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RSUME, a Small RWD-Containing Protein, Enhances SUMO Conjugation and Stabilizes HIF-1α during Hypoxia

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SUMMARY

SUMO conjugation to proteins is involved in the regulation of diverse cellular functions. We have identified a protein, RWD-containing sumoylation enhancer (RSUME), that enhances overall SUMO-1, -2, and -3 conjugation by interacting with the SUMO conjugase Ubc9. RSUME increases noncovalent binding of SUMO-1 to Ubc9 and enhances Ubc9 thioester formation and SUMO polymerization. RSUME enhances the sumoylation of IkB in vitro and in cultured cells, leading to an inhibition of NF-kB transcriptional activity. RSUME is induced by hypoxia and enhances the sumoylation of HIF-1α, promoting its stabilization and transcriptional activity during hypoxia. Disruption of the RWD domain structure of RSUME demonstrates that this domain is critical for RSUME action. Together, these findings point to a central role of RSUME in the regulation of sumoylation and, hence, several critical regulatory pathways in mammalian cells.

INTRODUCTION

Small ubiquitin-related modifiers (SUMOs) are small (12 kDa) proteins with low sequence identity to ubiquitin but very similar 3D structure. To date, four SUMO family members have been described in vertebrates: SUMO-1-4 (Melchior, 2000; Hay, 2001; Guo et al., 2004).

SUMO is conjugated to proteins in three enzymatic steps analogous to the ubiquitin conjugation cascade. First, SUMO is activated by forming a thioester bond with the catalytic cysteine of an E1 activating complex (SAE I + SAE II in mammalian cells) in an ATP-dependent process and is then transferred to the catalytic cysteine of the SUMO E2 enzyme, known as Ubc9, that conjugates SUMO to the ε-amino group of a lysine side chain on the substrate (Melchior, 2000). Unlike ubiquitination, which requires an E3 ligase to complete the final transfer from the E2 to the substrate protein, sumoylation can occur without E3 ligases in vitro, although somewhat inefficiently (Hay, 2005). However, several SUMO E3 ligases, such as RanBP2 (Pichler et al., 2002), whose mechanism of action has been described in detail (Pichler et al., 2002; Reverter and Lima, 2005), and the protein inhibitor of activated signal transducer and activator of transcription (PIAS) family (Johnson and Gupta, 2001; Hochstrasser, 2001) interact with Ubc9 and are essential for the correct sumoylation of proteins in vivo (Hay, 2005). SUMO is removed from substrates by specific cysteine proteases (SUMO isopeptidases) called SENPs (Melchior et al., 2003).

Sumoylation regulates diverse cellular functions, including transcription (Girdwood et al., 2004), nuclear translocation (Gill, 2004), stress response (Saitoh and Hinchey, 2000), and chromatin structure (Kim et al., 2006). In some proteins, SUMO conjugation can take place on the same lysine residues that are targets for ubiquitin conjugation. Sumoylated lysines cannot be ubiquitinated, thus resulting in a stabilization of the target protein. This can lead to functional consequences, as in the case of IκB, whose stabilization by SUMO results in the inhibition of NF-κB transcriptional activity (Desterro et al., 1998).

The RWD domain has been recently named after three major RWD-containing proteins—RING finger-containing proteins, WD-repeat-containing proteins, and yeast DEAD (DEXD)-like helicases—and proposed as a protein-protein interaction domain (Doerks et al., 2002), although functional studies have not been performed yet. The structure of the mouse GCN2 RWD domain was determined, revealing significant structural homology to the

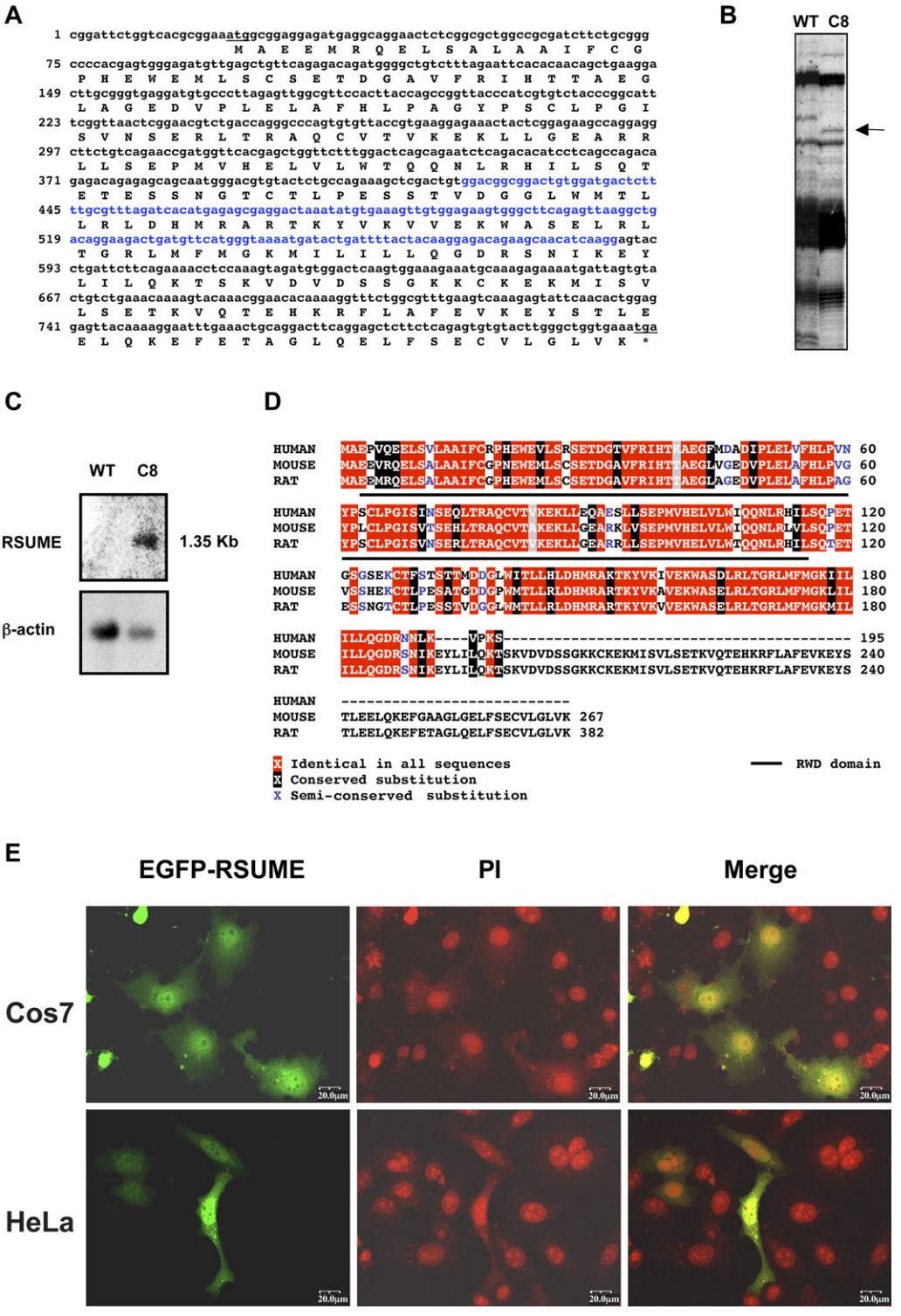


Figure 1. Cloning and Sequence Analysis of RSUME

(A) cDNA and deduced amino acid sequence of rat RSUME. The sequence of the fragment recovered from the differential display is highlighted in blue.

(B) mRNA differential display of pituitary GH3 cells stably transfected with a gp130 expression vector (C8) or the empty vector (WT). The arrow indicates the band identified as RSUME.

(C) Differential expression of RSUME was confirmed by northern blot analysis of mRNA obtained from GH3 control cells and C8 cells hybridized with the cloned band. Loading was assessed by β-actin.

mammalian SUMO conjugating enzyme Ubc9, despite a sequence similarity of only 13%. This core structure appears to be common among various RWD-containing proteins, although variations on the surface residues responsible for a putative protein-protein interaction specificity suggest specific roles for different RWD-containing proteins (Nameki et al., 2004).

In this work, we report the isolation and characterization of RSUME, a previously unknown gene coding for a small RWD domain-containing protein. RSUME was cloned from a pituitary cell line with an increased tumorigenic and angiogenic potential (Perez Castro et al., 2003). It is upregulated by cellular stress stimuli such as hypoxia and heat shock. RSUME enhances protein sumoylation through a direct interaction with Ubc9. These actions have functional implications on the NF-κB/IκB pathway and on the regulation of hypoxia inducible factor-1α (HIF-1α), both involved in angiogenesis and tumorigenic processes.

RESULTS

Identification of RSUME, an RWD-Containing Protein

We have previously generated stable clones from the rat pituitary lactosomatotrophic tumor cell line GH3 that show increased tumorigenic and angiogenic potential when injected into nude mice (Perez Castro et al., 2003). By differential display assay we compared one clone against a control GH3 clone stable for the empty vector. The differential bands were isolated, cloned, and sequenced. A previously uncharacterized mRNA (AF271158) coding for a putative protein of 267 amino acids (Figure 1A) appeared in two independent experiments (Figure 1B). A northern blot probed with the cloned sequence confirmed the differential expression (Figure 1C). According to the NCBI Conserved Domain Database, this protein has a single RWD domain spanning half its length (Figure 1D) and is thus referred to as RWD-domain-containing protein 3 (Rwdd3). For this reason and considering its function described in this work, we called this protein RSUME (for RWD-domain-containing sumoylation enhancer). RSUME is highly conserved in higher vertebrates (Figure 1D). Conserved hypothetical proteins with lower identity have been found in simpler organisms (*Drosophila*, NP_656300; zebrafish, XP702352.1; sea urchin, XP001200069.1). RSUME does not seem to belong to any previously known protein family.

The human RSUME protein is composed of 195 amino acids, with a putative molecular mass of 21 kDa. The sequence analysis revealed no consensus localization signal, and therefore by default the predicted localization of the protein was the cell's cytoplasm. This issue was addressed by subcloning RSUME as a GFP-fusion chimera and transfecting the construct into different cell lines. Despite lacking a nuclear localization signal, when observed under a confocal fluorescence microscope, N-terminal GFP-RSUME was found to be distributed in the nucleus and cytoplasm (Figure 1E). Similar results were obtained with the C-terminal GFP fusion protein.

RSUME Tissue Distribution and Expression

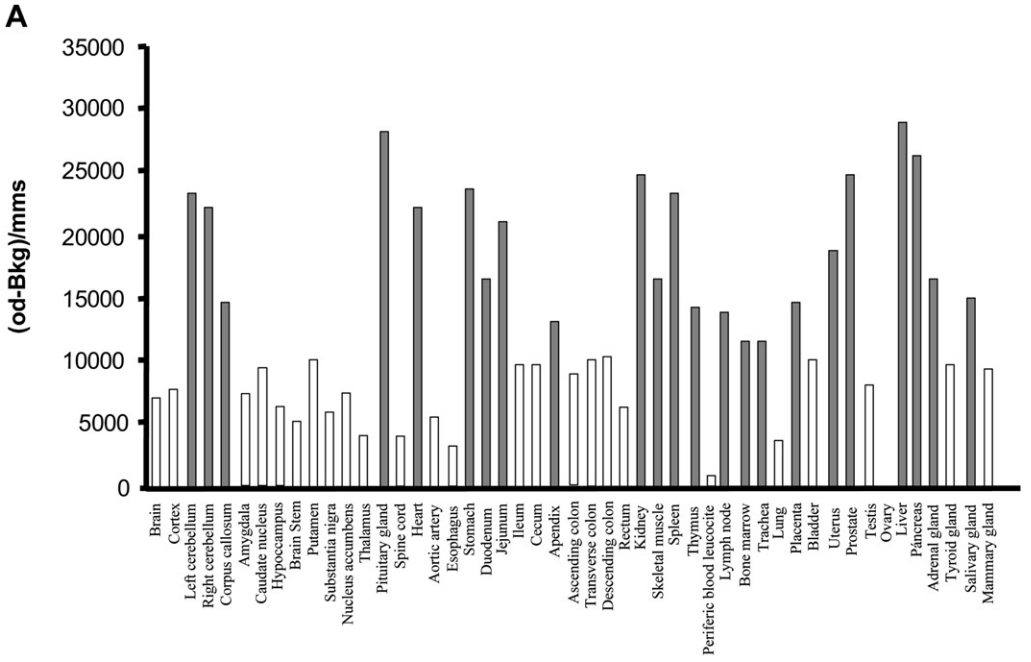
RSUME tissue distribution was studied in a commercial poly-A RNA dot blot membrane (Human Multiple Tissue Expression (MTE) Array, Clontech). RSUME is expressed in various tissues, with higher expression levels in cerebellum, pituitary, heart, kidney, liver, stomach, pancreas, prostate, and spleen (Figure 2A). Moreover, RSUME expression is induced in GH3 cells under stress conditions such as hypoxia, $CoCl_2$, and heat shock (Figure 2B). RSUME expression in pituitary tumors is increased by hypoxia (Figure 2C). Furthermore, it is expressed in glioma tumors (Figure 2C). In two gliomas in which two clearly different regions could be identified, we found RSUME expression in the necrotic inner zone but not in the peripheral zone of the tumor (Figure 2C).

RSUME Enhances Protein Sumoylation In Vitro and In Vivo

The RWD domain presents a significant structural similarity with the SUMO conjugase Ubc9 (Nameki et al., 2004), and heat shock and hypoxia, which induce RSUME, also induce SUMO-1 expression or increase protein sumoylation by SUMO-2/-3 (Saitoh and Hinchey, 2000; Shao et al., 2004). In view of the aforementioned, we tested whether RSUME is involved in protein sumoylation. Western blot (WB) against SUMO-1 showed increased protein sumoylation when RSUME was overexpressed in COS-7 cells (Figure 3A). RSUME did not change E1 levels (Figure 3A). Cotransfection with the SUMO hydrolase Senp-1 confirmed that the observed pattern corresponds to SUMO-conjugated proteins (Figure 3A). In a parallel control experiment, RSUME did not affect protein ubiquitination (Figure 3B).

We next characterized the action of RSUME on IκB. Recombinant RSUME was added to a cell-free in vitro sumoylation reaction. Addition of recombinant RSUME increased SUMO-1 conjugation, indicating a direct effect of RSUME on the core sumoylation machinery (Figure 3C). Despite its structural similarity with Ubc9, the RWD domain lacks a Cys residue structurally equivalent to Ubc9's Cys 93, and a sumoylation reaction performed

(D) Blast search to identify RSUME's protein homologs. Ortholog proteins with significant identity were found in humans, mouse, dog, chicken, and chimpanzee. A sequence alignment for human, mouse, and rat RSUME is shown. The RWD domain is underlined.
(E) Cellular distribution of N-terminal EGFP-RSUME visualized by confocal fluorescence microscopy (green). Nuclei were stained with propidium iodide (red). Merged images of both channels are included, with yellow representing overlapping signal. One representative image of 30 observations from two independent experiments with similar results is shown.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Pituitary gland
Spine cord
Heart
Aortic artery
Esophagus
Stomach
Duodenum
Jejunum
Ileum
Cecum
Apendix
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Transverse colon
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Skeletal muscle
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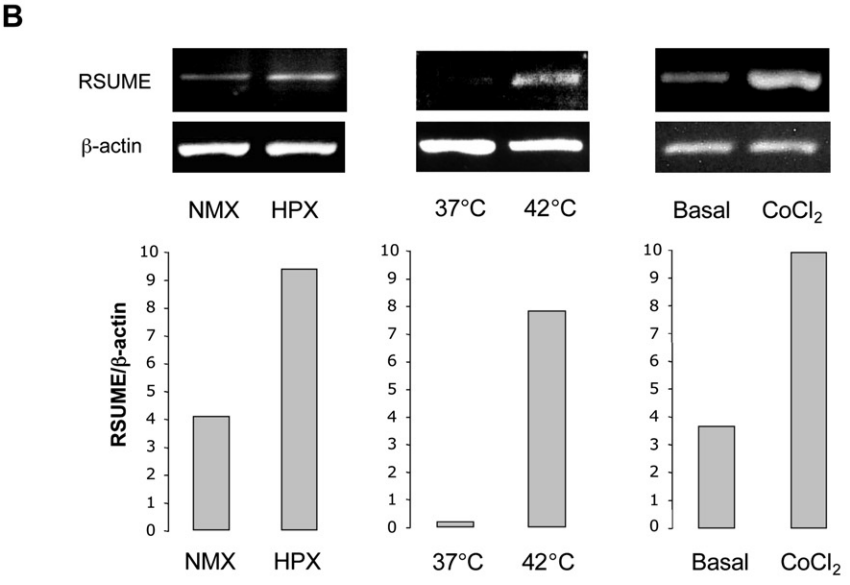
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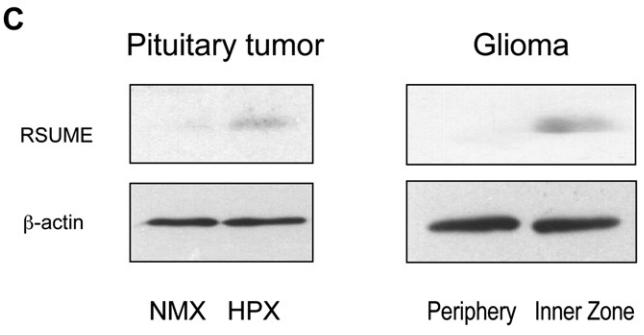
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without Ubc9 confirmed that RSUME lacks SUMO conjugase activity (Figure 3C).

Structural studies of the RWD domain revealed a highly conserved YPXXXP motif (Nameki et al., 2004). When we mutated this motif in RSUME in Tyr 61 and Pro 62 (two amino acids proposed to be critical to maintain the structural backbone of the domain structure) (Nameki et al., 2004) to alanine, sumoylation was no longer increased (Figure 3C). This suggests that the correct folding of RWD's domain is essential for RSUME activity.

To determine the functional relation of RSUME and Ubc9, we transfected COS-7 cells with RSUME together with Ubc9(C93S). This mutant was described previously to lack SUMO conjugase activity (Gong et al., 1997) and also to act as a dominant-negative by blocking endogenous Ubc9 (Giorgino et al., 2000), and is thus an ideal tool to address this question. As shown in Figure 3D, when Ubc9(C93S) was overexpressed, RSUME expression did not increase sumoylation, and enhanced the inhibitory effect of Ubc9(C93S). When Ubc9 and Ubc9 (C93S) were simultaneously overexpressed, the enhancing effect on Ubc9(C93S) disappeared only when similar amounts of both plasmids were transfected (Figure 3D, inset). These effects of RSUME were also manifest with SUMO-2 and SUMO-3, which use the same conjugase (Figure 3D). Ubc9(C93S) produced a slight decrease in RSUME levels, which did not affect the observed effects of RSUME (Figure 3D). These results indicate that (1) RSUME increases sumoylation by means of the catalytic activity of Ubc9 and that (2) RSUME also increases the negative effect of Ubc9(C93S) on sumoylation. These results support a direct role for RSUME facilitating the interaction of Ubc9 with the rest of the SUMO-conjugation complex.

To establish at which step RSUME acts, we performed a two-step in vitro sumoylation experiment, where RSUME was added either during the formation of the Ubc9-SUMO-1 thioester or during the transference of SUMO-1 from the thioester to a substrate (IκB). RSUME acted at both steps, although the strongest effect was seen when it was added during the formation of the thioester, in the absence of substrate (Figure 3E). Accordingly, RSUME enhanced Ubc9 thioester formation (Figure 3F). RSUME also enhanced Ubc9-mediated polymerization of SUMO-2 and SUMO-3 free chains (Figure 3G).

We next compared the activity of RSUME to that of the E3 ligase PIAS1, showing that when both were added together there was a stronger effect on overall sumoylation (Figure 3H).

RSUME Physically Interacts with Ubc9 and SUMO-1

The previous results suggested a direct interaction between RSUME and Ubc9. To address this hypothesis, we performed an in vitro pull-down experiment using recombinant GST-Ubc9 and RSUME, showing that RSUME interacts with Ubc9 (Figure 4A). Further immunoprecipitation experiments showed that RSUME could also interact with SUMO-1 (Figure 4B).

In competition experiments, RSUME enhanced SUMO-1 binding to Ubc9 (lower concentrations of SUMO-1 only interacted with Ubc9 when RSUME was present) (Figure 4C) in the presence or absence of E1 (see Figure S1 in the Supplemental Data available with this article online). When RSUME and Ubc9 assembly had been previously preformed, SUMO-1 did not displace RSUME binding to Ubc9 (left panel of Figure 4D). When all proteins competed simultaneously, higher concentrations of SUMO-1 prevented RSUME-binding to Ubc9 (lane 9 Figure 4A, lane 4 Figure 4C, and lane 3 right panel of Figure 4D; lane 5 Figure 4C and lane 4 right panel of Figure 4D).

To further understand RSUME's action, we studied its effect on the interaction of Ubc9 with E1 and found that RSUME increased the binding of Ubc9 and E1 (Figure 4E).

The interaction of RSUME to Ubc9 was confirmed in COS-7 cell extracts using GST-Ubc9 (Figure 4F). To explore whether this interaction was supported by the intracellular localization of RSUME and Ubc9, COS-7 and HeLa cells were cotransfected with EGFP-RSUME and RFP-Ubc9 constructs. A confocal microscopy examination showed a predominantly nuclear localization for Ubc9, as reported previously (Lee et al., 1998), and nuclear and cytoplasmic localization for RSUME. The merged image revealed defined nuclear spots where the colocalization of both proteins can be observed (Figure 4G). Together, these results strongly point to a direct interaction between RSUME and Ubc9.

RSUME Increases IκB Levels by Promoting Its Sumoylation and Inhibits NF-κB Transcriptional Activity and Target Expression

We next examined the functional consequences of the observed action of RSUME on IκB sumoylation in mammalian cells. Transfection of COS-7 cells with RSUME resulted in increased IκB sumoylation and protein stability (Figure 5A). The increased levels of IκB observed in Figure 5A are most likely due to a dynamic equilibrium of IκB with the increased sumoylated IκB pool that is refractory to degradation and leads to an increased stability of

Figure 2. Tissue Distribution and Expression of RSUME

(A) Tissue distribution of RSUME studied by hybridizing a commercial polyA mRNA dot blot membrane with the RSUME cDNA probe followed by a semiquantitative densitometric analysis. Tissues with the most significant expression levels are shaded in gray.

(B) RT-PCR to detect endogenous RSUME expression in pituitary GH3 cells under different stress conditions. Hypoxia: 1% O_2, 5% CO_2, 94% N_2 for 1 hr. Heat shock: 15 min at 42°C, then 1 hr at 37°. $CoCl_2$: 2% serum with 150 μM $CoCl_2$ for 3 hr. Densitometric values were normalized to β-actin levels. NMX, normoxia; HPX, hypoxia.

(C) Endogenous RSUME protein expression in brain tumors: one representative pituitary (nonfunctioning adenoma) and one glioma out of four tumors are shown. Periphery, peripheral well-vascularized zone; Inner, inner partially necrotic zone.

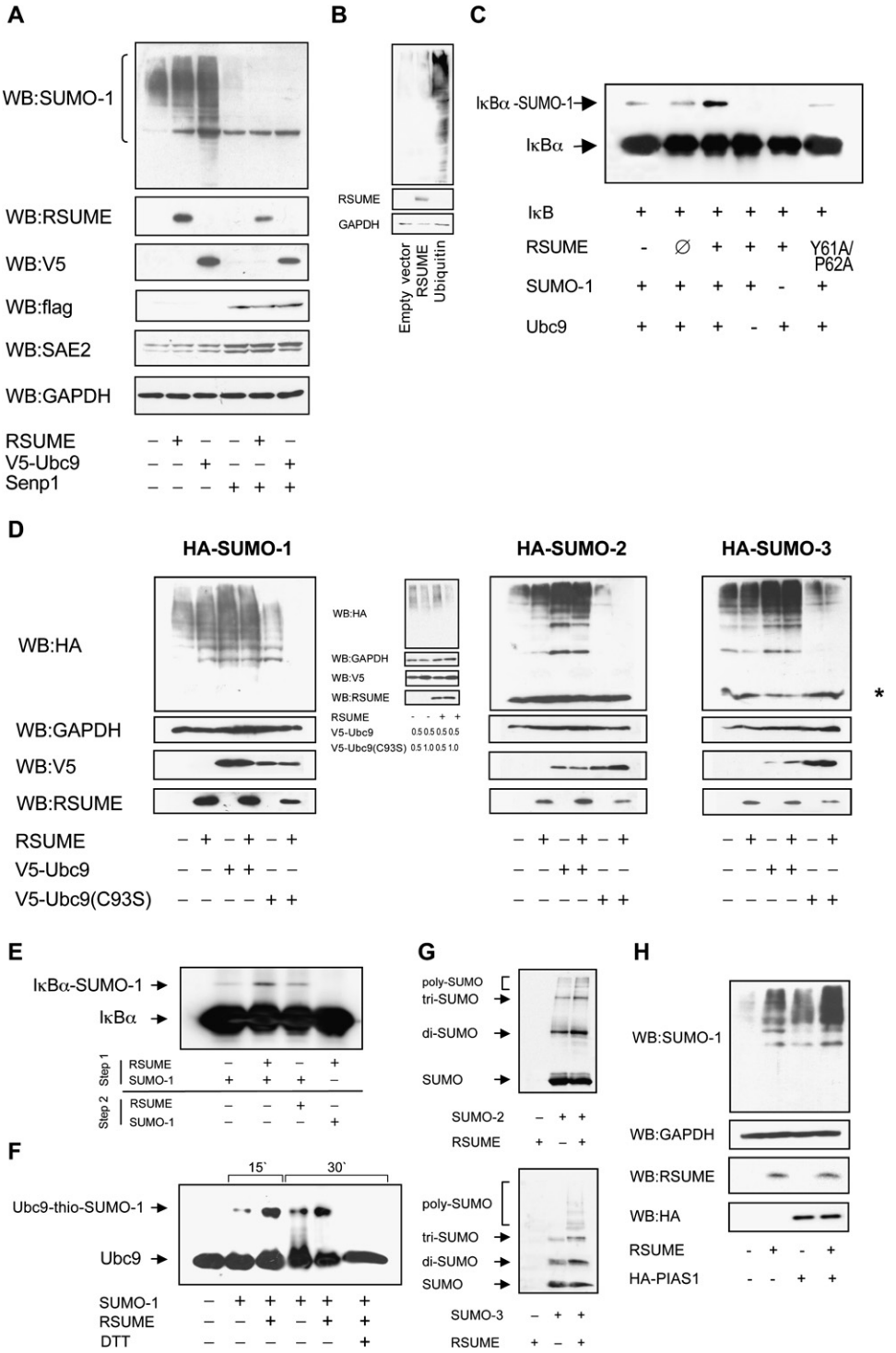

Figure 3. RSUME Enhances SUMO-Conjugation In Vivo and In Vitro

(A) COS-7 cells were transfected with 0.5 μg of RSUME, V5-Ubc9, and SENP-1 expression vectors. Forty-eight hours posttransfection, cell extracts were subjected to WB. GAPDH was used as loading control.

(B) COS-7 cells were transfected with 0.5 μg of RSUME or ubiquitin expression vectors. Forty-eight hours posttransfection, cell extracts were subjected to WB.

(C) SUMO-1 conjugation of recombinant IκB assayed in vitro in the presence or absence of 0.5 μg of recombinant RSUME protein or 0.5 μg of the Y61A/P62A mutant RSUME protein. Reaction mixes were subjected to WB with anti-IκB. ∅: control protein extract purified from *E.coli* transformed with the empty pQE30 vector.

the protein, as previously suggested (Desterro et al., 1998). Enhanced sumoylation of IκB by RSUME was abolished when IκB was mutated at lysines 21 and 22, both of which are SUMO-1 conjugation targets (Desterro et al., 1998) (Figure 5A). Immunoprecipitation experiments showed a direct interaction of RSUME with IκB (Figure 5B).

In order to determine whether there is a functional consequence of IκB's increased stability, we cotransfected RSUME with a κB-LUC reporter vector and studied its effects on TNF-α-induced transcriptional activity. RSUME inhibited TNF-α-induced reporter activity to levels similar to those obtained by overexpression of IκB or SUMO-1 (Figure 5C). Accordingly, small interference RNAs (siRNA) specifically directed against RSUME stimulated NF-κB transcriptional activity (Figure 5D). We then studied the action of RSUME on two NF-κB targets. RSUME inhibited interleukin-8 (IL-8) promoter activity (abolished when the NF-κB binding site of the promoter was mutated) (Figure 5E) and the expression of cyclooxigenase-2 (Cox-2) (Figure 5F).

Role of RSUME in HIF-1α Activation during Hypoxia

Hypoxia induces SUMO-1 expression (Shao et al., 2004), and sumoylation of HIF-1α leads to its stabilization (Bae et al., 2004). Given that RSUME is induced by hypoxia (Figures 2B and 2C) and plays a role in sumoylation and that sumoylation of some proteins with high molecular weight is increased by hypoxia (Figure S2), we hypothesized that RSUME might play a role in the cell's response to hypoxic stress.

HIF-1 is an heterodimer composed of HIF-1α and HIF-1β subunits. HIF-1α is rapidly degraded via the ubiquitin-proteasome pathway under normoxic conditions. Activation of HIF-1 by stabilization of HIF-1α during hypoxia regulates adaptive responses to changes in oxygen tension and is associated with various physiological and pathological processes (Semenza, 2003; Pouyssegur et al., 2006). We first transfected COS-7 cells with an RSUME expression vector and determined HIF-1α protein levels. After 16 hr hypoxic stress, RSUME expression increased HIF-1α protein levels, without affecting Ubc9 levels (Figure 6A). Reciprocally, we observed an increase of RSUME transcripts and promoter activity by hypoxia (bar 3 versus bar 1 and bar 4 versus bar 2) and by HIF-1α in normoxia (bar 2 versus bar 1) (Figures 6B and 6C). However, the effect of HIF-1α was less pronounced during hypoxia (bar 4 versus bar 3) where HIF-1α levels are already high (Figures 6B and 6C). Deletion in the RSUME promoter of the consensus sequence (RCGTG) reported to be critical for HIF-1α binding (Semenza et al., 1996; Bae et al., 2006) abolished the observed effects (Figure 6C).

The role of endogenous RSUME levels on HIF-1α activation was studied using siRNA specifically directed against RSUME. Downregulation of endogenous RSUME, confirmed by WB, resulted in the inhibition of endogenous HIF-1α stabilization during hypoxia (Figure 6D). Accordingly, a significant inhibition by RSUME siRNA was observed on HIF-1α transcriptional activity when measured in an HRE-LUC reporter system (Figure 6E). Immunoprecipitation experiments detected HIF-1α in an extract where RSUME was immunoprecipitated (Figure 6F), indicating an interaction between RSUME and HIF-1α during hypoxia that could account for the observed effects. Moreover, RSUME enhanced HIF-1α sumoylation both in vitro (Figure 6G) and in cells, where RSUME siRNA diminished HIF-1α sumoylation (Figure 6H). This supports a mechanism in which RSUME participates in HIF-1α stabilization, in line with the siRNA experiments shown in Figure 6D. As a functional consequence of HIF-1α stabilization, during hypoxia RSUME enhanced induction of promoter activity (Figure 6I), and mRNA and protein expression (Figure 6J) of a well-characterized HIF-1α target (Richard et al., 1999), VEGF, further supporting the functional relevance of the interaction between RSUME and HIF.

DISCUSSION

The RWD domain has been shown to have significant structural similarity with the SUMO conjugase Ubc9 (Nameki et al., 2004) and has been proposed to have a general function as protein-protein interaction motif. Nevertheless, there is no previous experimental evidence for its function in mammalian cells, and a specific biochemical function for this domain has only been described for the eIF2α kinase GCN2 in yeast (Kubota et al., 2000). No function has been determined for Rwdd1 and Rwdd2 either. In this work, we performed a detailed experimental analysis of RSUME, a small protein containing an RWD domain. We found that RSUME enhances SUMO

(D) 0.3 μg HA-SUMO-1, -2, and -3 expression vectors were cotransfected with 0.5 μg of RSUME, V5-Ubc9, and V5-Ubc9(C93S) expression vectors. Forty-eight hours posttransfection, cell extracts were subjected to WB with anti-HA to detect sumoylated proteins. *: free SUMO, which was not detected in the case of SUMO-1. RSUME expression was confirmed with anti-RSUME antibodies, and Ubc9 or Ubc9(C93S) expression was confirmed with anti-V5 antibodies. GAPDH was used as loading control.

(E) Two-step sumoylation assay for IκB. Step 1: thioester formation reactions with GST-Ubc9 in the absence of substrate at 30°C for 10 min. Step 2: the GST-Ubc9 thioester obtained in step 1 was incubated with recombinant IκB at 30°C for 5 min. 0.5 μg of RSUME was added either in step 1 or step 2 as indicated. Reaction mixes were subjected to WB with anti-IκB.

(F) Ubc9-SUMO1 thioester formation reactions were performed in vitro in the presence or absence of 0.3 μg of recombinant RSUME protein for the indicated times. Reactions were stopped with nonreducing gel loading buffer and subjected to WB with anti-Ubc9.

(G) Assays of poly-SUMO-2 and poly-SUMO-3 chain formation were performed with 5 μg of SUMO-3 or 8 μg GST-SUMO-2 in the presence or absence of 0.3 μg of recombinant RSUME. Reaction mixes were subjected to WB.

(H) COS-7 cells were transfected with 0.5 μg of RSUME and HA-PIAS1 expression vectors as indicated. Forty-eight hours posttransfection, cell extracts were subjected to WB. GAPDH was used as loading control.

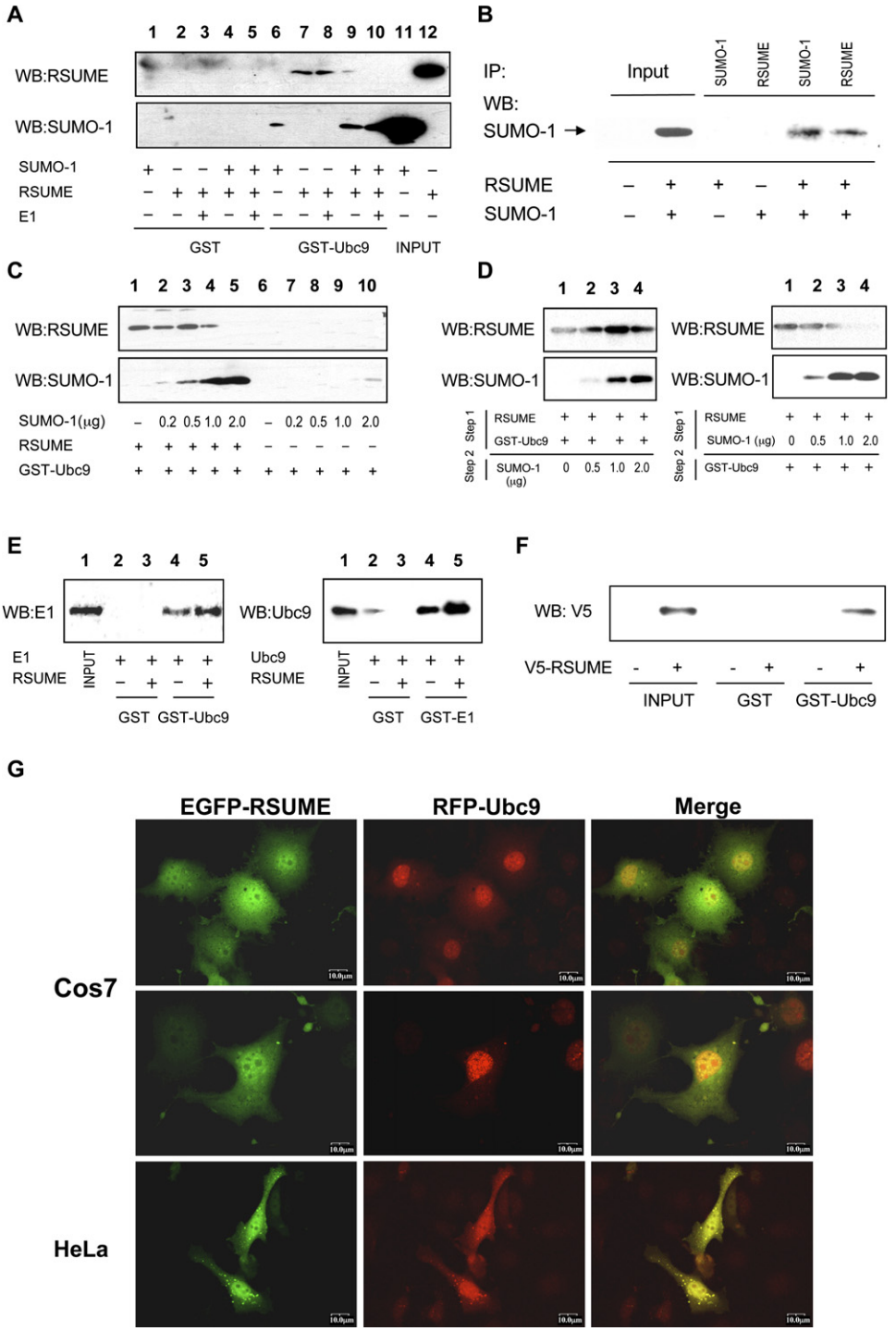

Figure 4. RSUME Directly Interacts with Ubc9

(A) 0.5 μg of recombinant RSUME was coprecipitated by GST-Ubc9 in vitro. Where indicated, 1.0 μg of SUMO-1 was included. Pull-down experiments were performed, and samples were subjected to WB.

(B) 0.5 μg of recombinant RSUME and 0.5 μg of recombinant SUMO-1 were coimmunoprecipitated in RIPA with anti-RSUME or anti-SUMO-1 antibodies. The immunoprecipitated fractions were subjected to WB with SUMO-1 antibodies.

(C) 0.5 μg of recombinant RSUME and 0.2–2.0 μg of recombinant SUMO-1 were coprecipitated by GST-Ubc9 in vitro pull-down experiments. Samples were subjected to WB.

(D) Two-step competition experiment. Step1: indicated mixes were incubated at 25°C for 1 hr. Step 2: SUMO-1 or GST-Ubc9 was added as indicated. Samples were precipitated by GST-Ubc9 as in vitro pull-down experiments, and reaction mixes were subjected to WB.

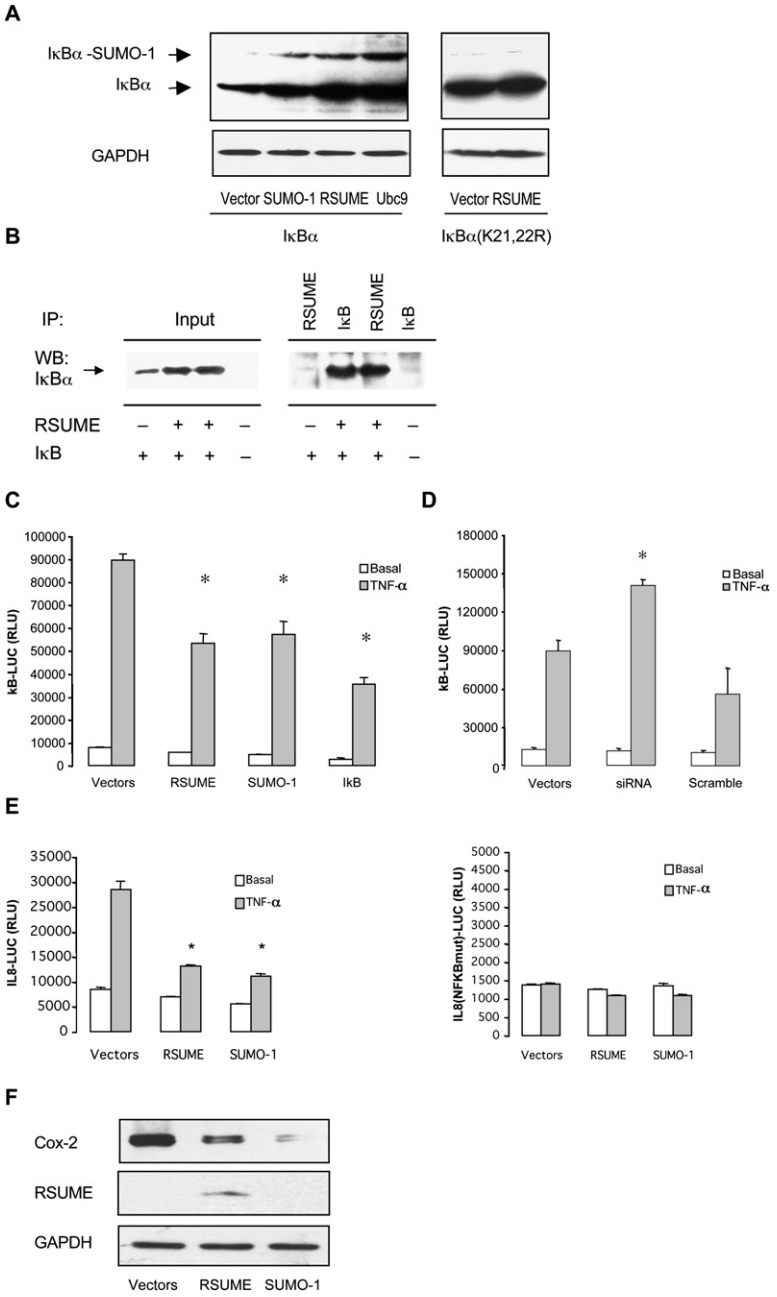

Figure 5. RSUME Stimulates IκB Sumoylation and Inhibits NF-κB Transcriptional Activity

(A) COS-7 cells were cotransfected with 0.5 μg of IκB, IκB (K21,22R), and the indicated expression vectors to analyze in vivo IκB sumoylation. Forty-eight hours posttransfection, cell extracts were subjected to WB with anti-IκB antibodies.

(B) 0.5 μg of recombinant RSUME and 0.5 μg of recombinant IκB were coimmunoprecipitated in RIPA with anti-RSUME or anti-IκB antibodies and subjected to WB.

(C and D) HepG2 cells were transfected with 0.5 μg of $(NF\text{-}\kappa B)_3$-LUC reporter vector and 0.5 μg of β-galactosidase control plasmid, with 0.5 μg of RSUME, HA-SUMO1, or IκB expression vectors (C) or 20 μM of siRNAs directed against RSUME or a scrambled siRNA as a control (D). After 24 hr, cells were stimulated with 10 ng/ml TNF-α for 6 hr, and luciferase (LUC) activity was measured in the cell extracts. Each value was normalized to β-galactosidase activity, and results are expressed as mean ± SEM of triplicates of one representative experiment of three experiments with similar results. *$p < 0.05$ compared with cells transfected with the empty vector stimulated with TNF-α (ANOVA with Scheffé's test).

(E) HepG2 cells were transfected with 0.5 μg of IL8-LUC or IL8(NFκBmut)-LUC reporter vector, 0.5 μg of RSUME, or HA-SUMO1 expression vectors and 0.5 μg of β-galactosidase control plasmid. After 24 hr, cells were stimulated with 10 ng/ml TNF-α for 6 hr, and LUC activity was measured in the cell extracts. Each value was normalized to β-galactosidase activity, and results are expressed as mean ± SEM of triplicates of one representative experiment of three experiments with similar results. *$p < 0.05$ compared with cells transfected with the empty vector stimulated with TNF-α (ANOVA with Scheffé's test).

(F) Glial 1321 cells were transfected with 0.5 μg V5-RSUME or HA-SUMO-1 expression vectors. Cells were subjected to 24 hr washout without serum and stimulated with 2.5 ng/ml IL-1 for 3 hr. Cell extracts were subjected to WB. GAPDH was used as loading control.

conjugation; this action depends on an intact RWD domain. Our study provides experimental evidence of a specific function for the RWD domain in a mammalian protein.

E1 and E2 have been shown to be sufficient for sumoylation of various substrates in vitro. However, most sumoylation reactions require E3 activity in vivo. RSUME has functional similarities to SUMO ligases: it enhances sumoylation in vitro and in vivo, colocalizes with Ubc9, and interacts with GST-Ubc9 in pull-down experiments. However, RSUME also increases the overall protein

(E) E1 or Ubc9 were coprecipitated by GST-Ubc9 or GST-E1, respectively, in vitro in the presence or absence of 0.5 μg RSUME. Pull-down experiments were performed, and samples were subjected to WB.

(F) Pull-down of RSUME transfected COS-7 extracts with GST-Ubc9. COS-7 cells transfected with V5-RSUME and HA-SUMO-1 expression vectors were lysed in GST binding buffer and precipitated with GST-Ubc9. Precipitates were subjected to WB.

(G) COS-7 and HeLa cells transfected with 0.5 μg EGFP-RSUME and 0.5 μg RFP-Ubc9 expression vectors were fixed 48 hr posttransfection. Colocalization of EGFP-RSUME (green) and RFP-Ubc9 (red) was observed by confocal fluorescence microscopy. Merged images of both channels are included, with yellow representing overlapping signal. One representative image of 30 observations from two independent experiments with similar results is shown.

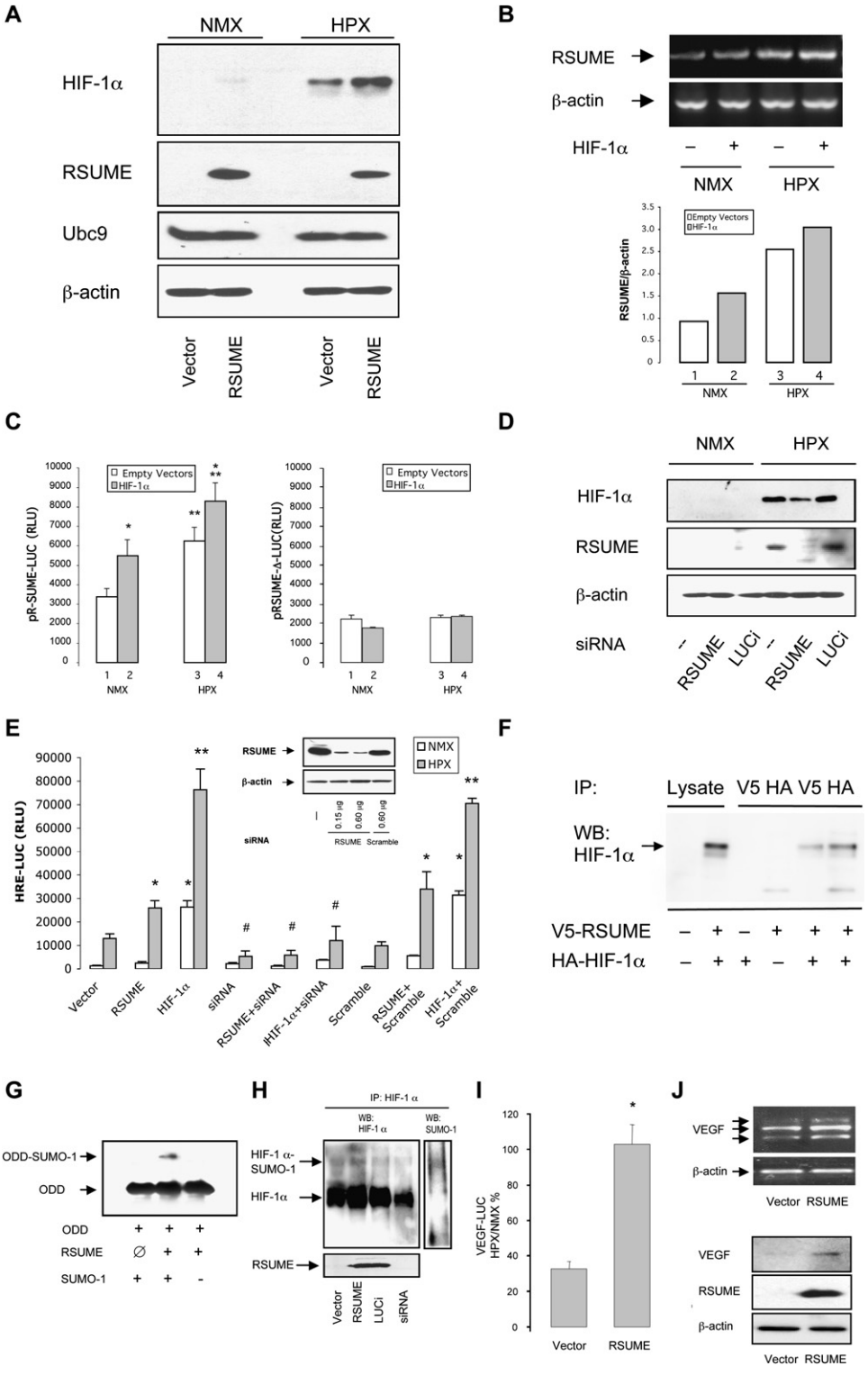

Figure 6. Role of RSUME during Hypoxia

(A) COS-7 cells were transfected with 0.5 μg of V5-RSUME or the corresponding empty vector. Twenty-four hours posttransfection, cells were subjected to hypoxic conditions (1% O_2, 5% CO_2, 94% N_2) for 16 hr. Cell extracts were subjected to WB. NMX, normoxia; HPX, hypoxia. β-actin was used as loading control.

sumoylation and the SUMO targeting of several proteins, thus pointing to a more general function as an enhancer of SUMO conjugation. The stimulation by RSUME of the dominant-negative effect of Ubc9(C93S) provides further support for this notion. Moreover, the two-step in vitro sumoylation experiment shows that RSUME has a stronger effect during the thioester formation step but also enhances transfer to the substrate. Thus, RSUME acts on two different phases of the sumoylation reaction: it enhances Ubc9-SUMO thioester formation and also the sumoylation of specific substrates (i.e., IκB and HIF-1α, with which RSUME interacts either directly or via Ubc9), with a stronger action in the first phase (Figure 7). In contrast to what is found in the ubiquitin pathway, in which E3s are the primary determinants of target specificity, in the SUMO pathway other alternatives may occur, for example the E2 Ubc9 also directly recognizes several targets, such as RanGAP1.

Even though only a small fraction of a given SUMO substrate protein is sumoylated in vivo at any given time as a result of dynamic sumoylation/desumoylation cycles, deficient or excessive sumoylation can have dramatic effects on the target protein function, making specific and general regulators of SUMO conjugation essential. The adenoviral protein Gam1 inhibits the SUMO pathway by interfering with the activity of E1 (SAE1/SAE2). Gam1 inhibits the formation of the E1-SUMO thioester complex and induces the disappearance of SAE1, SAE2, and Ubc9 in vivo, resulting in an overall inhibition of protein sumoylation (Boggio et al., 2004). Whereas Gam1 is a viral protein inhibitor of protein sumoylation, RSUME is a cellular enhancer of the sumoylation machinery, which does not affect E1 and Ubc9 levels and participates in specific cellular contexts such as hypoxia.

Unlike the ubiquitination pathway that can use several E2-conjugating enzymes, Ubc9 is the only known protein that can function as a SUMO E2 (Desterro et al., 1997; Gong et al., 1997). Accordingly, Ubc9-deficient mice die at the early postimplantation stage (Nacerddine et al., 2005). RSUME not only binds to Ubc9 and increases noncovalent SUMO-1 binding but also enhances Ubc9 activity. RSUME also interacts with SUMO-1. SUMO-1 prevents RSUME interaction with Ubc9 only in the higher concentrations in certain dynamic situations in which the heterodimer RSUME-Ubc9 has not yet assembled. Under these conditions, SUMO-1 may also bind to additional sites in RSUME that could partially overlap with the RSUME-Ubc9 binding site, which, in analogy with the Mms2-Ubc13 complex further discussed below, would include the N-terminal helix (VanDemark et al., 2001; Eddins et al., 2006).

The results showing the interaction of RSUME with the SUMO conjugation proteins and the enhancement of the formation of the E2-SUMO-1 thioester are in agreement with RSUME acting as a sumoylation enhancer (Figure 7). Noncovalent ubiquitin-like (Ubl) protein-E2 complexes are

(B) COS-7 cells were transfected with HIF-1α expression vector or the corresponding empty vector. Twenty-four hours posttransfection, cells were subjected to hypoxic conditions as in (A) for 16 hr, and RNA was extracted and amplified by RT-PCR to detect endogenous RSUME. Densitometric values were normalized to β-actin levels.

(C) COS-7 cells were transfected with 0.5 μg of pRSUME-LUC or pRSUME-Δ-LUC reporter vector, 0.5 μg of HIF-1α expression vector, and 0.5 μg of β-galactosidase control plasmid. Twenty-four hours posttransfection, cells were subjected to hypoxic conditions as in (A) for 16 hr, and LUC activity was measured in the cell extracts. Each value was normalized to β-galactosidase activity, and results are expressed as mean ± SEM of triplicates of one representative experiment of three experiments with similar results. *$p < 0.05$ compared with cells transfected with the empty vector. **$p < 0.05$ compared with the corresponding transfected cells under normoxic conditions (ANOVA with Scheffé's test).

(D) COS-7 cells were transfected with 20 μM siRNA directed against RSUME or Firefly Luciferase (LUCi) as a control. Twenty-four hours posttransfection, cells were subjected to hypoxic conditions as in (A) for 16 hr. Cell extracts were subjected to WB. β-actin was used as loading control.

(E) COS-7 cells were transfected with 0.5 μg of HRE(V4R)-LUC reporter vector, 0.5 μg of HIF-1α or 0.5 μg of RSUME expression vector, 20 μM of siRNAs directed against RSUME or a scrambled siRNA as a negative control, and pRL-TK control plasmid. Twenty-four hours posttransfection, cells were subjected to hypoxic conditions as in (A) for 16 hr, and LUC activity was measured in cell extracts. Each value was normalized to *Renilla* luciferase (pRL-TK) activity, and results are expressed as mean ± SEM of triplicates of one representative experiment of three experiments with similar results. *$p < 0.05$ and **$p < 0.01$ compared with the corresponding cells transfected with the empty vector; #$p < 0.05$ compared with the corresponding transfected cells not treated with siRNA directed against RSUME (ANOVA with Scheffé's test). Inset: specificity of siRNA directed against RSUME tested by WB.

(F) COS-7 cells were transfected with 1 μg of V5-RSUME and 1 μg of HA-HIF-1α expression vectors. Forty-eight hours posttransfection, cells were lysed in RIPA buffer and immunoprecipitated with anti-HA or anti-V5 antibodies. The immunoprecipitated fractions and the whole lysates were subjected to WB with anti-HIF-1α antibodies.

(G) SUMO-1 conjugation of recombinant GST-ODD domain (amino acids 378–603) of HIF-1α was assayed in vitro in the presence or absence of 0.5 μg of recombinant native RSUME protein. Reaction mixes were subjected to WB with anti-GST.

(H) COS-7 cells were transfected with 0.5 μg of RSUME expression vector, 20 μM of siRNAs directed against RSUME, or LUCi as a negative control. Twenty-four hours posttransfection, cells were subjected to hypoxic conditions as in (A) for 16 hr. Cells were lysed in RIPA buffer and immunoprecipitated with anti- HIF-1α antibodies. The immunoprecipitated fractions were subjected to WB with anti-HIF-1α. Anti-SUMO-1 WB is shown as control for the identity of the sumoylation band.

(I) COS-7 cells were transfected with 0.5 μg of VEGF-LUC reporter vector, 0.5 μg of RSUME, and 0.5 μg of β-galactosidase control plasmid. Twenty-four hours posttransfection, cells were subjected to hypoxic conditions as in (A) for 16 hr, and luciferase activity was measured in the cell extracts. Each value was normalized to β-galactosidase activity and results expressed as HPX/NMX % (mean ± SEM of triplicates of one representative experiment of three experiments with similar results). *$p < 0.05$ compared with cells transfected with the empty vector (ANOVA with Scheffé's test).

(J) HeLa cells were transfected with 0.5 μg of RSUME expression vector or with the corresponding empty vector. Twenty-four hours posttransfection, VEGF expression was assessed by RT-PCR and WB. β-actin was used as loading control. The three bands observed in the RT-PCR correspond to three VEGF isoforms.

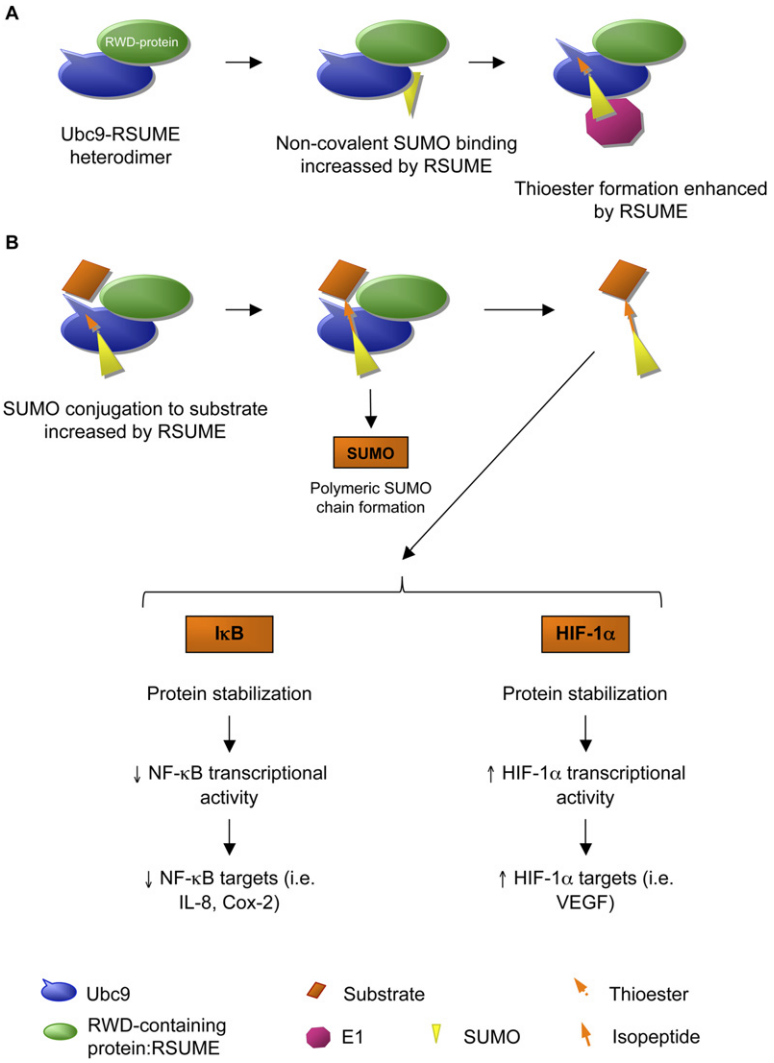

Figure 7. Schematic Model for the Actions of RSUME on the Sumoylation Process

(A) RSUME action on Ubc9-SUMO thioester formation.

(B) RSUME action on the transference of SUMO to the substrate and physiological consequences.

conserved platforms with important and differentiated functions in the Ubl pathways (Capili and Lima, 2007; Duda et al., 2007). The structure and functional implications of the noncovalent binding of SUMO to Ubc9 have been described in detail very recently (Capili and Lima, 2007; Duda et al., 2007; Knipscheer et al., 2007). The analysis of several Ubc9 loss-of-function mutants has shown that a reduction of noncovalent SUMO binding may in some cases also perturb E1 interaction and can result in different degrees of alteration of E2-thioester formation (Capili and Lima, 2007; Knipscheer et al., 2007). Conversely, the presence of RSUME increases E1 and SUMO binding to Ubc9. Given the importance of the E2-E1 interaction in the progression of the transfer cascade (Huang et al., 2007), this effect of RSUME can underlie the observed increase in thioester formation. Future studies on the crystal structure of the RSUME-Ubc9 complex and their interacting proteins will provide high-resolution insights into these mechanisms.

Considering the structural similarity of the RWD domain to UEVs (ubiquitin E2 variant proteins) and to E2s (Nameki et al., 2004) and the fact that RSUME binds to Ubc9, these two proteins (RSUME and Ubc9) could form a heterodimer analogous to the one found in the ubiquitination pathway where the catalytically active Ubc13 binds the structurally similar but catalytically inactive UEV Mms2 (VanDemark et al., 2001; Eddins et al., 2006). In both cases, the catalytically inactive member of the heterodimer enhances the activity of the E2 conjugase and consequently enhances polymeric Ubl chain synthesis or, like the UEV-domain-containing protein tumor susceptibility gene 101 (TSG101), monoubiquitination. Beside UEVs, some ubiquitin-E2 noncovalent interactions have also been implicated in polyubiquitin chain assembly (VanDemark et al., 2001; Brzovic et al., 2006). In view of the aforementioned structural homologies, the RSUME-Ubc9 heterodimer enhances polysumoylation very likely via a platform similar to that recently modeled for a Ubc9-Ubc9

homodimer (Knipscheer et al., 2007), based on the Mms2-Ubc13 heterodimer that mediates ubiquitin chain formation (VanDemark et al., 2001; Eddins et al., 2006). The conservation described between the ubiquitin and SUMO pathways is further expanded by the structural and functional analogy of RSUME and UEVs. RSUME could constitute an additional regulatory point for the control of poly- or monosumoylation under specific physiological conditions, such as hypoxia.

RSUME regulates two central players in mammalian angiogenesis and tumorigenesis. It inhibits NF-κB activity through the stabilization of IκB, which leads to the inhibition of two of its targets, Cox-2 and IL-8, and activates HIF-1α and consequently VEGF, one of its targets for vascularization. NF-κB signaling plays a role in cancer development and progression, controlling tumor angiogenesis, invasiveness, and resistance to apoptosis (Karin, 2006). Activation of HIF-1α is also a strong angiogenic stimulus associated with a variety of tumorigenic processes and has been suggested as a target for anticancer therapies (Lee et al., 2004). RSUME is induced by hypoxia in tumors. The finding of its expression in the necrotic zone of gliomas underscores the importance of RSUME during hypoxia in tumorigenesis. Moreover, NF-κB and HIF-1α have been proposed to interact in the regulation of angiogenesis, since cells lacking HIF-1α show an increase in NF-κB reporter activity in colon cancer cells (Mizukami et al., 2005). Understanding the diverse pathways involved in angiogenesis and tumorigenesis in which sumoylation and its enhancer RSUME participate is essential to develop better combined therapeutic strategies in the future.

EXPERIMENTAL PROCEDURES

Plasmids, Cloning, and Mutagenesis

Cloning and mutagenesis were performed with standard PCR-based techniques. The nucleotide sequences of all constructs obtained by PCR were confirmed by DNA sequencing. pAMP1-hRSUME, containing the hRSUME cDNA (clone ID: DKFZp566K023), was obtained from the Resource Center for the German Human Genome Project. The following plasmids, were kindly provided by: $(NF\text{-}\kappa B)_3$-LUC by Dr. M. Bell; IκB and IκB(K21R,K22R) expression vectors, V5-hUbc9, V5-hUbc9(C93S), HA-SUMO-1, HA-SUMO-2, and HA-SUMO-3 by Dr. R. Hay; HA-PIAS1 expression vector by Dr. C. Abate-Shen; Flag-SENP1 by Dr. E. Yeh; HIF-1α expression vector by Dr. J. Pouyssegur; pBI-GLV4R HRE-reporter plasmid by Dr. E. Van Meir; VEGF-LUC reporter vector by Dr. E. Ng; IL8-LUC and IL8-LUC (NF-κBmut) by Dr. N. Mukaida.

All plasmids used are described in detail in the Supplemental Experimental Procedures.

Cell Culture

Unless otherwise stated, cell culture and reagents were obtained from Invitrogen, Sigma Chemical Co., and MERCK. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (pH 7.3) supplemented with 10% FCS, 2.2 g/liter $NaHCO_3$, 10 mM HEPES, 4 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Tumors were processed for WB as previously described (Paez-Pereda et al., 2003).

For hypoxia, cells were incubated in 2% serum at 37°C, 5% CO_2, and 1% O_2 balanced with N_2 using a sealed chamber. For $CoCl_2$ stimulation, cells were incubated in 2% serum with 150 μM $CoCl_2$ for 3 hr.

Transfections

Transfection assays with lipofectamine reagent (Invitrogen) were performed as described (Kovalovsky et al., 2002). RSV-β-gal, coding for the bacterial β-galactosidase gene under the control of the viral RSV promoter, or pRL-Tk (Promega), coding for the *Renilla* luciferase gene under the control of the viral thymidin kinase promoter, were used as second reporter control plasmids.

Differential Display

A tumorigenic pituitary GH3 cell clone stably expressing gp130 (Perez Castro et al., 2003) was used for differential display compared to GH3 cells transfected with the empty vector. Total RNA was isolated as described (Arzt et al., 1992), and mRNA differential display was performed with an RNAimage kit (Gene Hunter).

In Vitro SUMO Conjugation Assays

SUMO conjugation assays were performed following the manufacturer's instructions using the Sumoylation Kit from LAE Biotech International. Briefly, assays were carried out in a final volume of 20 μl in reaction buffer containing 20 mM HEPES, 5 mM $MgCl_2$, 2 mM ATP, 7.5 μg/ml E1, 50 μg/ml Ubc9, 50 μg/ml SUMO-1, 1.0 μg of the corresponding substrate protein, and 0.5 μg of recombinant RSUME where indicated. Reaction mixes were incubated at 30°C for 1 hr and stopped by addition of 20 μl of a 2× Laemmly sample buffer.

Two-Step Sumoylation Assay

First step: reaction mixes containing 20 mM HEPES, 5 mM $MgCl_2$, 2 mM ATP, 7.5 μg/ml E1, 50 μg/ml GST-E2 (Ubc9) bound to glutathione Sepharose beads, and 50 μg/ml SUMO-1, but without substrate, were incubated at 30°C for 10 min. The reaction was stopped by washing the beads twice with 20 mM HEPES, 5 mM EDTA, 50 mM NaCl, 1% Tween-20. Second step: the beads with the GST-Ubc9 thioester were incubated in 20 mM HEPES, 5 mM EDTA, 50 mM NaCl with recombinant IκB at 30°C for 5 min, and the reaction was stopped by addition of 1 volume of a 2× Laemmly sample buffer. 0.5 μg of RSUME was added either in the first or second step, as indicated.

Proteins were separated by SDS-PAGE and detected by WB with the appropriate antibodies as described (Paez-Pereda et al., 2003).

Formation of Poly-SUMO-2 and Poly-SUMO-3 Chains

Assays were performed at 37°C in buffer containing 20 mM HEPES, 10 mM $MgCl_2$, 50 mM NaCl, 7 mM ATP, 1 mM DTT, and an ATP-regenerating system. Reaction mixtures contained 10 μg/ml E1, 12.5 μg/ml Ubc9, 5 μg of SUMO-3, or 8 μg GST-SUMO-2. When indicated, 0.3 μg of recombinant RSUME was added. Proteins were separated by SDS-PAGE and the SUMO-2- or SUMO-3-conjugated chains were detected by WB with anti-SUMO-2/-3 antibodies. Bands of the top of the gel indicated as poly-SUMO chains may include higher-order SAE II conjugates that occur as a side effect of the reaction as described (Knipscheer et al., 2007).

Thioester Formation Assay

Assays were carried out in a final volume of 15 μl in a buffer containing 20 mM HEPES, 10 mM $MgCl_2$, 50 mM NaCl, 7 mM ATP, 7.5 μg/ml E1, 12.5 μg/ml E2 (Ubc9), and 50 μg/ml SUMO-1, without substrate and with 0.3 μg of recombinant RSUME when indicated. Reaction mixes were incubated at 37°C for 15 or 30 min and stopped by incubating at 37°C for 30 min with 20 μl of nonreducing 2× sample buffer with 4 M urea or 10 mM DTT. Proteins were separated by SDS-PAGE, and the thioester was detected by WB with anti-Ubc9 antibodies.

GST Pull-Down Assay

Three micrograms of GST or GST fusion proteins immobilized on Sepharose beads were incubated with either 1 μg of the indicated recombinant proteins or COS-7 cell lysates transfected with an RSUME expression vector for 1 hr at 4°C in GST-binding buffer (20 mM Tris-HCl [pH 7.4], 100 mM KCl, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1 mM

DTT, 0.5% NP-40, 1 mM PMSF). Samples were washed four times in lysis buffer, boiled at 95°C for 5 min in 2× Laemmly sample buffer, and separated by SDS-PAGE.

Two-Step Competition Assay

First step: reaction mixes containing 0.5 μg of RSUME and 3 μg GST-Ubc9 bound to glutathione Sepharose beads or 0.5 μg of RSUME and 0–2 μg of SUMO-1 in 10 μl of binding buffer (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 5% glycerol, 50 mM NaCl, 1 mM DTT, 0.5 mM PMSF) were incubated at 25°C for 1 hr to pre-form protein complexes. Second step: 0–2 μg of SUMO-1 or 3 μg GST-Ubc9 and 500 μl of binding buffer were added to the reaction mixes and incubated at 25°C for 30 min with agitation. Sepharose beads and bound proteins were then pelleted, washed, and analyzed as in the GST pull-down assays.

Coimmunoprecipitation

COS-7 cells were cotransfected in 6-well plates with 1 μg V5-RSUME and 1 μg HIF-1α expression vectors. Cells were subjected to hypoxia for 16 hr, washed twice with ice-cold PBS, lysed on ice with modified RIPA buffer, and immunoprecipitated with the indicated antibodies as previously described (Paez-Pereda et al., 2003). For coimmunoprecipitation with recombinant proteins, 0.5 μg of each indicated recombinant protein were precleared in RIPA buffer with 15 μl of protein A-agarose beads for 1 hr and immunoprecipitated as indicated for cell extracts. Beads were collected by centrifugation, washed four times with 500 μl modified RIPA buffer, and boiled at 95°C for 5 min in 2× Laemmly sample buffer before loading in SDS-PAGE gels. WB analyses were performed with the indicated antibodies.

Statistics

Statistics were determined using ANOVA in combination with Scheffé's test.

Further experimental information can be found in the Supplemental Data.

Supplemental Data

The Supplemental Data for this article, including Supplemental Figures and Experimental Procedures, can be found online at http://www.cell.com/cgi/content/full/131/2/309/DC1/.

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