7 cells were transfected with WT HSF4b, S299A, or empty plasmid (M), labeled with ^{32}P, and exposed to heat shock (HS; 1 h at 42°C) or left untreated (C). HSF4 was immunoprecipitated, and phosphorylation was detected by autoradiography. Quantification was made by phosphorimager (Fuji) from three independent experiments. Equal loading is shown by Western blotting with anti-HSF4 antibodies. (*B*) Phosphoamino acid analysis of the ^{32}P-labeled HSF4b shows phosphorylation on serine residues in nonstressed (C) and heat-shocked (HS) cells. (*C*) Two-dimensional phosphopeptide maps of WT HSF4b and S299A from untreated (C) and heat-shocked (HS; 1 h at 42°C) samples. Arrows indicate phosphopeptides missing from the maps of HSF4b S299A. (*D* and *E*) Cos-7 cells were transfected with empty plasmid (mock), WT HSF4b, K294R, or S299A, together with GFP-SUMO-1 or His-SUMO-2 as indicated. Cells were exposed to heat shock (HS; 15 min at 42°C) or left untreated (C). HSF4b sumoylation was detected by Western blotting with anti-HSF4 antibodies. Hsc70 is shown for equal loading. (*F*) Phosphopeptide maps of ^{32}P-labeled GATA-1 and MEF2A WT and S→A mutants were made as in *C*. Arrows indicate phosphopeptides missing from the maps of the S→A mutants. (*G* and *H*) Cos-7 cells were transfected with GATA-1-MycHis (WT, S142A, or S142D) or MEF2A-MycHis (WT, S408A, or S408D), together with GFP-SUMO-1 as indicated. Cells were lysed in Laemmli sample buffer. Sumoylated species were detected by Western blotting with anti-Myc antibodies. Hsc70 shows equal loading. (*I*) *In vitro* sumoylation of GATA-1. Recombinant SUMO-1, SAE1/2, Ubc9, and PIASy were incubated with ^{35}S-labeled reticulocyte-lysate translated GATA-1 (WT or S142D) at 30°C for 2 h. After SDS/PAGE separation, sumoylation of GATA-1 was detected by fluorography.

Finally, we tested whether phosphorylation of the ΨKxExxSP motif acts directly by promoting sumoylation. To this end, we conducted *in vitro* sumoylation of reticulocyte lysate-translated GATA-1 by purified E1, E2, and E3 enzymes. Consistent with the possibility that phosphorylation directly promotes sumoylation, rather than inhibits desumoylation, GATA-1 with an acidic substitution (S142D) was more efficiently sumoylated *in vitro* than was the unphosphorylated WT GATA-1 (Fig. 2*I*). This finding is in agreement with our earlier observation that a corresponding mutation increases the sumoylation of HSF1 *in vitro* (4). Because the reticulocyte lysate, however, may contain some isopeptidase activity, we cannot exclude the possibility that phosphorylation protects from desumoylation. Taken together, the phosphorylation-dependent sumoylation seems to be a general regulatory mechanism in substrates containing the ΨKxExxSP motif. For conciseness, the motif is called phosphorylation-dependent sumoylation motif and is abbreviated as PDSM.

SUMO Modification of the PDSM Represses HSF4b and HSF1. To functionally characterize the PDSM, we analyzed the transactivating capacity of HSF4b in a GAL4-driven luciferase reporter assay. The GAL4 DNA-binding domain was fused to HSF4b lacking its own DNA-binding domain and oligomerization domain, HR-A/B. WT HSF4b displayed prominent transactivity when compared with mock transfectants (Fig. 3*A*) (14). Intriguingly, mutation of the SUMO target lysine K294 caused an ≈10-fold increase in the HSF4b-mediated transcription (Fig. 3*A*). In addition, the S299A mutation markedly increased the activity of HSF4b (Fig. 3*A*), which suggests that the phosphorylation-dependent sumoylation of the PDSM strongly represses HSF4b. We also used a dominant-negative Ubc9 (dnUbc9) and SUMO-specific protease SENP1, which interfere with the SUMO conjugation and desumoylates substrates, respectively (5, 30). As expected, dnUbc9 and SENP1 clearly derepressed (≈3-fold) the activity of WT HSF4b, but not K294R (Fig. 3*B*), verifying that the repression of transactivating capacity of HSF4b was indeed mediated by SUMO modification.

The repressive effect of sumoylation on HSF4b prompted us to study whether the regulatory function is conserved between HSF4b and HSF1. Indeed, the transactivity of the GAL4-HSF1 chimera was significantly increased when HSF1 sumoylation was prevented by mutating either the SUMO target lysine (K298) or the regulatory phosphorylation site (S303) (Fig. 3*C*). In contrast to S303, mutation of an adjacent serine 307, which is phosphorylated without affecting HSF1 sumoylation (4), did not activate HSF1 (Fig. 3*C*). Similarly to HSF4b, dnUbc9 or SENP1 derepressed the WT HSF1 without affecting the activity of the K298R mutant (Fig. 3*D*). The DNA-binding activities of the GAL4 chimeras were confirmed by EMSA to be equal in the WT

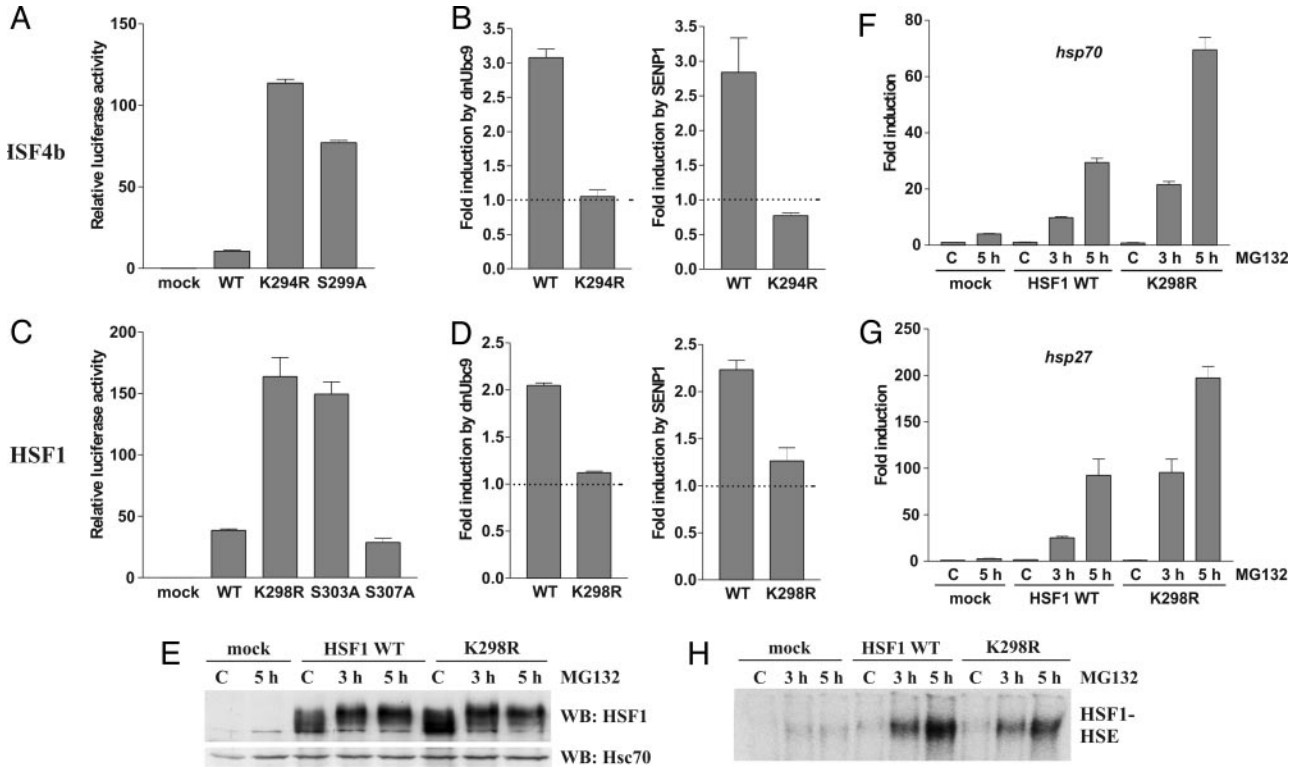

Fig. 3. SUMO modification of the PDSM represses HSF4b and HSF1. (*A*) K562 cells were transfected with GAL4-driven luciferase, RSV-β-galactosidase, and WT GAL-HSF4b, K294R, S299A, or empty plasmid (mock) as indicated. The luciferase activity was normalized against the β-galactosidase activity. The data showing mean values (mean $\pm$ standard error of the mean) from a single experiment with duplicates are representative of four independent experiments. (*B*) The luciferase assay was performed as in *A* except that dnUbc9, SENP1, or empty plasmid was cotransfected with WT GAL-HSF4b and K294R. β-Galactosidase-normalized relative luciferase activity values were calculated as above. The data are presented to show the fold derepression caused by coexpression of dnUbc9 or SENP1. The luciferase activities of WT GAL-HSF4b and K294R without dnUbc9 or SENP1 have been given the value 1. (*C* and *D*) The analyses were performed as in *A* and *B*, but WT GAL-HSF1, K298R, S303A, and S307A mutants were used. (*E*) HSF1-deficient MEFs were transfected with WT HSF1, K298R, or empty plasmid (mock) as indicated. The cells were left untreated or treated with 10 μM MG132 for 3 or 5 h. Expression of HSF1 was analyzed by Western blotting. Hsc70 is shown for equal loading. (*F* and *G*) HSF1-deficient MEFs were transfected and treated as in *E*, and the relative amounts of *hsp70* and *hsp27* mRNAs were measured by quantitative real-time RT-PCR. The values are presented as a fold induction compared with the mock-transfected control sample. The data showing mean values (mean $\pm$ standard error of the mean) from a single experiment with duplicates of two separate PCR runs are representative of four independent experiments. (*H*) HSF1-deficient MEFs were transfected and treated as in *E*. Heat-shock element-binding activity (HSF1-HSE) was analyzed by EMSA.

and nonsumoylatable mutants of HSF4b and HSF1 (data not shown).

Because we have previously shown that HSF1 sumoylation has no severe effect on Hsp70 protein accumulation in response to stress (4), we used a more quantitative method to analyze HSF1 transactivity. WT and K298R HSF1 were transfected into *hsf1*$^{-/-}$ MEFs and activated by the proteasome inhibitor MG132 (Fig. 3*E*) (31). Subsequently, the expression of the endogenous HSF1 target genes *hsp70* and *hsp27* was examined by quantitative real-time RT-PCR. As expected, in the presence of WT HSF1 a robust increase in the mRNA levels was observed (Fig. 3 *F* and *G*). However, when HSF1 sumoylation was prevented by the K298R mutation, the MG132-induced accumulation of *hsp70* and *hsp27* mRNA was consistently enhanced by 2- to 3-fold when compared with the induction by the WT HSF1 (Fig. 3 *F* and *G*). EMSA confirmed that the increased heat-shock gene expression in the absence of HSF1 sumoylation was not due to enhanced DNA-binding activity (Fig. 3*H*). In conclusion, our results demonstrate that sumoylation of the PDSM negatively regulates the transactivating capacity of HSF1 *in vivo*.

Discussion

We have identified a recurrent motif PDSM (ΨKxExxSP) involved in the phosphorylation-mediated SUMO modification of several transcriptional regulators, including HSFs, GATA-1, and MEF2A. In addition to the many PDSM-containing proteins that have already been reported to undergo sumoylation, we identified here three substrates, HSF4b, ERRγ, and Smad nuclear interacting protein 1 (SNIP-1), which are sumoylated on the PDSM. Therefore, the conserved PDSM can be considered a useful tool in predicting new SUMO substrates.

Within PDSM, the spacing between the SUMO target and phosphorylation sites is likely to be crucial, because known SUMO substrates or transcriptional regulators were not highly represented in searches using alternate spacing between these sites. In addition, the close proximity of the sumoylation and phosphorylation sites suggests that the PDSM affects directly the interaction between the substrate and the SUMO-conjugating machinery. Accordingly, replacement of the regulatory serine by a phosphomimetic amino acid stimulated GATA-1 sumoylation *in vitro*, providing evidence that the phosphorylation-mediated increase in steady-state levels of sumoylated substrate is due to enhanced SUMO conjugation. Further detailed analyses are, however, required to understand the structural basis for the function of PDSM. Another important aspect is to identify the regulatory signals and kinase pathways involved in phosphorylation of the PDSM. Interestingly, many proteins listed in Table 1 contain a mitogen-activated protein kinase consensus site, PxSP, within the PDSM. On the other hand, the PDSM in MEF2 was earlier shown to be phosphorylated by Cdk5 (32). Therefore,

it is plausible that the kinases phosphorylating the PDSM are substrate-specific.

In most cases sumoylation impairs the transactivation capacity of the substrate (33), which also applies to the proteins containing PDSM. In addition to HSFs, c-Myb, MEF2 transcription factors, peroxisome proliferator-activated receptor γ, Sox3, and sterol regulatory element-binding protein 2 are repressed by sumoylation (22, 24–27). The biological consequences of sumoylation display some substrate specificity also in the case of proteins with the PDSM. For example, sumoylation does not affect the transactivation capacity of GATA-1 (21), whereas sumoylation of nuclear receptor coactivator 2 (glucocorticoid receptor interacting protein 1) is needed for its action to enhance androgen receptor-mediated transcription (23). Our sumoylation assays show that, upon overexpression, the PDSM can mediate phosphorylation-dependent conjugation of both SUMO-1 and SUMO-2. Whether both SUMO paralogs can endogenously be conjugated to PDSM remains to be explored.

Despite the conserved PDSM in HSF1 and HSF4b, their stress-inducible sumoylation differs markedly. HSF4b is constitutively modified by SUMO, whereas HSF1 sumoylation is induced in a stress-dependent manner (Fig. 1*E*) (4). This difference is likely to reflect the different oligomerization states of these HSFs, because a constitutively trimeric mutant of HSF1 was extensively SUMO-modified also in the absence of stress (Fig. 5, which is published as supporting information on the PNAS web site) (34). HSF4b seems to function as a transcriptional activator or repressor, depending on the target genes and cellular context (16). Therefore, it is tempting to speculate that sumoylation would be involved in switching HSF4b from activator to repressor. Although they are contradictory to the data of Hong *et al*. (12), who reported that sumoylation activates HSF1, our results are well in line with other studies showing that phosphorylation of S303 represses HSF1 activity (35, 36). It seems plausible that the repressive effect of S303 is predominantly due to the phosphorylation-dependent sumoylation of the PDSM. It was also demonstrated earlier that the repression mediated by S303 phosphorylation is relieved by exposure to prolonged severe stress (43°C, 3 or 4 h; refs. 35 and 36). In agreement, the kinetics of HSF1 sumoylation appears to depend on the stress dose; under modest stress conditions (41°C) HSF1 sumoylation is sustained, whereas severe stress (44°C) leads to a more transient modification (Fig. 6, which is published as supporting information on the PNAS web site). Therefore, we propose that the sumoylation of the PDSM provides a buffer capacity for HSF1 activity upon moderate stress, which is removed under severe stress conditions when maximal HSF1 activity is needed (for a model, see Fig. 7, which is published as supporting information on the PNAS web site). Forthcoming studies are aimed to unravel the complex biological ramifications of HSF1 sumoylation, including analyses of genome-wide gene expression patterns and the biological role of HSF1 sumoylation in the development and physiology of vertebrates.

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