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PARP-1 transcriptional activity is regulated by sumoylation upon heat shock

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Heat shock and other environmental stresses rapidly induce transcriptional responses subject to regulation by a variety of post-translational modifications. Among these, poly(ADP-ribosyl)ation and sumoylation have received growing attention. Here we show that the SUMO E3 ligase PIASy interacts with the poly(ADP-ribose) polymerase PARP-1, and that PIASy mediates heat shock-induced poly-sumoylation of PARP-1. Furthermore, PIASy, and hence sumoylation, appears indispensable for full activation of the inducible *HSP70.1* gene. Chromatin immunoprecipitation experiments show that PIASy, SUMO and the SUMO-conjugating enzyme Ubc9 are rapidly recruited to the *HSP70.1* promoter upon heat shock, and that they are subsequently released with kinetics similar to PARP-1. Finally, we provide evidence that the SUMO-targeted ubiquitin ligase RNF4 mediates heat-shock-inducible ubiquitination of PARP-1, regulates the stability of PARP-1, and, like PIASy, is a positive regulator of *HSP70.1* gene activity. These results, thus, point to a novel mechanism for regulating PARP-1 transcription function, and suggest crosstalk between sumoylation and RNF4-mediated ubiquitination in regulating gene expression in response to heat shock.
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Introduction

The cellular response to sudden environmental stress is characterized by a rapid activation phase, which is invariably followed by attenuation of the response, despite the persistent presence of the inducing signal. Many transcriptional regulatory mechanisms for this involve post-translational modifications, because their usually transient nature permits both rapid amplification and subsequent extinction of the transduced signals. The heat-shock response represents a well-characterized model system for the study of transcriptional responses to environmental stress. In mammals, a major consequence of heat shock is the activation of a number of heat-shock factors (HSFs) that drive the transcriptional activation of heat-shock protein (HSP) genes that encode protein chaperones involved in protecting cellular functions from the deleterious effects of misfolded, aggregated, or mislocalized proteins (Morimoto, 1998, 2008). The factors that impinge on the regulation of HSP genes are, therefore, a subject of intense scrutiny.

Among the proteins now known to play a key role in this regulation is the cellular sensor of DNA damage, poly(ADP-ribose) polymerase 1 (PARP-1, reviewed by Schreiber *et al*, 2006). PARP-1 is the most abundant and founding member of a super-family of proteins defined by their homology to the catalytic domain of PARP-1 that is responsible for the synthesis of linear or branched polymers of ADP-ribose (PAR) from nicotinamide adenine dinucleotide (NAD^+; Schreiber *et al*, 2006; Hakme *et al*, 2008). Poly(ADP-ribosyl)ation, besides being strongly induced by DNA-damaging agents, such as reactive oxygen (e.g. H_2O_2), has been shown to exert major effects on chromatin structure and hence on the regulation of, particularly, transcriptionally active loci (for review, see Kraus, 2008). In *Drosophila* polytene chromosomes, for example, these PAR-containing loci are readily visible as puffs of de-compacted chromatin, thus providing perhaps the most striking evidence for the association of poly(ADP-ribosyl)ation with chromatin de-condensation (Tulin and Spradling, 2003). The concomitant rapid nucleosome loss, even prior to transcriptional onset, from the *Drosophila HSP70* promoter region, has been shown to require PARP activity (Petesch and Lis, 2008). On nucleosomal DNA templates, PARP-1 was shown to occupy a position between nucleosomes, consistent with *in vivo* results showing that PARP-1 and linker histone H1 occupy distinct and mutually exclusive chromosomal regions (Kim *et al*, 2004; Krishnakumar *et al*, 2008). Ouararhni *et al* (2006) have extended these findings by showing that on the *HSP70.1* promoter, DNA-bound PARP-1 is held in place and is enzymatically inactive by interaction with the variant histone macroH2A (mH2A). Perturbation of this interaction results in rapid PARP-1 activation and PARP-1 clearance from the *HSP70.1* promoter. The precise mechanism for this release, however, is still unclear.

In eukaryotes, modification by the ubiquitin (Ub)-like SUMO proteins has been shown to exert profound effects

on the activity of numerous transcription factors and cofactors (Verger *et al*, 2003; Müller *et al*, 2004; Gill, 2005). Like Ub, SUMO is covalently conjugated to its targets employing a cascade of E1, E2 (Ubc9), and E3 enzymes, including the PIAS (protein inhibitor of activated stats) proteins (Hay, 2005; Geiss-Friedlander and Melchior, 2007). The modification is reversible through the action of de-sumoylating enzymes called SENPs (Mukhopadhyay and Dasso, 2007). SUMO-2 and -3, but not SUMO-1, can form polymeric chains through a specific lysine, K11, which is part of a consensus modification motif (Tatham *et al*, 2001). While, unlike ubiquitination, sumoylation does not directly target its substrates to proteosomal degradation, recent genetic and biochemical evidence has uncovered an intriguing crosstalk mechanism with the Ub-proteasome system. This mechanism involves ubiquitination and degradation of poly-SUMO-modified proteins by the RING domain-containing Ub ligase of the Slx5/Slx8 (*Saccharomyces cerevisiae*; Wang *et al*, 2006; Burgess *et al*, 2007; Ii *et al*, 2007; Sun *et al*, 2007; Uzunova *et al*, 2007; Xie *et al*, 2007; Mullen and Brill, 2008), Slx8/Rfp1/2 (*Saccharomyces pombe*; Kosoy *et al*, 2007; Prudden *et al*, 2007; Sun *et al*, 2007), and RNF4 (mammals; Häkli *et al*, 2005; Lallemand-Breitenbach *et al*, 2008; Tatham *et al*, 2008) family. These SUMO-targeted Ub ligases contain multiple SUMO-interaction motifs (SIMs), thus providing an efficient binding interface only with SUMO substrates bearing SUMO chains (Tatham *et al*, 2008). To date, only two proteins, PML and PEA3, have been shown to be subject to poly-SUMO-mediated ubiquitination and degradation by RNF4 (Lallemand-Breitenbach *et al*, 2008; Tatham *et al*, 2008; Guo and Sharrocks, 2009).

A wide range of environmental stresses has been shown to lead to a massive and rapid increase in global sumoylation, preferentially with SUMO-2/SUMO-3, indicating involvement of a large number of protein targets (Saitoh and Hinchey, 2000; Blomster *et al*, 2009; Golebiowski *et al*, 2009). In the present work, we demonstrate that heat shock leads to rapid recruitment of the SUMO machinery on the *HSP70.1* promoter and induces PIASy-dependent sumoylation of PARP-1, necessary for full activation of the inducible *HSP70.1* gene. Furthermore, we show that PARP-1 is subject to heat-shock-induced, RNF4-mediated ubiquitination, and that, like PIASy, RNF4 controls the amount of modified PARP-1 and is necessary for full activation of *HSP70.1* transcription. Altogether, these results functionally link two important post-translational modifications in regulating PARP-1-mediated transcriptional activation in response to stress.

Results

PARP-1 is a direct binding partner of the SUMO E3 ligase PIASy

The SUMO E3 ligase PIASy has been shown to play important roles in numerous cellular processes such as senescence, apoptosis, and transcription (Sachdev *et al*, 2001; Bischof *et al*, 2006; Sharrocks, 2006). To gain further insight into PIASy function, we used a biochemical purification approach to identify interaction partners of PIASy (Martin *et al*, 2008). Among the interacting proteins identified were the signalling protein FIP200 (Martin *et al*, 2008), the DNA-repair factor Ku70, the heat-shock chaperone HSP70, the arginine methyltransferase PRMT5, and PARP-1 (data not shown). Focusing on PARP-1, we confirmed the *in vivo* interaction between endogenous PIASy and PARP-1 in a reciprocal experiment in which PIASy was co-immunoprecipitated with PARP-1 from HeLa extracts (Figure 1A). Consistent with these *in vivo* results, immobilized GST–PIASy specifically bound ^{35}S-methionine-labelled PARP-1, but not an unrelated control protein, in an *in vitro* GST pull-down assay (Figure 1B, top and middle panels, respectively). A similar experiment carried out with purified baculovirus-produced PARP-1 further confirmed this interaction (Figure 1B, lower panel) and indicated that PIASy and PARP-1 interact directly.

To assess the impact of poly(ADP-ribosyl)ation on PIASy–PARP-1 interaction, we carried out a similar *in vitro* binding assay using auto-poly(ADP-ribosyl)ated PARP-1. Both poly(ADP-ribosyl)ated and non-poly(ADP-ribosyl)ated PARP-1 bound immobilized PIASy without apparent discrimination (Figure 1C). In contrast, PIASy–PARP-1 co-immunoprecipitation *in vivo* was drastically reduced upon induction of poly(ADP-ribosyl)ation by treatment of cells with hydrogen peroxide, an effect that could be reversed by treatment of the cells with the poly(ADP-ribosyl)ation inhibitor 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1-(2H)-isoquinolinone (DPQ) (Supplementary Figure S1). Given that poly(ADP-ribosyl)ation does not affect PIASy–PARP-1 binding *in vitro*, these results suggest that stress-induced poly(ADP-ribosyl)ation can modulate the PIASy–PARP-1 interaction *in vivo*. Whether hydrogen peroxide-induced poly(ADP-ribosyl)ation of other substrates, or sequestration of poly(ADP-ribosyl)ated PARP-1 into PIASy inaccessible subcellular sites accounts for the reduced interaction, remains to be determined.

PIASy and PARP-1 contain several well-defined structural domains (Figure 1D). To determine whether the PIASy SP-RING finger domain is involved in the association, we expressed FLAG–HA-tagged PIASy wild-type (WT) or a derivative mutated in the SP-RING finger motif (Cys342Phe, abolishing E3 ligase activity; Bischof *et al*, 2006) in HeLa cells for immunoprecipitation experiments. Endogenous PARP-1 was detectable only in anti-HA immunoprecipitates from cells overexpressing WT PIASy (Figure 1E, compare lanes 5 and 6), suggesting that the interaction requires the integrity and/or ligase function of the PIASy RING finger. To next map the regions of PARP-1 responsible for interaction with PIASy, a series of PARP-1 truncation mutants fused to GST were expressed in HeLa cells and purified on glutathione beads. As shown in Figure 1F, both the PARP-1 N-terminus that encompasses the DNA-binding domain, as well as the auto-modification (BRCT) domain, bound ^{35}S-labelled PIASy protein in GST pull down assays, suggesting that these domains, either together or separately, are critical for PIASy interaction.

PARP-1 is SUMO-modified and PIASy is poly(ADP-ribosyl)ated

PARP-1 has been shown to interact with the E2 SUMO-conjugating enzyme Ubc9 (Masson *et al*, 1997) and, more recently, to be modified by SUMO (Blomster *et al*, 2009; Golebiowski *et al*, 2009; Messner *et al*, 2009), suggesting that PARP-1 could be a substrate for PIASy SUMO E3 ligase activity. To test this, we first confirmed that PARP-1 is modified by SUMO-1 and SUMO-2 *in vitro* (Figure 2A), and *in vivo*, under overexpression conditions (Figure 2B, lanes 2

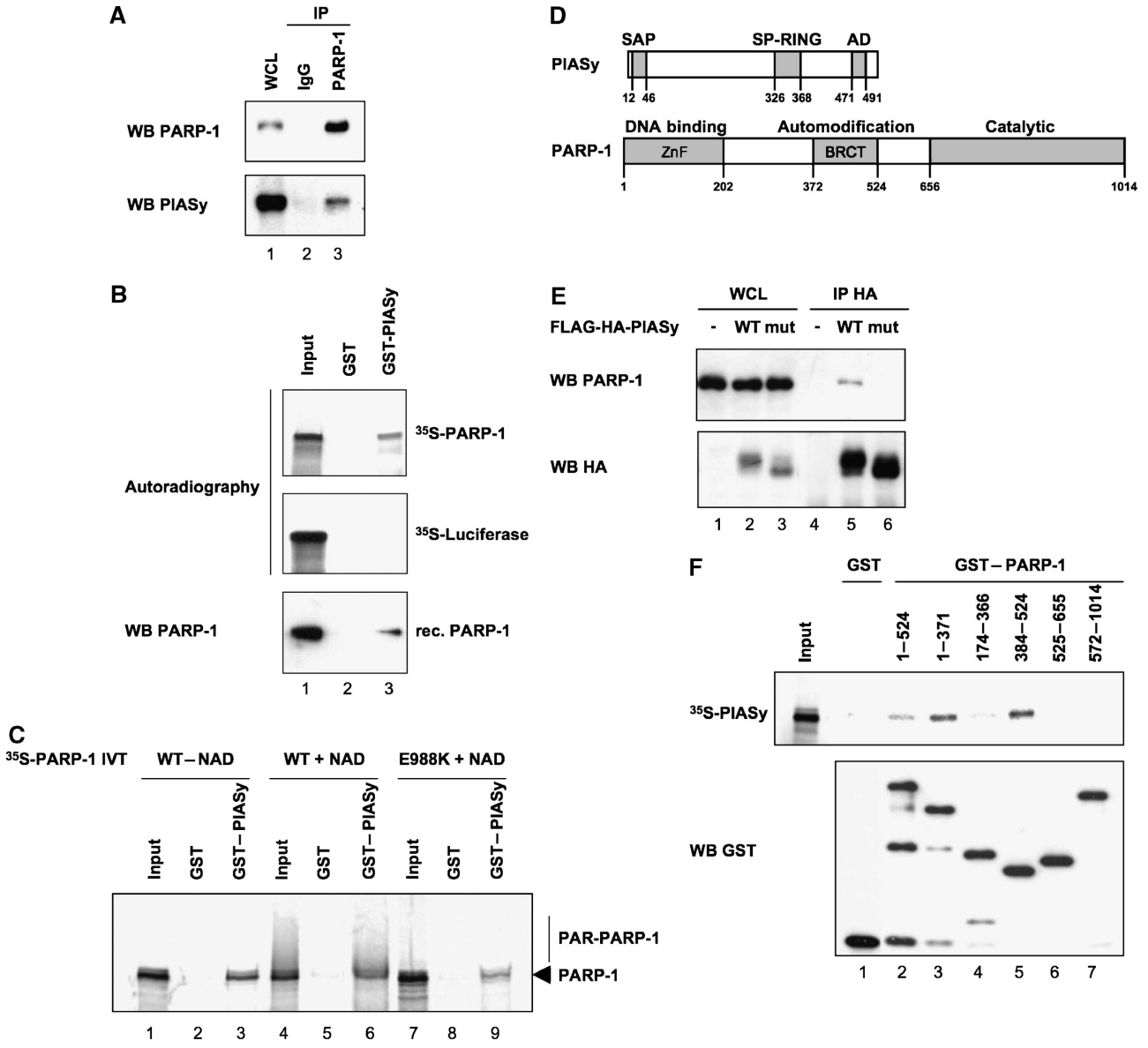


Figure 1 PIASy interacts with PARP-1. (**A**) Endogenous PIASy and PARP-1 interact *in vivo*. HeLa cell lysates were immunoprecipitated (IP) with mouse anti-PARP-1 or control (IgG) antibodies and probed with anti-PARP-1 and anti-PIASy antibodies. WCL, whole-cell lysate, 2% of amount used in IP. (**B**) PIASy and PARP-1 interact *in vitro*. Pull-down experiment with GST, GST–PIASy, and ^{35}S-labelled *in vitro* translated PARP-1 and luciferase or recombinant PARP-1. Bound proteins revealed by autoradiography or by anti-PARP-1 antibody. Input: 20% of amount used in binding assays. (**C**) PIASy interacts with both unmodified and poly(ADP-ribosyl)ated PARP-1 *in vitro*. GST pull down with ^{35}S-labelled *in vitro* translated PARP-1 (WT, wild type; E988K, catalytically inactive) after incubation of PARP-1 in a poly(ADP-ribosyl)ation reaction containing (+) or not (−) NAD. Bound proteins revealed by autoradiography. Input: 20% of amount used in the binding reaction. PAR–PARP-1, poly(ADP-ribosyl)ated PARP-1. (**D**) Major protein domains of PIASy and PARP-1. SAP, SAF-A/B, Acinus, and PIAS domain; SP-RING, Siz/PIAS-RING domain; AD, acidic domain; ZnF, zinc fingers; BRCT, BRCA1 C-terminus domain. (**E**) Integrity of PIASy SP-RING domain is required for PARP-1 interaction. Co-immunoprecipitation (IP) with endogenous PARP-1 and WT or C342F (mut) FLAG–HA–PIASy expressed in HeLa cells; WCL, whole-cell lysate, 5% of amount used in IP. (**F**) PARP-1 N-terminus and auto-modification domains interact with PIASy. Immobilized GST or GST–PARP-1 domains, expressed in HeLa cells, were incubated with ^{35}S-Met-labelled, *in vitro*-translated PIASy. Bound proteins were revealed by autoradiography (top panel) and purified GST or GST–PARP-1 proteins were detected with anti-GST antibody (bottom panel). Input: 20% of amount used in binding reactions.

and 5). Moreover, simultaneous overexpression of Ubc9 stimulated (lane 3), whereas Senp1 abrogated (lane 4), SUMO-1 modification of PARP-1. Western blotting of extracts from HeLa cells overexpressing SUMO-1, or left untransfected, showed that endogenous PARP-1 also is SUMO-modified (Figure 2C, lanes 1 and 2). Similarly, immunoprecipitation of untransfected HeLa cell extracts with anti-SUMO-1 (lane 5) and anti-SUMO-2 (lane 8) antibodies, but not control antibodies (lanes 4 and 7), revealed the presence of endogenously SUMO-modified PARP-1.

To then test whether PIASy acts as a SUMO E3 ligase for PARP-1, we added bacterially produced GST–PIASy to an *in vitro* sumoylation reaction. As shown in Figure 2D, GST–PIASy enhanced the sumoylation of PARP-1 by both SUMO-1 (compare lanes 2 and 3) and SUMO-2 (compare lanes 7 and 8). Similarly, use of FLAG–PIASy, expressed in

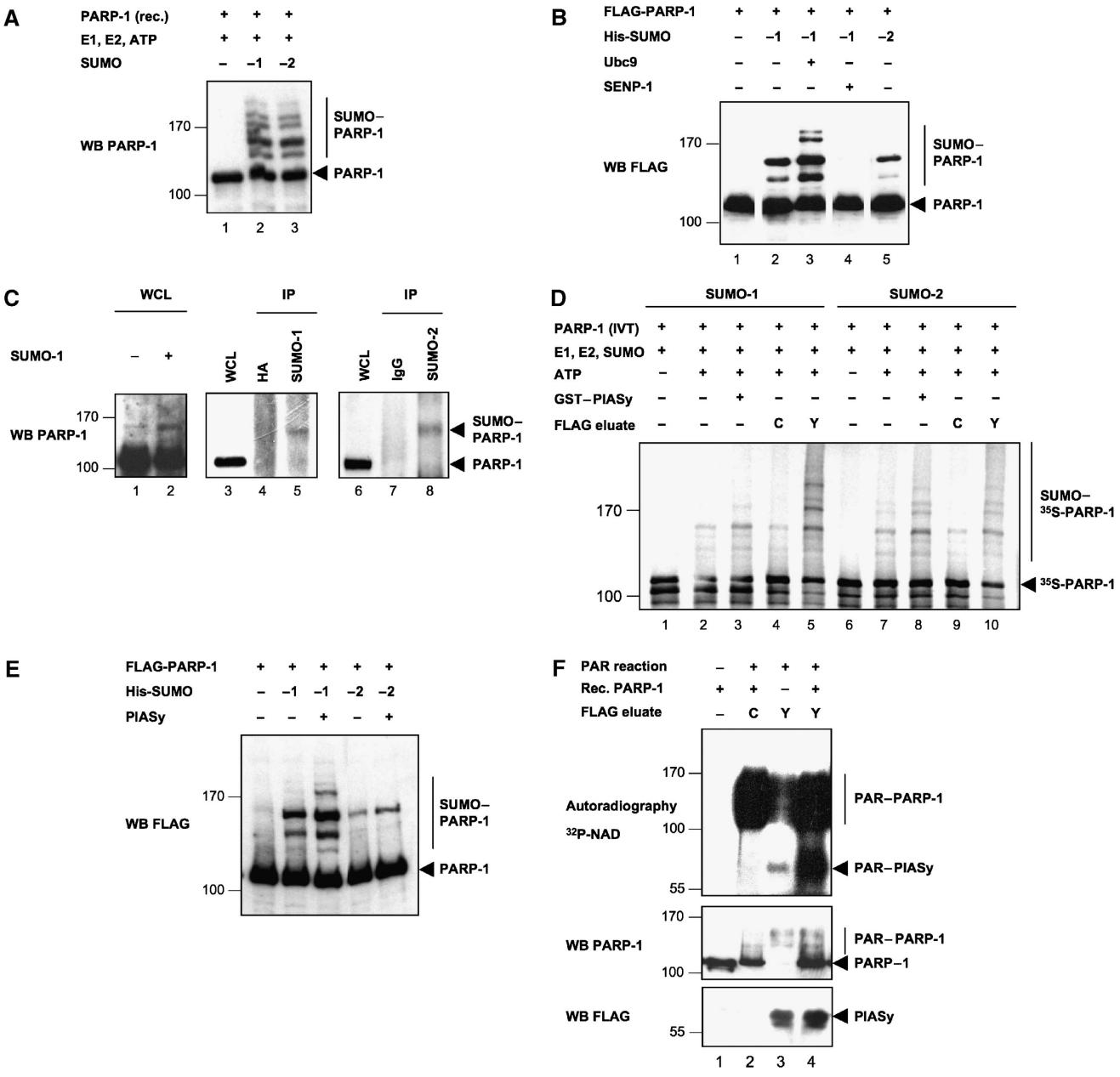


Figure 2 Cross-modification between PIASy and PARP-1. (**A**) Sumoylation of PARP-1 *in vitro*. Sumoylation of recombinant PARP-1 with SUMO-1 or SUMO-2 after 15 min reaction revealed with anti-PARP-1 antibody. (**B**) Sumoylation of PARP-1 *in vivo*. HeLa cells were cotransfected with the indicated expression vectors and analysed by western blot with anti-FLAG antibody. Composite figure from a multi-lane, single blot with enhanced contrast. (**C**) PARP-1 is sumoylated *in vivo* at the endogenous level. Anti-PARP-1 antibody was used in western blotting to probe whole-cell lysates (WCL) or anti-SUMO-1, anti-SUMO-2, or control (HA, IgG) immunoprecipitates (IP) from untransfected HeLa cells (lanes 1, 3–8), or from cells transfected with SUMO-1 (lane 2). (**D**) PIASy acts as a SUMO E3 ligase for PARP-1 *in vitro*. *In vitro* sumoylation of ^{35}S-labelled *in vitro* translated (IVT) PARP-1 in the absence (−) or presence (+) of ATP or of PIASy, added as GST fusion or as FLAG eluate from HeLa cells transfected with FLAG–PIASy (Y) or empty vector (C). Reaction products were detected by autoradiography. (**E**) PIASy acts as a SUMO E3 ligase for PARP-1 *in vivo*. Lysates from HeLa cells transfected with the indicated expression vectors were probed with anti-FLAG antibody. (**F**) PARP-1 poly(ADP-ribosyl)ates PIASy *in vitro*. FLAG eluates from mock-transfected (C) or FLAG–PIASy-transfected (Y) HeLa cells were used as substrates in a poly(ADP-ribosyl)ation reaction catalysed by recombinant PARP-1 and ^{32}P-NAD$^+$. After SDS–PAGE, reaction products were visualized by autoradiography (top panel) and identified by western blotting as indicated (middle and bottom panels).

HeLa cells and immunoprecipitated with anti-FLAG antibody, also led to marked enhancement of PARP-1 modification by SUMO-1 (lane 5) and SUMO-2 (lane 10), whereas a mock eluate had no effect (lanes 4 and 9). To confirm these results *in vivo*, we next coexpressed FLAG–PARP-1 together with PIASy and SUMO. PIASy stimulated the modification by both SUMO isoforms (Figure 2E, lanes 3 and 5). PIAS1, PIASxα, and PIASxβ, but not PIAS3, also showed a stimulating effect on PARP-1 sumoylation *in vitro*. Moreover, PIASxα also interacts with PARP-1 *in vivo* (Supplementary Figure 2), suggesting that other PIAS family members may function as SUMO E3 ligases for PARP-1 under these experimental conditions.

The finding that PIASy mediates sumoylation of PARP-1 prompted us to investigate whether, reciprocally, PARP-1

could poly(ADP-ribosyl)ate PIASy. For this, we incubated a FLAG–PIASy eluate with ^{32}P-labelled NAD$^+$ and DNAseI-treated DNA in the presence or absence of recombinant PARP-1. As seen in Figure 2F, PARP-1 was efficiently auto-poly(ADP-ribosyl)ated under these conditions (top and middle panels, lanes 2 and 4). Addition of FLAG–PIASy eluate led to the appearance of a second major band corresponding in size to (ADP-ribosyl)ated PIASy (top panel, lane 4). Remarkably, even in the absence of added recombinant PARP-1, weaker signals corresponding to (ADP-ribosyl)ated PIASy (lane 3) and PARP-1 (top and middle panels, lane 3) could be detected, suggesting the presence of endogenous PARP-1 activity in the FLAG–PIASy eluate. Finally, a GST–PIASy fusion protein could also be (ADP-ribosyl)ated by recombinant PARP-1 (data not shown). Taken together, these results indicate that PIASy and PARP-1 cross modify each other, suggesting a possible interplay between these two types of protein modifications.

Lysine 486 and 203 are the principal SUMO-acceptor sites of PARP-1

Inspection of the human PARP-1 amino-acid sequence revealed the presence of numerous (>20) putative sumoylation sites, of which five conformed most faithfully to the classical ΨKxE motif (Rodriguez *et al*, 1999; Figure 3A). One of these (K486) could be confirmed by mass spectroscopy analysis (data not shown). Mutation of these lysine residues to arginine showed two of these, K486 and K203, to be critical for PARP-1 sumoylation, although mutation of either alone, or both (2KR), failed to abolish PARP-1 sumoylation entirely, both *in vitro* (Figure 3B and Supplementary Figure S3) and *in vivo* (Figure 3C). Of note, *in vitro* modification with SUMO-1 (Figure 3B, odd-numbered lanes) revealed the existence of additional sites, whereas modification with SUMO-2 additionally led to the formation of high-molecular-weight (MW) polymeric SUMO-2 chains (lanes 6 and 8). Taken together, these results show PARP-1 to be SUMO-modified

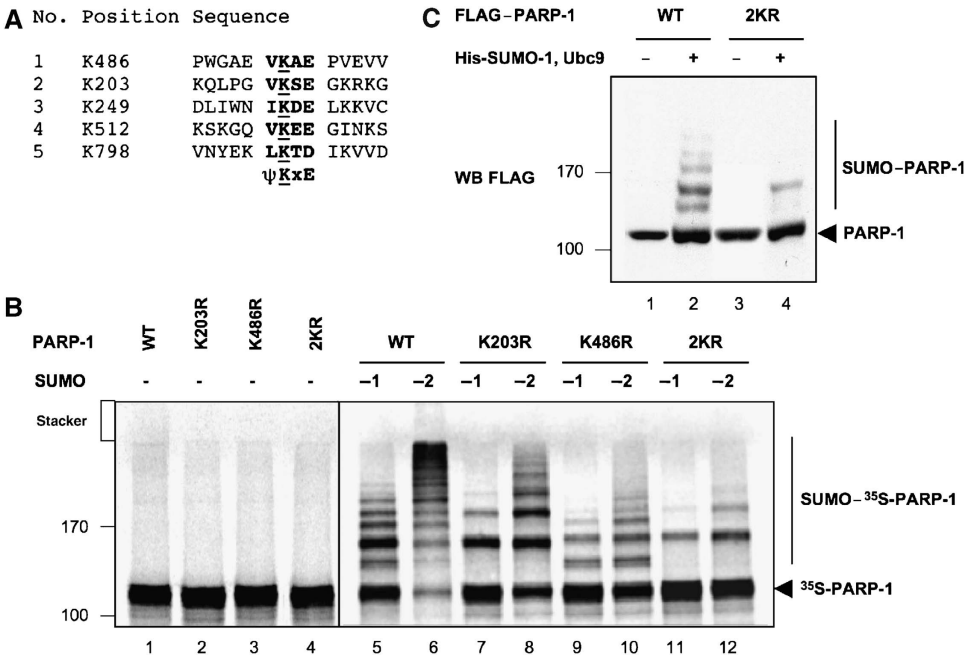


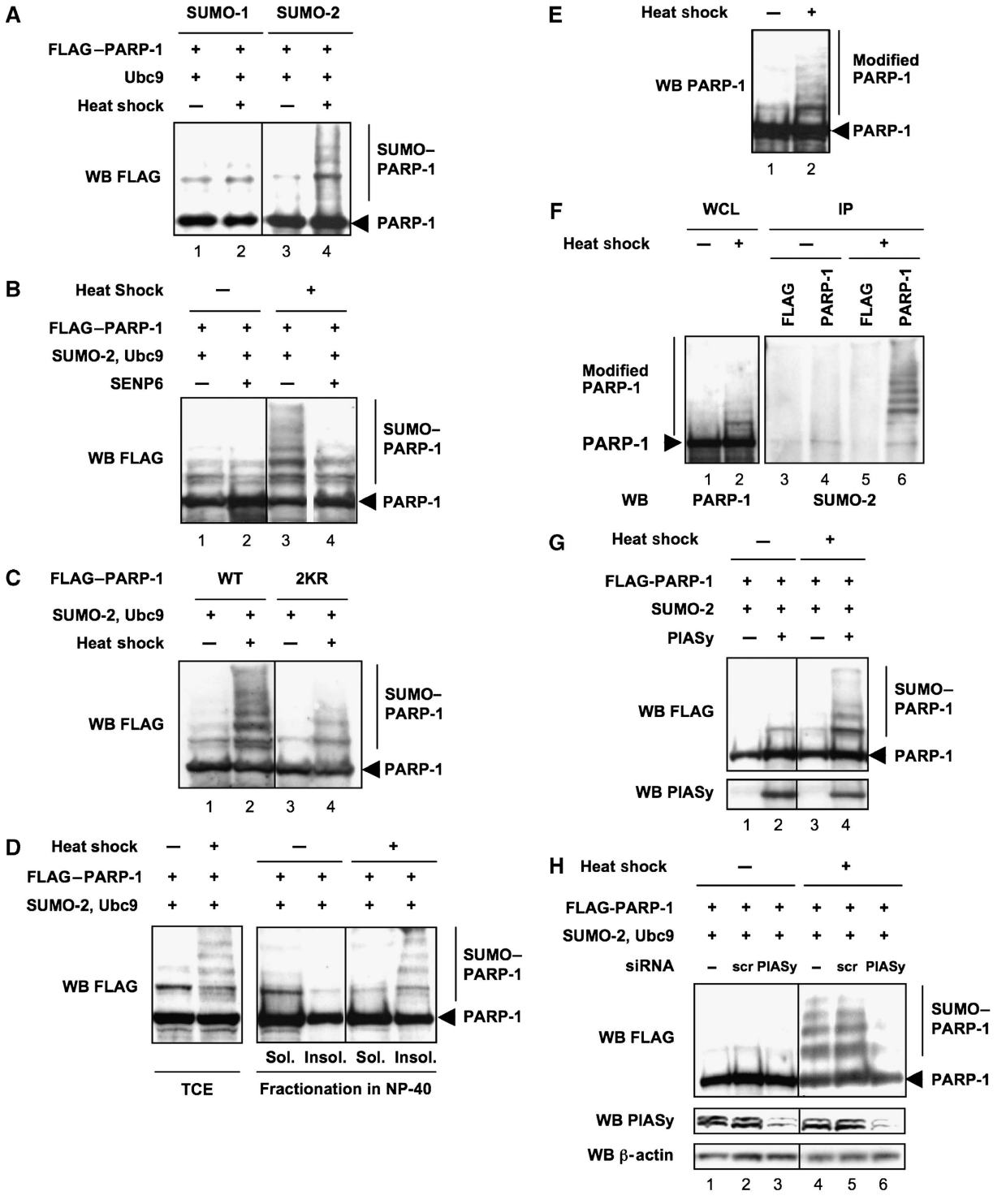
Figure 3 Mapping of the SUMO-acceptor sites of PARP-1. (**A**) Five most probable ΨKxE sumoylation consensus motifs of human PARP-1 protein. (**B**) *In vitro* sumoylation of ^{35}S-labelled *in vitro* translated WT, K203R, K486R, or K203R/K486R (2KR) PARP-1 with SUMO-1 or SUMO-2 revealed by autoradiography after long reaction time (60 min). (**C**) Sumoylation of FLAG–PARP-1 WT or 2KR mutant *in vivo*. Lysates from HeLa cells transfected with the indicated expression vectors were probed with anti-FLAG antibody.

Figure 4 Heat shock induces PIASy-dependent sumoylation of PARP-1. (**A**) Heat shock induces preferential SUMO-2 modification of PARP-1. Whole-cell lysates of HeLa cells transfected as indicated, untreated or heat shocked (30 min, 43°C), were western blotted with anti-FLAG antibody. (**B**) Heat shock induces poly-modification of PARP-1 by SUMO-2. Whole-cell lysates of HeLa cells transfected as indicated, untreated or heat shocked (30 min, 43°C), were western blotted with anti-FLAG antibody. Composite figure from a multi-lane, single blot with enhanced contrast. (**C**) High sumoylation of PARP-1 upon heat shock is impaired by the K203R/K486R (2KR) mutation. Whole-cell lysates of HeLa cells transfected as indicated, untreated or heat shocked (30 min, 43°C), were western blotted with anti-FLAG antibody. (**D**) Modified PARP-1 partitions to the detergent-insoluble fraction. HeLa cells transfected as indicated, untreated, or heat shocked (30 min, 43°C) were either lysed directly in SDS sample buffer (TCE, total cell extract, left panel), or extracted in NP-40-containing Chris buffer, separated into soluble (Sol.) and insoluble (Insol.) fractions by centrifugation (right panels), and western blotted with anti-FLAG antibody. (**E**) Heat shock induces accumulation of modified forms of endogenous PARP-1 in HeLa cells. Whole-cell lysates of HeLa cells, untreated or heat shocked (30 min, 43°C), were western blotted with anti-PARP-1 antibody. (**F**) Heat shock induces sumoylation of endogenous PARP-1 in HeLa cells. FLAG (control) or PARP-1 immunoprecipitates from untransfected HeLa cells untreated or heat shocked (30 min, 43°C) were analysed by western blot using anti-SUMO-2 antibody (right panel). The corresponding whole-cell lysates were analysed using anti-PARP-1 antibody (left panel). (**G**) PIASy enhances heat-shock-induced PARP-1 sumoylation. HeLa cells were transfected as indicated and left untreated or heat shocked (30 min, 43°C). Whole-cell lysates were probed with the indicated antibodies. (**H**) PIASy is required for the heat-shock-induced increase in PARP-1 sumoylation. HeLa cells were transfected with scrambled control (scr) or PIASy siRNA and re-transfected 24 h later with FLAG–PARP-1, SUMO-2, and Ubc9. After 24 h, cells were either left untreated or heat shocked (30 min, 43°C). Whole-cell lysates were probed with the indicated antibodies.

on lysine 486 and 203, as well as on other, non-consensus or promiscuous modification sites.

Heat shock induces PARP-1 sumoylation

Environmental stresses such as heat shock, osmotic, or oxidative stress are known to induce the preferential conjugation of SUMO-2/SUMO-3 to numerous target proteins (Saitoh and Hinchey, 2000). In addition, PARP-1 was shown to regulate the expression of the heat-shock-inducible *HSP70.1* gene (Ouararhni *et al*, 2006). These findings prompted us to examine whether heat shock could induce the sumoylation of PARP-1. Consistent with recently published results (Blomster *et al*, 2009; Golebiowski *et al*, 2009), coexpression of FLAG–PARP-1 and Ubc9 together with either SUMO-1 or SUMO-2 in HeLa cells exposed to heat shock (43°C, 30 min) resulted in the appearance of slower migrating PARP-1 species in the presence of SUMO-2 but not of SUMO-1 (Figure 4A). In contrast, simultaneous coexpression of



SENP6, a de-sumoylating enzyme with specificity for poly-SUMO chains (Mukhopadhyay *et al*, 2006), led to disappearance of these high-MW PARP-1 species, demonstrating that heat shock promotes the formation of PARP-1–poly-SUMO-2 conjugates (Figure 4B, compare lanes 3 and 4), the abundance of which was significantly reduced when the PARP-1 2KR mutant was expressed instead of WT (Figure 4C).

Fractionation of cell extracts from PARP-1-, Ubc9-, and SUMO-2-overexpressing cells further revealed enhanced association of modified PARP-1 with the detergent (Nonidet P-40 (NP-40))-insoluble fraction under heat shock (Figure 4D), suggesting that the induced sumoylation of PARP-1 is preferentially associated with the chromatin and/or nuclear matrix compartment. Non-transfected HeLa cells similarly displayed accumulation of modified endogenous PARP-1 species upon heat shock (Figure 4E). Immunoprecipitation with anti-PARP-1 antibody (Figure 4F, lanes 4 and 6), or anti-FLAG control antibody (lanes 3 and 5), from extracts of unstressed or heat shocked HeLa cells confirmed that these endogenous higher-MW PARP-1 species correspond to polymeric or multiply modified PARP-1–SUMO-2 conjugates.

Consistent with previous *in vitro* results, overexpression of PIASy (Figure 4G), PIASxα, or PIASxβ (Supplementary Figure S2B, lanes 10 and 11) stimulated heat-shock-induced PARP-1 sumoylation under cotransfection conditions. Conversely, siRNA-mediated knockdown of PIASy expression in HeLa cells almost completely abolished the heat-shock-induced sumoylation of PARP-1 (Figure 4H, compare lanes 5 and 6), whereas cells transfected with a scrambled control siRNA behaved like mock-transfected cells (compare lanes 4 and 5), suggesting that PIASy occupies a privileged position as a SUMO E3 ligase for PARP-1 under heat shock. Taken together, these results show that heat shock strongly upregulates PARP-1 sumoylation, in both quantity as well as quality (SUMO-2 polymers), and further, that PIASy appears to play a critical role in this process *in vivo*.

Role of PARP-1 sumoylation in HSP70.1-promoter activation

Given the role of PARP-1 in the transcriptional regulation of the *HSP70.1* gene, we next asked whether PARP-1 sumoylation could affect *HSP70.1* transcription. To this end, we retrovirally infected murine embryonic fibroblasts (MEFs) derived from PARP-$1^{-/-}$ mice with the control vector or vectors expressing either WT PARP-1 or its sumoylation-defective K203R/K486R derivative (PARP-1^{2KR}). Littermate-matched WT (PARP$^{+/+}$) cells were infected with empty vector as control. These cell populations were subsequently heat shocked and *HSP70.1* gene expression was monitored by quantitative RT–PCR. As seen in Figure 5A, PARP-1 and PARP-1^{2KR} were expressed at similar levels in restored PARP-$1^{-/-}$ MEFs, although still less than that in the PARP$^{+/+}$ control cells. In the absence of PARP-1, *HSP70.1* gene transcription was significantly reduced and could be restored by expression of WT PARP-1, albeit only partially (due to the lower exogenous expression level achieved). Remarkably, the enhancement attributable to PARP-1 was reduced by 60% when the PARP-1^{2KR} mutant was used instead, suggesting that PARP-1 sumoylation plays a measurable role in *HSP70.1*-promoter activation. The fact that PARP-1 sumoylation could not be completely abrogated in the PARP-1^{2KR} mutant (Figures 3B and C, and 4C) may, in part, explain the residual activity of this mutant in this rescue experiment. Non-heat shocked cells displayed a similar activation profile, further suggesting that PARP-1 sumoylation also affects transcriptional activity under non-stress conditions. Differential poly(ADP-ribosyl)ation activity of PARP-1^{2KR} versus PARP-1 WT is unlikely to account for their differential transactivation capacity, since both possess the same enzymatic activity *in vitro* (Supplementary Figure S4). Also, a possible role for other modifications targeting lysine 203 or 486 cannot be formally ruled out.

Given that PIASy appears responsible for much, if not all, heat-shock-induced sumoylation of PARP-1 (Figure 4H), we next sought to determine whether suppression of PIASy expression would affect transcription of the *HSP70.1* gene under heat shock. For this, HeLa cells were transfected with siRNA oligonucleotides directed against PIASy or scrambled control to monitor the expression of the *HSP70.1* gene in response to heat shock. As shown in Figure 5B, PIASy knockdown reduced heat-shock induction of the endogenous *HSP70.1* gene expression by more than 50%. A similar reduction was obtained upon PIASy depletion using a transfected *HSP70.1* promoter–luciferase reporter construct (Figure 5C), indicating that presence of PIASy is necessary for the full *HSP70.1* transcriptional response under heat shock. The incomplete inhibition of PIASy expression in cells transfected with the specific PIASy siRNA (Figure 4H), or its possibly redundant role vis-à-vis other PIAS SUMO E3 ligases (Supplementary Figure S2), may, in part, account for the residual induction observed upon heat shock.

These findings, thus, raised the question of whether PIASy-mediated sumoylation of PARP-1 may occur and exert its role directly on the *HSP70.1* promoter. A direct mechanism would predict the co-occupancy of components of the SUMO machinery together with PARP-1 on the *HSP70.1* promoter. To test this, we carried out chromatin immunoprecipitation (ChIP) experiments using antibodies against PIASy, PARP-1, Ubc9, and SUMO-2. All four antibodies, but not control antibodies, immunoprecipitated detectable amounts of *HSP70.1* promoter fragments (Figure 5D), indicating that the corresponding proteins are bound to the promoter in normal conditions. To next examine the effect of heat shock on the promoter occupancy of these proteins, we carried out a ChIP time-course experiment upon prolonged heat-shock treatment. Consistent with previously published results (Ouararhni *et al*, 2006), we observed mild and transient enrichment of PARP-1 during the first 5 min of heat shock, followed by marked release from the promoter thereafter (Figure 5E). By contrast, both PIASy (Figure 5F) and Ubc9 (Figure 5G), present at low levels at the outset, exhibited very pronounced recruitment to the *HSP70.1* promoter within the first 5 min, with a 23-fold and five-fold increase in promoter-associated PIASy and Ubc9, respectively. Upon longer heat-shock treatment, the amount of PIASy and Ubc9 bound to *HSP70.1* promoter significantly decreased. Altogether, these findings support a key role of PIASy and of PARP-1 sumoylation in *HSP70.1*-promoter activation. To further characterize other possible effects on *HSP70.1*-promoter activation, we also tested the interaction of PIASy with other factors known to be present on this promoter. These included the DNA-repair factors Ku70 and Ku80, the arginine methyltransferase

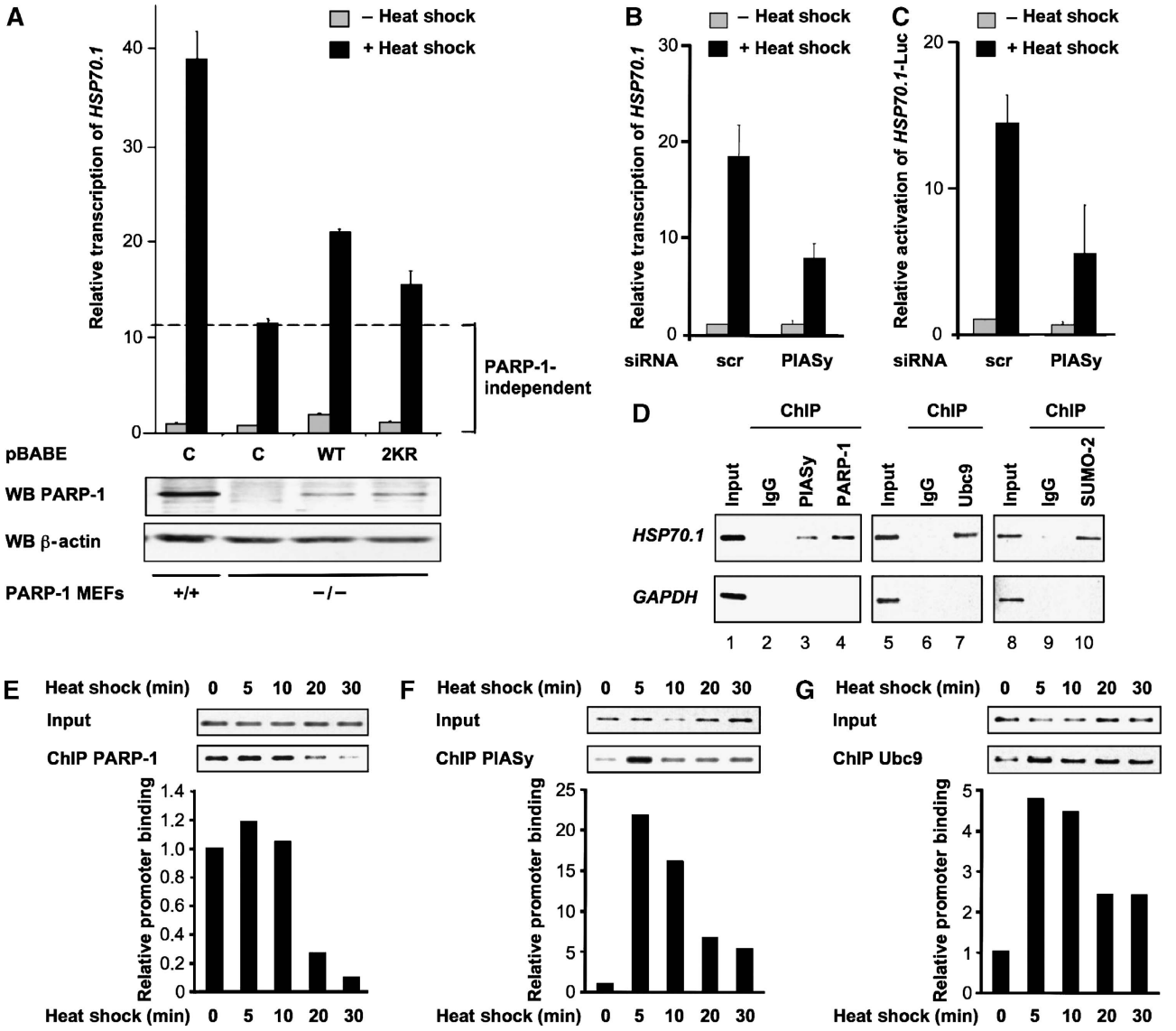


Figure 5 PARP-1 sumoylation is necessary for full *HSP70.1*-promoter activation. (**A**–**C**) Impairment of PARP-1 sumoylation reduces *HSP70.1* gene transcription. (A) PARP-$1^{+/+}$ and PARP-$1^{-/-}$ MEFs were infected with retroviruses expressing WT PARP-1, K203R/K486R mutant (2KR) PARP-1, or control (C, empty vector). Cells were left untreated or heat shocked (1 h, 43°C). After recovery at 37°C for 30 min, *HSP70.1* gene expression, normalized against *GAPDH*, was determined by quantitative RT–PCR. *HSP70.1* transcript levels ± s.e. were then plotted relative to control cells before heat shock. Protein expression was checked by western blotting using the indicated antibodies. (B, C) PIASy knockdown reduces *HSP70.1* gene transcription. (B) HeLa cells were transfected with scrambled control (scr) or PIASy siRNA. Heat shock and quantification of *HSP70.1* gene expression were as described in panel A. (C) HeLa cells were transfected with siRNA oligos as in panel B and re-transfected 24 h later with *HSP70.1* promoter–luciferase reporter and CMV–β-gal plasmid for an additional 24 h, and then heat-shocked (1 h, 43°C), or left untreated, as indicated. After a further 12 h, luciferase and β-gal activities were determined. Values for luciferase activity were corrected for β-gal activity. Plotted values in panels B and C represent means ± s.e. for three independent experiments, with non-heat shocked scrambled control value set to 1. (**D**–**F**) Occupancy of the *HSP70.1* promoter by PARP-1 and the SUMO machinery. (D) ChIP from Jurkat cells with antibodies against PARP-1, PIASy, Ubc9, SUMO-2, or isotypic control antibodies (IgG) using primers targeting the *HSP70.1* or *GAPDH* (control) gene promoters. (E–**G**) Time course of the association of PARP-1, PIASy and Ubc9 with the *HSP70.1* promoter upon heat shock. Jurkat cells heat shocked at 43°C for the indicated times were subjected to ChIP using antibodies against PARP-1 (**E**), PIASy (**F**), or Ubc9 (G). ChIP products were analysed by semi-quantitative PCR with primers against the *HSP70.1* promoter. Bar graphs: Densitometry of ChIP PCR normalized to input with time zero set to 1. Results representative of several independent experiments are given.

PRMT5 (Ouararhni *et al*, 2006), and the tumour suppressor MEN1, a homologue of the *Drosophila* Menin protein, that is recruited to the *HSP70* promoter upon heat shock (Papaconstantinou *et al*, 2005). Indeed, all four of these proteins interacted with PIASy *in vivo* (Supplementary Figure S5A–C), with Ku70, Ku80 (Gocke *et al*, 2005; Yurchenko *et al*, 2006), as well as PRMT5 (Supplementary Figure S5D) also being SUMO substrates themselves. These results suggest that PIASy and, by extension, sumoylation target several other factors, besides PARP-1, present on the *HSP70.1* promoter.

Involvement of the SUMO-targeted Ub ligase RNF4 in HSP70.1 gene activation

The observation that PARP-1 is modified by multiple and polymeric SUMO molecules upon heat shock (Figure 4),

raised the possibility that sumoylated PARP-1 could be targeted by the poly-SUMO-specific Ub E3 ligase RNF4 and subsequently tagged for degradation by the Ub proteasome system. To explore this possibility, we, thus, tested whether PARP-1 and RNF4 interact *in vivo*. For this, HeLa cells were cotransfected with vectors expressing FLAG–PARP-1, SUMO-2, and either WT or RING-finger mutant (mut; C136/139/177/180S; Häkli *et al*, 2005) FLAG–RNF4, or appropriate empty vectors. As shown in Figure 6A, detectable amounts of WT (row d, lane 4) or mut (row d, lane 8) RNF4 co-immunoprecipitated with an anti-PARP-1 antibody, demonstrating that RNF4 interacts with PARP-1 *in vivo* and that, as shown for PML (Häkli *et al*, 2005), this interaction does not require the integrity of the RNF4 RING domain. Moreover, this experiment also demonstrated that overexpression of WT, but not mut FLAG-RNF4, reduced the amount of PARP-1, both in whole-cell extracts and in anti-PARP-1 immunoprecipitates (compare lanes 4 and 8 in rows a and c), indicating that RNF4 induces PARP-1 degradation in a manner dependent on its Ub E3 ligase activity. To further rule out that this observed disappearance of PARP-1 was possibly due to RNF4-induced apoptosis and the consequent cleavage of PARP-1 (a hallmark of apoptosis; Soldani and Scovassi, 2002), we carried out a similar experiment to also monitor the amount of cleaved PARP-1. As before, expression of WT (but not mut) FLAG–RNF4 led to consistent disappearance of coexpressed FLAG–PARP-1 (Figure 6B, compare lanes 4, 5, and 6 in row a). Furthermore, WT FLAG–RNF4 also led to the concomitant disappearance, not accumulation, of cleaved PARP-1 (compare lanes 4, 5, and 6 in row b), thus demonstrating that the disappearance of full-length PARP-1 observed here is not a consequence of enhanced, RNF4-induced apoptosis and PARP-1 cleavage. To then test whether PARP-1 degradation induced by RNF4 is mediated by the proteasome, HeLa cells coexpressing FLAG–PARP-1, SUMO-2, and FLAG–RNF4, as before, were treated with the proteasome inhibitor MG-132. This treatment restored the amount of PARP-1 to levels detected in the absence of coexpressed FLAG–RNF4 (Figure 6C, compare lanes 2 and 3 with lanes 5 and 6 in row a), indicating that RNF4 targets PARP-1 for proteasomal degradation.

To next ask whether the effect of RNF4 on PARP-1 stability depends on the sumoylation of PARP-1, we carried out an *in vitro* binding assay to determine, first, whether RNF4 preferentially binds sumoylated PARP-1. As shown in Figure 6D, immobilized GST–RNF4 binds *in vitro* translated ^{35}S-labelled SUMO-2-modified PARP-1, presumably through polymeric SUMO-2 chains (Tatham *et al*, 2008), whereas SUMO-1-modified PARP-1 conjugates show little affinity, as does the non-modified PARP-1. Consistent with this finding, we found that upon simultaneous overexpression of FLAG–PARP-1, Ubc9, SUMO-2 (i.e. conditions permitting the ready detection of overexpressed, sumoylated PARP-1 *in vivo*), and T7-RNF4, RNF4 expression led to the preferential disappearance of the modified species of PARP-1 (Figure 6E). Moreover, their intrinsic instability could be further confirmed in a time-course experiment using FLAG–PARP-1, SUMO-2, and Ubc9-overexpressing HeLa cells incubated with the protein synthesis inhibitor cycloheximide, a treatment that did not induce global instability of proteins conjugated to SUMO in our experimental conditions (Supplementary Figure S6). By contrast, heat shock of HeLa cells subjected to siRNA-mediated knockdown of RNF4 increased the abundance of higher MW endogenous PARP-1 conjugates (Figure 6F), suggesting that the modified and unmodified forms of PARP-1 show differential stability in an RNF4-dependent manner *in vivo*.

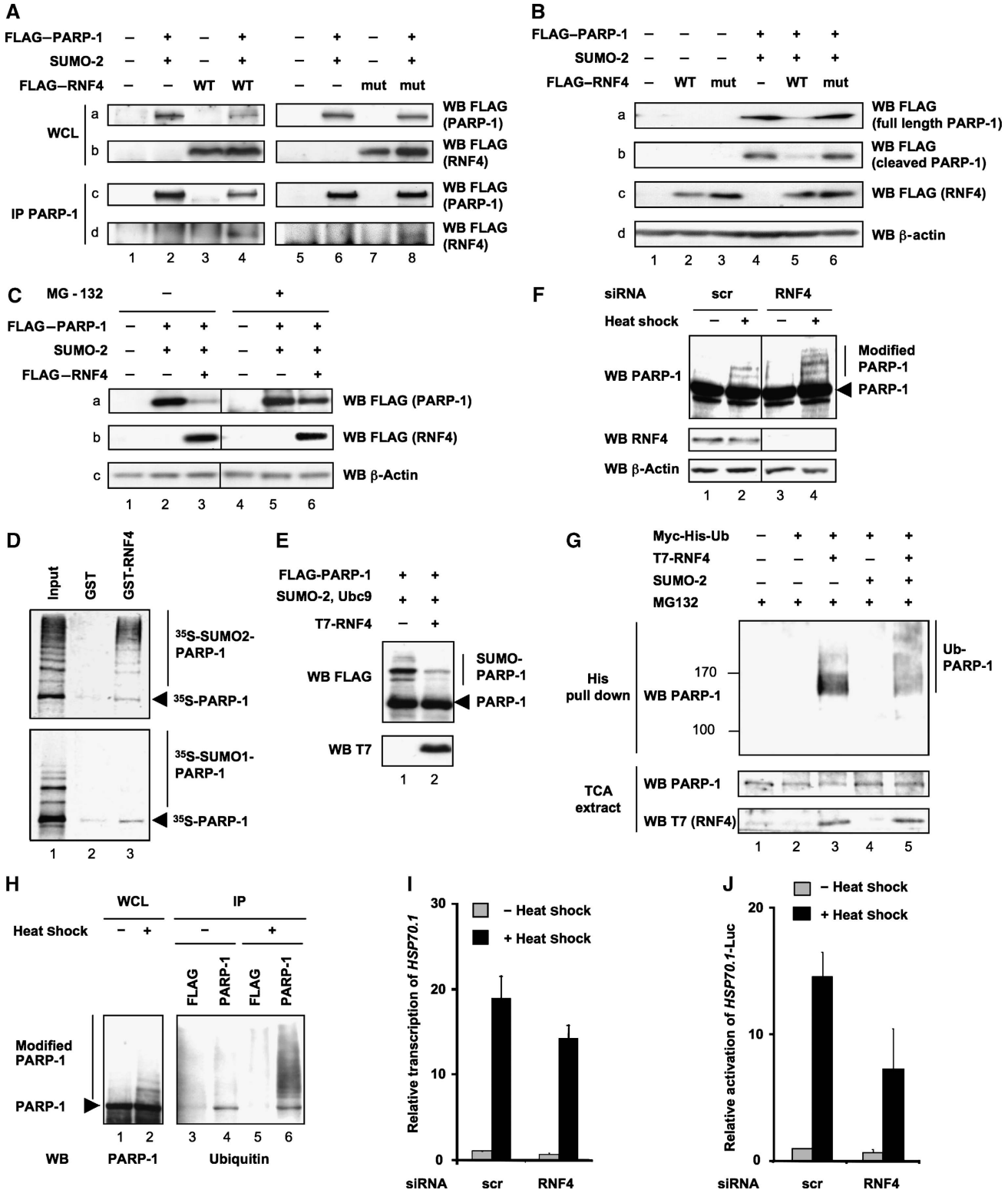
To next examine whether RNF4 mediates the ubiquitination of PARP-1 or its SUMO conjugated forms, we coexpressed Myc–His-tagged Ub and PARP-1 for subsequent purification of Ub conjugates by nickel-ion affinity chromatography. As shown in Figure 6G, expression of PARP-1 and Myc–His–Ub alone yielded no detectable PARP-1–Ub conjugates (lane 2). Addition of RNF4, however, led to the appearance of a characteristic smear corresponding to PARP-1–Ub conjugates (lane 3). Further addition of SUMO-2 extended this smear to even higher MW species (lane 5), an effect that could not be seen in the absence of added RNF4 (lane 4),

Figure 6 Involvement of RNF4 in SUMO-dependent PARP-1 ubiquitination and heat-shock-promoter activation. (**A**) RNF4 interacts with PARP-1 *in vivo*. Co-immunoprecipitation (IP) experiment with the indicated expression vectors (mut FLAG-RNF4: C136/139/177/180S mutant) transfected in HeLa cells. WCL, whole-cell lysate, 5% of amount used in IP. (**B**) RNF4 induces PARP-1 degradation through its Ub E3 ligase activity. Whole-cell lysates of HeLa cells transfected as indicated were analysed by western blot with the indicated antibodies. Cleaved PARP-1, PARP-1 N-terminal fragment produced by caspase-dependent cleavage. (**C**) RNF4-induced PARP-1 degradation is dependent on the proteasome. HeLa cells transfected as indicated were left untreated or treated with 50 μM MG132 for 8 h. Protein levels were then checked by western blot using the indicated antibodies. (**D**) Preferential binding of SUMO-2-modified PARP-1 to RNF4. GST pull down with ^{35}S-labelled PARP-1 *in vitro* modified by SUMO-1 and SUMO-2. Input: 5% of modified PARP-1 used in the binding reactions. (**E**) RNF4 reduces PARP-1-SUMO-2 conjugate levels. Western blots of extracts from HeLa cells transfected as indicated. (**F**) RNF4 knockdown enhances heat-shock-induced PARP-1 conjugate levels. Western blots of HeLa cells transfected with scramble (scr) or RNF4 siRNA, left untreated, or heat shocked (30 min, 43°C) 48 h later. (**G**) RNF4 acts as a Ub E3 ligase for PARP-1. HeLa cells overexpressing FLAG–PARP-1 together with the indicated proteins were treated at 40 h post transfection with 50 μM MG132 for 8 h and whole-cell extracts (WCE) were prepared under denaturing conditions. His–Ub conjugates were affinity purified by nickel-ion chromatography and probed with anti-PARP-1 antibody (top panel). WCE, cleared of guanidine by TCA precipitation, were probed with the indicated antibodies (middle and bottom panels). Positions and sizes (in kDa) of MW marker proteins are indicated. (**H**) Heat shock induces ubiquitination of endogenous PARP-1 in HeLa cells. FLAG (control) or PARP-1 immunoprecipitates from untransfected HeLa cells untreated or heat shocked (30 min, 43°C) were analysed by western blot using anti-Ub antibody (right panel). The corresponding whole-cell lysates were analysed using anti-PARP-1 antibody (left panel). (**I**, **J**) RNF4 knockdown attenuates *HSP70.1* gene expression. (**I**) HeLa cells were transfected with scrambled control (scr) or RNF4 siRNA and left untreated or heat shocked (1 h, 43°C) 48 h later. After recovery at 37°C for 30 min, *HSP70.1* gene expression, normalized against *GAPDH*, was determined by quantitative RT–PCR. (**J**) Hela cells were transfected with scrambled control (scr) or RNF4 siRNA and re-transfected 24 h later with *HSP70.1* promoter–luciferase reporter and CMV–β-gal control plasmid. They were then left untreated or heat shocked (1 h, 43°C) after an additional 24 h. After a further 12 h, luciferase and β-gal activities were determined. Values for luciferase activity were corrected for β-gal activity. Plotted values in panels E and F represent means ± s.e. for three independent experiments, with non-heat shocked scrambled control value set to 1.

indicating that RNF4 and SUMO-2 enhance the ubiquitination of PARP-1.

The finding that heat shock greatly enhances PARP-1 sumoylation and that RNF4 acts as a Ub E3 ligase for PARP-1, predicts that heat shock would similarly enhance PARP-1 ubiquitination. To test this at the endogenous protein level, we carried out an anti-PARP-1 (or anti-FLAG control) immunoprecipitation from extracts of unstressed or heat shocked HeLa cells. As shown in Figure 6H, probing such immunoprecipitates with an anti-Ub antibody revealed the characteristic high-MW poly-Ub smear from extracts of heat shocked, but not unshocked, cells (compare lanes 4 and 6).

To next evaluate the role of RNF4 in *HSP70.1* gene activity in response to heat shock, we used siRNA to ablate RNF4



expression in HeLa cells. As seen in Figure 6I, RNF4 knockdown led to consistent, albeit modest, reduction (25%) in basal and heat-shock-induced activity of the endogenous *HSP70.1* gene. A similar, but more pronounced, result (50%) was obtained using instead an *HSP70.1* promoter–luciferase reporter construct (Figure 6J), indicating that, like PIASy (Figure 5B and C), RNF4 appears to be necessary for full activity of this heat-shock-inducible promoter.

Taken together, these results support the involvement of the SUMO-specific Ub E3 ligase RNF4 in regulating both the abundance of SUMO-modified PARP-1 and the activity of the heat-shock-inducible *HSP70.1* promoter.

Discussion

In this report, we have described the association of the SUMO E3 ligase PIASy with the poly(ADP-ribosyl)polymerase PARP-1 and explored its functional consequences in the regulation of the heat-shock-inducible *HSP70.1* gene. As discussed below, our results are consistent with a model whereby heat shock induces rapid PARP-1 multi- and poly-sumoylation, which leads to RNF4 recruitment, ubiquitination, and subsequent degradation, thus likely contributing to PARP-1 clearance from a heat-shock-inducible promoter (Figure 7).

Poly(ADP-ribosyl)ation and sumoylation

Together with three recent reports (Blomster *et al*, 2009; Golebiowski *et al*, 2009; Messner *et al*, 2009), the present work adds sumoylation to the list of post-translational modifications affecting the activity of PARP-1. Besides poly(ADP-ribosyl)ation, previous work has also shown that PARP-1 is subject to acetylation (Hassa *et al*, 2005; Messner *et al*, 2009), phosphorylation (Kauppinen *et al*, 2006), and K48-linked ubiquitination (Wang *et al*, 2008). Indeed, Messner *et al* (2009) have shown that PARP-1 mono-sumoylation at K486 inhibits p300-mediated acetylation at lysines proximal to this modification site, confirming the existence of cross-talk mechanisms between these different modifications. Our demonstration here that PIASy may be poly(ADP-ribosyl)ated, besides confirming the physical interaction with PARP-1, could furthermore suggest that the activity of PIASy, like that of PARP-1 itself, is regulated by poly(ADP-ribosyl)ation. This could, for example, affect the DNA or chromatin binding of PIASy, as has been shown for p53 (Mendoza-Alvarez and Alvarez-Gonzalez, 2001), or its SUMO E3 ligase activity. Conversely, recent *in vitro* results suggest that sumoylation does not affect poly(ADP-ribosyl)ation of PARP-1 (Messner *et al*, 2009). Nonetheless, given our finding that poly(ADP-ribosyl)ated PARP-1 exhibits reduced binding to PIAS *in vivo*, but not *in vitro*, it will be interesting to further explore the possible interplay between poly(ADP-ribosyl)ation and sumoylation.

The steady-state level of sumoylated PARP-1 in non-stressed cells is very low. For this reason, perhaps, Messner *et al* (2009) report on only mono-sumoylated PARP-1 under their experimental conditions. Unlike these authors, we found PIASy to stimulate PARP-1 sumoylation in both unstressed, as well as heat shocked cells. This role for the members of the PIAS family proteins in the stimulation of sumoylation under thermal stress appears to be evolutionarily conserved, as it has also been described in plants (Kurepa *et al*, 2003; Yoo *et al*, 2006; Miura *et al*, 2007; Saracco *et al*, 2007). Heat shock greatly stimulates the formation of high-MW PARP-1 species, which consist principally of poly-SUMO-2/3 conjugates (this work and Blomster *et al*, 2009;

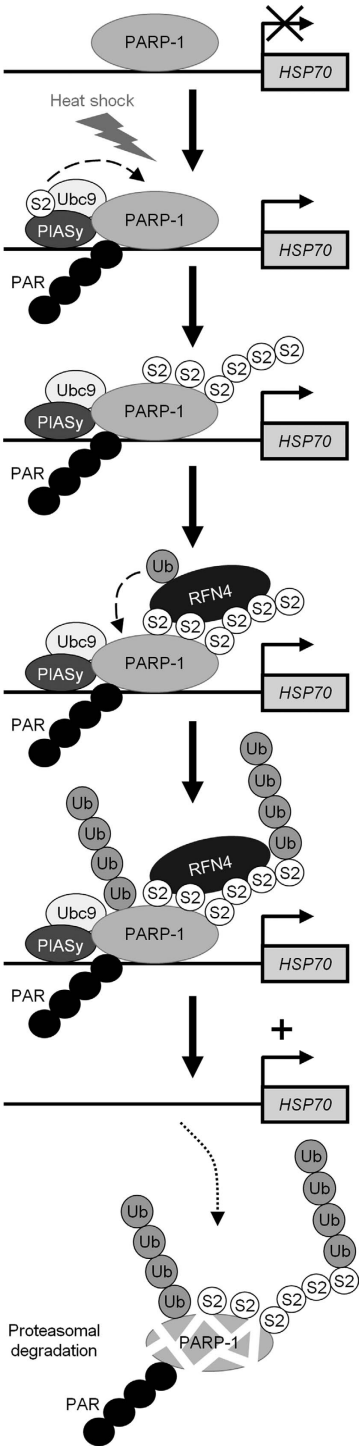


Figure 7 Role of PARP-1 sumoylation and ubiquitination in the regulation of *HSP70.1*-promoter activation. PARP-1, present on the *HSP70.1* promoter under normal conditions, but repressed by sequestration in the mH2A1.1 complex, is poly(ADP-ribosyl)ated upon heat shock (not shown; Ouararhni *et al*, 2006). In parallel, Ubc9 and PIASy are rapidly recruited to catalyse PARP-1 poly- and multi-sumoylation, in turn recruiting RNF4, which catalyses ubiquitination and subsequent degradation of PARP-1, thereby contributing to gene activation by PARP-1 clearance from promoter. For clarity, other possible modifications and chromatin factors discussed in the text have been omitted.

Golebiowski *et al*, 2009). Like for arsenic-induced hyper-sumoylation of PML (Lallemand-Breitenbach *et al*, 2008; Tatham *et al*, 2008), or that of other proteins under different stresses (Saitoh and Hinchey, 2000), the effectors regulating the sumoylation of PARP-1 and of numerous other proteins under heat shock remain to be identified.

PARP-1 ubiquitination and degradation

Our finding that heat shock induces the hyper-sumoylation of PARP-1, principally by SUMO-2/3, raised the possibility that PARP-1 is targeted by the E3 Ub ligase RNF4. In support of this, we found that RNF4 overexpression enhances PARP-1 ubiquitination and proteasome-mediated degradation. Furthermore, consistent with a role of RNF4, the highly poly-sumoylated forms of PARP-1 displayed reduced stability, whereas conversely, RNF4 depletion led to their stabilization. Finally, we show that PARP-1 ubiquitination, like sumoylation, is strongly enhanced by heat shock. Altogether, these results link the sumoylation and ubiquitination of PARP-1 and, moreover, provide evidence for a novel, caspase-independent pathway for PARP-1 degradation.

RNF4, in possessing four SIMs, has been shown to target only poly-SUMO-2/3-modified substrates with high affinity (Tatham *et al*, 2008). That PARP-1 likely possesses many more possible sumoylation sites besides the two principal sites described here (K203 and K486), raises the possibility that not only poly-sumoylation, but also multi-sumoylation of PARP-1, could lead to RNF4 recruitment, even by the non-chain forming SUMO-1. Such a mechanism has been suggested recently (Ulrich, 2008) and may account for our finding that RNF4 leads to the ubiquitination of PARP-1 even without heat shock (Figure 6G), that is, under conditions in which formation of poly-SUMO-2 chains is presumably minimal.

Transcriptional regulation

Sumoylation of transcription factors and cofactors is generally associated with repression mechanisms (for reviews, see Verger *et al*, 2003; Girdwood *et al*, 2004; Müller *et al*, 2004; Gill, 2005). Where sumoylation has been shown to contribute to activation, as in the case of p53 (Gostissa *et al*, 1999; Rodriguez *et al*, 1999; Müller *et al*, 2004; Bischof *et al*, 2006) or Tcf4 (Yamamoto *et al*, 2003), the mechanisms involved remain obscure. In this context, members of the HSF (heat shock factor) family of transcription factors are modified by SUMO (Goodson *et al*, 2001; Hong *et al*, 2001; Hietakangas *et al*, 2003, 2006; Hilgarth *et al*, 2004; Anckar *et al*, 2006). The precise function, here, of sumoylation in activating or repressing gene transcription, however, appears to be complex and may involve regulation of response duration or intensity, rather than simple on/off switching (Hietakangas *et al*, 2003).

Similarly, the role of PARP-1 and poly(ADP-ribosyl)ation in transcriptional regulation is multi-faceted and context-dependent. In some cases, such as in NF-κB-mediated activation, poly(ADP-ribosyl)ation appears dispensable (Hassa *et al*, 2003) or may even repress activity (Meisterernst *et al*, 1997). Where it does contribute to activation, it is generally seen as leading to chromatin decompaction (Poirier *et al*, 1982; Kim *et al*, 2004; Wacker *et al*, 2007), possibly mediated by electrostatic repulsion between poly(ADP-ribosyl)ated proteins (e.g. PARP-1 and histones) and the DNA. The finding that PARP-1 poly(ADP-ribosyl)ation activity is held in check by interaction with the variant histone mH2A (Ouararhni *et al*, 2006; Nusinow *et al*, 2007), has also provided further evidence that PARP-1 and poly(ADP-ribosyl)ation exert their function in a context-dependent manner. In the case of a constitutively silent promoter, such as that of an inactive X (Xi)-linked transgene, PARP-1 is indispensable for silencing (Nusinow *et al*, 2007), whereas for the *HSP70.1* promoter, it is required for inducible activation (Ouararhni *et al*, 2006). Yet, even in the absence of PARP-1, heat shock promotes significant promoter activation, thus suggesting the existence of PARP-1-independent mechanisms. Nevertheless, PIASy, sumoylation, and RNF4 appear critically involved, as reducing their activity also reduces PARP-1-dependent promoter activation. That this occurs also in the absence of heat shock may suggest that the sumoylation of PARP-1 plays a similar role under basal conditions, but that, in absolute terms, sumoylation exerts its most significant effect upon heat shock.

PARP and poly(ADP-ribosyl)ation have been shown to be required for rapid nucleosome remodelling that precedes transcriptional onset upon heat shock in *Drosophila* cells (Petesch and Lis, 2008). Yet interestingly, poly(ADP-ribosyl)ation by itself does not appear to be sufficient for the release of PARP-1 from the condensed mH2A1.1 chromatin (Ouararhni *et al*, 2006), suggesting that additional factors, such as chromatin remodellers (e.g. SWI/SNF; Pavri *et al*, 2005), sumoylation, or other post-translational modifications, are critically required. The results obtained to date do not provide sufficient temporal resolution to unravel the order, if any, with which poly(ADP-ribosyl)ation and sumoylation occur upon heat shock, but it is highly likely that sumoylation, like poly(ADP-ribosyl)ation, plays an important role in the removal of PARP-1 from the promoter. Consistent with this, we show that PARP-1, PIASy, and Ubc9 leave the promoter with similar kinetics upon prolonged heat shock. Moreover, our finding that sumoylated PARP-1 is associated with the insoluble cellular fraction is again consistent with a role of sumoylation in the differential localization of the protein.

Sumoylation-coupled ubiquitination and degradation may also be necessary for the enhanced or prolonged clearance of PARP-1 from a heat-shock-induced promoter in that sustained transcriptional activation or its rapid extinction upon stimulus withdrawal may require the rapid recycling of PARP-1. A similar model has also been invoked for the sumoylation of PEA3 during synergistic activation of target genes with CBP (Guo and Sharrocks, 2009). Our finding that other factors associated with HSP promoters are sumoylated or are PIASy-binding partners (e.g. MEN1, Ku70/80, and PRMT5) suggests that SUMO-triggered, RNF4-mediated ubiquitination may similarly play a wider role by regulating the activity of other proteins besides PARP-1.

Materials and methods

Plasmids and siRNAs

FLAG–HA–PIASy was inserted into the pcDNA3 vector (Invitrogen); T7-PIAS1, T7-PIAS3, T7-PIASxα, T7-PIASxβ, and T7-PIASy into the pSG5 vector (Stratagene); PARP-1 into pFLAG-CMV-6c (Sigma), pSG5, pBS, and pBABE vectors; and RNF4 into the pGEX2T (GE Healthcare) and pcDNA3 vectors by standard procedures. Point mutant derivatives of PARP-1 (K203R, K249R, K486R, K512R, K798R, E988K, and K203R/K486R double mutant) were constructed by site-directed mutagenesis (QuikChange XL kit; Stratagene).

GST–PIASy, FLAG–HA–PIASy WT, and mut (C342F); GST–PARP-1, SUMO-1, His–SUMO-1, SUMO-2, His–SUMO-2, Ubc9, SENP-1, T7-PIASy, and CMV–β-galactosidase plasmids were described previously (Masson *et al*, 1998; Sachdev *et al*, 2001; Bischof *et al*, 2006). Plasmid for His–Myc–Ub was kindly provided by C Neuveut; for FLAG–RNF4 WT and mut (C136/139/177/180S) by J Palvimo; for SENP-6 by R Hay; for VSV-MEN1 by CX Zhang; for FLAG–PRMT5 by C Sardet; and for *HSP70.1* promoter–luciferase reporter by O Bensaude. All constructions were verified by DNA sequencing. siRNAs used were as follows: PIASy sense sequence: CAAGACAGGUGGAGUUGAUUU; RNF4 sense sequence: GAAUGGA CGUCUCAUCGUUUU, as well as scrambled controls (Dharmacon).

Cell culture, infection, transfection, and reporter assays

HeLa cells and PARP-$1^{+/+}$ and PARP-$1^{-/-}$ MEFs were grown in DMEM and Jurkat cells in RPMI medium under standard culture conditions. Poly(ADP-ribosyl)ation (without heat shock) was induced by treatment with 1 mM H_2O_2 (Gifrer) for 10 min and/or inhibited with 30 mM DPQ (Alexis Biochemicals) for 1.5 h. Protein stability was analysed by treating the cells with 50 μM MG-132 (Sigma) for 8 h or 50 μg/ml cycloheximide (Sigma) for the times indicated. Infections of MEFs by retrovirus-mediated gene transfer were performed with Phoenix packaging cells. At 24 h post-infection, cells were selected with 4 μg/ml puromycin for 4 days. Transfections of plasmids and siRNAs in HeLa cells were performed with Lipofectamine and with Oligofectamine (Invitrogen), respectively. Five days after the end of selection, or 48 h after transfection, cells were heat shocked at 43°C if needed and protein or RNA extraction was performed. For some *in vivo* sumoylation assays and for reporter assays, HeLa cells were transfected with siRNAs and re-transfected 24 h later with expression vectors or *HSP70.1* promoter–luciferase reporter and CMV–β-gal control plasmids. Cells were heat shocked at 43°C after a further 24 h, either lysed directly for *in vivo* sumoylation assays or 12 h later for luciferase and β-gal assays. Luciferase and β-gal activities were determined using the Luciferase reporter assay system (Promega) and the Galacto-star kit (Tropix).

Protein extraction, immunoprecipitation, and His pull down

For sumoylation and ubiquitination studies, cells were washed in PBS supplemented with 10 mM *N*-ethylmaleimide (NEM; Sigma). For direct western blots, cells were lysed directly in sample buffer containing 2% sodium dodecyl sulphate (SDS). For co-immunoprecipitation of PIAS with PARP-1, MEN1, Ku70/Ku80, and PRMT5, cells were scraped in PBS and lysed in Chris buffer (50 mM Tris, pH 8.0, 0.5% NP-40, 200 mM NaCl, 0.1 mM EDTA, 10% glycerol, and protease inhibitors (Complete EDTA-free; Roche)). For co-immunoprecipitation of PARP-1 with RNF4, cells were scraped in PBS and lysed in RIPA buffer (50 mM Tris, pH 8.0, 1% Triton X-100, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, protease inhibitors, 10 mM NEM). For immunoprecipitation of PARP-1 conjugates under untreated or heat-shock conditions, cells were lysed in SDS sample buffer, diluted 10-fold in Chris buffer. Total cell lysates were then incubated for 2 h at 4°C with the appropriate antibody and immune complexes were collected by incubation for 1 h at 4°C with Protein G plus/Protein A agarose (Calbiochem) and washed three times in lysis buffer. In some cases, bound proteins were then eluted by incubating the beads for 45 min at 20°C with FLAG peptide (Sigma). His pull downs from transfected HeLa cells were carried out as described previously (Kirsh *et al*, 2002).

Immunoblotting and antibodies

Western blots were prepared on Hybond C-extra membranes (Amersham) and revealed using CDP-Star (Tropix). Antibodies used were as follows: mouse anti-PARP-1 (C2-10; Trevingen), rabbit anti-PARP-1 (H-250; Santa Cruz), mouse anti-poly(ADP-ribose) (10H; Alexis), mouse anti-HA (16B12; Covance), mouse anti-GST (B-14; Santa Cruz), mouse anti-T7 (Novagen), mouse anti-FLAG (M2; Sigma), rabbit anti-FLAG (Sigma), mouse anti-VSV (P5D4; Sigma), mouse anti-Ku70 (N3H10; Abcam), mouse anti-Ku80 (111; Abcam), mouse anti-β-actin (Sigma), mouse and rabbit IgGs (Upstate), mouse anti-Ubc9 (50; Pharmingen), mouse anti-Ub (FK2; Biomol), rabbit anti-PIASy (Bischof *et al*, 2006), mouse anti-SUMO-1 (Zymed), mouse anti-SUMO-2 (8A2; Zhang *et al*, 2008), and rabbit anti-RNF4 (a gift from J Palvimo; Häkli *et al*, 2005).

RNA isolation and RT–PCR analysis

Total RNA was extracted using the RNeasy RNA isolation kit (Qiagen) and reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). cDNAs were added to the SYBR Green PCR master mix (Applied Biosystems) using the following oligonucleotide pairs: 5′-CCAAGGTGCAGGTGAACTACAA -3′ and 5′-CAGCACCATGGACGAGATCTC-3′ for *HSP70.1* and 5′-GC AAAGTGGAGATTGTTGCCA-3′ and 5′-ATTTGCCGTGAGTGGAGT CAT-3′ for *GAPDH*. Real-time quantitative PCR was performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and normalized to *GAPDH* signal.

Chromatin immunoprecipitation

ChIP was carried out as previously described (Bischof *et al*, 2006). Chromatin immunoprecipitated DNA was analysed by PCR with the following primers: 5′-GGCGAAACCCCTGGAATATTCCCGA-3′ and 5′-AGCCTTGGGACAACGGGAG-3′ for *HSP70.1* promoter and 5′-GG ACCTGACCTGCCGTCTAGAA-3′ and 5′-GGTGTCGCTGTTGAAGTCAG AG-3′ for *GAPDH* promoter.

Protein expression and in vitro sumoylation assays

GST, GST–PIASy, and GST–RNF4 were produced in BL21(DE3) pLysS cells and PARP-1 in Sf9 cells and purified under native conditions using standard protocols. ^{35}S-methionine-labelled, *in vitro* translated proteins were prepared using the T7 or Sp6 TNT-coupled reticulocyte lysate kit (Promega). *In vitro* sumoylation assays were carried out by incubating recombinant or ^{35}S-methionine-labelled *in vitro* translated PARP-1 or PRMT5 with recombinant Aos1/Uba2 (370 nM), Ubc9 (630 nM), and SUMO (7 μM) in 30 mM Tris, 5 mM ATP, 10 mM $MgCl_2$, pH 7.5, at 33°C as previously described (Kirsh *et al*, 2002). Recombinant GST–PIASy (at a final concentration of 500 nM), a FLAG eluate, or an *in vitro* translated PIAS was added in this reaction.

In vitro poly(ADP-ribosyl)ation assays

For *in vitro* poly(ADP-ribosyl)ation of PARP-1, unlabelled, or ^{35}S-methionine-labelled, *in vitro* translated PARP-1 was incubated in 20 μl of activity buffer (50 mM Tris, pH 7.5, 4 mM $MgCl_2$, 200 μM dithiothreitol (DTT), 0.1 μg/μl BSA, 4 ng/μl DNaseI-activated calf thymus DNA, and 400 μM NAD^+). To test PIASy poly(ADP-ribosyl) ation by PARP-1, FLAG eluates or GST fusion proteins were incubated with 100 ng of recombinant PARP-1 in activity buffer supplemented with 1 μCi ^{32}P-NAD^+. After 2 min at 20°C, reactions were stopped by dilution in SDS sample buffer, resolved by gel electrophoresis, and transferred to nitrocellulose membrane for visualization of (ADP-ribosyl)ated products by autoradiography or western blot.

GST pull down

Recombinant PARP-1, ^{35}S-methionine-labelled, *in vitro* translated proteins, or products of an *in vitro* sumoylation or poly(ADP-ribosyl)ation assay were incubated with the relevant GST-fusion protein bound to 10 μl of glutathione–Sepharose beads (Amersham). After 4 h incubation at 4°C and five washes in GST binding buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 0.1% Triton-X100, 10% glycerol, 1 mM DTT, and protease inhibitors), bound proteins were eluted with SDS sample buffer, resolved by gel electrophoresis, and visualized by immunoblotting with PARP-1 antibody or by direct autoradiography.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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