Ubc9 acetylation modulates distinct SUMO target modification and hypoxia response

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While numerous small ubiquitin-like modifier (SUMO) conjugated substrates have been identified, very little is known about the cellular signalling mechanisms that differentially regulate substrate sumoylation. Here, we show that acetylation of SUMO E2 conjugase Ubc9 selectively downregulates the sumoylation of substrates with negatively charged amino acid-dependent sumoylation motif (NDSM) consisting of clustered acidic residues located downstream from the core ψ -K-X-E/D consensus motif, such as CBP and Elk-1, but not substrates with core ψ -K-X-E/D motif alone or SUMO-interacting motif. Ubc9 is acetylated at residue K65 and K65 acetylation attenuates Ubc9 binding to NDSM substrates, causing a reduction in NDSM substrate sumoylation. Furthermore, Ubc9 K65 acetylation can be downregulated by hypoxia via SIRT1, and is correlated with hypoxia-elicited modulation of sumoylation and target gene expression of CBP and Elk-1 and cell survival. Our data suggest that Ubc9 acetylation/deacetylation serves as a dynamic switch for NDSM substrate sumoylation and we report a previously undescribed SIRT1/Ubc9 regulatory axis in the modulation of protein sumoylation and the hypoxia response.

The EMBO Journal (2013) 32, 791–804. doi:10.1038/emboj.2013.5; Published online 8 February 2013

Subject Categories: proteins

Keywords: acetylation; hypoxia; SIRT1; sumoylation; Ubc9

Introduction

Protein conjugation by a SUMO peptide on the lysine residue has recently emerged as an important post-translational control in modulating protein function (Geiss-Friedlander and Melchior, 2007; Gareau and Lima, 2010). The SUMO conjugation to protein substrates is initially activated by E1-activating enzyme (SAE1/SAE2), then transferred from E1 to the E2 conjugase (Ubc9) (Geiss-Friedlander and Melchior, 2007; Gareau and Lima, 2010). In some cases, E3 ligases are required to enhance substrate sumoylation (Pichler *et al*, 2002; Zhao *et al*, 2005). Sumoylation generally occurs on lysine residue within a consensus motif ψ -K-X-E/D (where ψ is a large hydrophobic residue and X is any residue) of protein substrates, which mediates a direct interaction with Ubc9 (Rodriguez *et al*, 2001; Sampson *et al*, 2001). In addition, the flanking residues surrounding this canonical four amino acid SUMO motif have been shown to promote substrate sumoylation via additional interaction with Ubc9 such as the negatively charged amino acid-dependent SUMO motif (NDSM) that comprises clustered negatively charged residues located C-terminal to the core ψ -K-X-E/D motif (Yang *et al*, 2006), the phosphorylation-dependent SUMO motif (PDSM) that includes a phosphorylation site located downstream from the core ψ -K-X-E/D motif (Hietakangas *et al*, 2006), and the hydrophobic cluster sumoylation motif (HCSM) that contains several hydrophobic residues located N-terminal to the ψ -K-X-E/D motif (Matic *et al*, 2010). In addition to Ubc9 interaction mediating substrate sumoylation, the substrate SUMO-interacting motif (SIM) can, likewise, facilitate sumoylation (Lin *et al*, 2006; Meulmeester *et al*, 2008; Zhu *et al*, 2008). The SIM-dependent sumoylation was thought to be achieved by binding of SIM to the SUMO moiety of the Ubc9-SUMO thioester complex, permitting Ubc9 to conjugate substrate lysine residue(s) not necessary within the consensus SUMO motif. Similarly to the ability of phosphorylated PDSM to enhance substrate sumoylation, we recently demonstrated that SIM phosphorylation upregulated Daxx selectivity for SUMO-1 binding and conjugation (Chang *et al*, 2011). These findings illustrated that the two current models of phosphorylation-enhanced protein sumoylation occur via increased affinity of substrate towards Ubc9 or SUMO.

In addition to regulation of substrate sumoylation by phosphorylation, post-translational modifications on the SUMO machinery also control sumoylation of global substrates or selective targets. For instance, reactive oxygen species inhibit global protein sumoylation through cross-linking the catalytic cysteines of SAE2 and Ubc9 (Bossis and Melchior, 2006). Likewise, the avian CELF adenoviral protein Gam-1 blocks cellular sumoylation by enhancing ubiquitination of SUMO E1 and Ubc9 enzymes (Boggio *et al*, 2004). In contrast to altering global substrate sumoylation, SUMO modification of Ubc9 was reported to enhance Sp100 sumoylation via additional interaction

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Received: 3 August 2012; accepted: 9 January 2013; published online: 8 February 2013

between Sp100 SIM and the SUMO moiety of sumoylated Ubc9 (Knipscheer *et al*, 2008). Currently, it is not clear whether other post-translational modifications also modify Ubc9 and alter its affinity towards a selective panel of protein substrates, thereby modulating substrate sumoylation levels.

Here, we identify an acetylation site of Ubc9 at residue K65 and demonstrate that acetylation of Ubc9 K65 significantly reduced Ubc9 binding to NDSM substrates but not to substrates with SIM or with a typical SUMO motif, which is correlated with decreased sumoylation levels of protein substrates with NDSM such as Elk-1, CBP and calpain-2. We further show that SIRT1 can deacetylate Ubc9, and that activation of SIRT1 activity increased NDSM substrate sumoylation. Conversely, inhibition of SIRT1 activity or depletion of SIRT1 expression decreased NDSM substrate sumoylation. Moreover, hypoxia induced Ubc9 deacetylation via SIRT1 and increased CBP and Elk-1 sumoylation levels, correlating with a decrease in CBP and Elk-1 regulated promoter activity and gene expression as well as cell survival.

Results

HDAC inhibitor treatment downregulates NDSM protein substrate sumoylation levels

We examined the effect of HDAC inhibitors on protein sumoylation levels using different substrates, including proteins with the core ψ -K-X-E/D consensus motif, NDSM or SIM. Treatment with HDAC inhibitors TSA and NAM reduced sumoylation levels of NDSM substrate Elk-1, but not SIM-dependent substrate Daxx or the typical ψ -K-X-E/D containing substrate TCF4 (Figure 1B, D and E, lanes 2 versus 3). Given that NDSM exhibits negatively charged amino-acid residues flanking the ψ -K-X-E/D consensus motif, these results raised the possibility that these negatively charged amino acids might contribute to the effect of HDAC inhibitors on NDSM substrate sumoylation. As expected, we found that sumoylation levels of the Elk-1 E3A mutant, in which three acidic residues located C-terminal to the core ψ -K-X-E/D motif were replaced with Ala (Figure 1A), were insensitive to HDAC inhibitor treatment (Figure 1B, lanes 4 and 5). Notably, the acidic residue mutant E3A reduced the potential of Elk-1 for sumoylation (lanes 4 versus 2), consistent with a previous report (Yang *et al*, 2006). These results suggest an important role for negatively charged amino-acid residues, within the NDSM substrate, in responding to HDAC inhibitor treatment. Our data also showed that the sumoylation levels of two other NDSM substrates, CBP and calpain-2, were sensitive to HDAC inhibitor treatment (Figure 1C; Supplementary Figure S1, lanes 2 and 3), further supporting the specificity of NDSM substrate sumoylation modulated by HDAC inhibitors. Likewise, mutation of the negatively charged amino-acid residues of CBP and calpain-2 to Ala also attenuated the effect of HDAC inhibitor on sumoylation of both factors (Figure 1C; Supplementary Figure S1, lanes 4 and 5). These results suggest that treatment of HDAC inhibitors preferentially modulates NDSM substrate sumoylation.

Because the negatively charged amino-acid residues flanking the ψ -K-X-E/D motif enhance substrate for Ubc9 interaction (Yang *et al*, 2006), we next tested whether treatment of HDAC inhibitors modulates the interaction between Ubc9 and NDSM substrates. As expected, HDAC inhibitor treatment reduced the levels of NDSM substrates precipitated by Ubc9

(such as Elk-1 and CBP), albeit having no significant effect on Ubc9 interaction with Daxx or TCF4 (Figure 2A). These results suggest that acetylation regulates the interaction between Ubc9 and NDSM substrates.

We next investigated whether acetylation of NDSM substrate and/or Ubc9 affects the Ubc9-NDSM substrate interaction. If NDSM substrate acetylation is involved in the regulation of its own sumoylation, then the acidic residue mutation within the NDSM should affect substrate acetylation in cells. However, both Elk-1 WT and acidic residue mutant E3A gave comparable acetylation levels in cells treated with HDAC inhibitors (Supplementary Figure S2A, lanes 4 and 6). Similar observation was made with CBP_{900–1100} WT and E4A mutant (Supplementary Figure S2B). These results suggest the negatively charged residues in these factors are unrelated to NDSM substrate acetylation. By contrast, Ubc9 acetylation level was induced in HDAC inhibitor-treated cells (Figure 1B–E), suggesting that Ubc9 acetylation may attenuate its interaction with NDSM substrates.

Acetylation of Ubc9 K65 attenuates NDSM substrate interaction

To evaluate the effect of Ubc9 acetylation on NDSM substrate interaction, we first mapped the Ubc9 acetylation site(s). Mass spectrometry analysis revealed that Ubc9 is acetylated at K65 (Supplementary Figure S2C), consistent with a recent large scale mass spectrometry study of cellular factor acetylation (Choudhary *et al*, 2009). We further showed that mutation of Ubc9 K65 to R significantly reduced HDAC inhibitor-elicited Ubc9 acetylation levels (Figure 2B), suggesting that K65 was the major site of Ubc9 acetylation. We next tested whether acetylation of Ubc9 K65 directly affects Ubc9-NDSM substrate interaction by subjecting K65 acetylated (K65-Ac) Ubc9 recombinant proteins to *in vitro* binding studies, based on a strategy developed by Chin and coworkers (Neumann *et al*, 2009), to generate a site-specific acetylated Ubc9 recombinant protein. K65-Ac Ubc9 recombinant protein was purified from *E. coli* and acetylation levels investigated by western analysis (Figure 2C). GST pull-down assays showed reduced levels of K65-Ac Ubc9 protein precipitated by GST-Elk-1 or -CBP fragment recombinant protein compared to WT Ubc9 (Figure 2D and E). In contrast, K65-Ac Ubc9 pulled down by GST fusion proteins of Daxx or RanGAP1, another ψ -K-X-E/D motif containing factor, was comparable to Ubc9 WT (Figure 2F and G). These data suggest that acetylation of Ubc9 at K65 reduces its interaction with NDSM substrates.

Acetylation of Ubc9 K65 attenuates NDSM substrate sumoylation

To test whether Ubc9 K65 acetylation directly affected NDSM substrate sumoylation, *in vitro* sumoylation assays using K65-Ac Ubc9 recombinant protein were then performed. As expected, K65-Ac Ubc9 protein yielded reduced Elk-1, CBP and calpain-2 sumoylation levels, compared to Ubc9 WT protein (Figure 3A and B; Supplementary Figure S3A), while both K65-Ac and WT Ubc9 conferred sumoylation levels similar to Daxx, TCF4 and RanGAP1 (Figure 3C and D; Supplementary Figure S3B). Along with the results of GST pull-down assays, these data suggest that Ubc9 K65 acetylation decreases NDSM substrate interaction, thereby causing a reduction in NDSM substrate sumoylation. In line with this

Figure 1 HDAC inhibitor treatment reduces NDSM substrate sumoylation. (A) Sequence alignment of the NDSM substrates used in this study. The sumoylation consensus motif ψ -K-X-E/D is boxed and the flanking acidic residues mutated to Ala are indicated in bold. (B–E) Western blots show the sumoylation levels of indicated epitope-tagged substrates in 293T cells cotransfected with pEGFP-SUMO-1, HA- or Flag-tagged Ubc9 and treated with HDAC inhibitors (HDACi), a mixture of 5 μ M TSA and 10 mM NAM, for 8 h. WCL, whole cell lysates. Asterisk, arrow and arrowhead: SUMO-1-modified and -unmodified protein and acetylated Ubc9, respectively. Short exposure shows a similar level of unmodified proteins in each transfected sample. The intensity of SUMO-modified and -unmodified bands was quantified by densitometry, and the ratio of modified to unmodified protein is indicated after normalization to untreated WT protein sumoylation levels. Source data for this figure is available on the online supplementary information page.

notion, an Ubc9 acetylation-mimic mutant (Lys-65 to Gln substitution; K65Q) was created and analysed for NDSM substrate sumoylation in cells. Reduced sumoylation levels of NDSM substrates Elk-1, CBP and calpain-2 were noted in cells expressing Ubc9 K65Q, compared to Ubc9 WT-trans-

fected cells (Figure 3E and F; Supplementary Figure S3C, lanes 4 and 5). In contrast, Ubc9 K65Q was comparable to WT in terms of Daxx and TCF4 sumoylation (Figure 3G and H). These data further support Ubc9 acetylation at K65 in the downregulation of NDSM substrate sumoylation.

Figure 2 K65 acetylation attenuates the binding of Ubc9 towards NDSM substrates. (A) Immunoblotting shows indicated protein substrates precipitated by Flag-Ubc9 in 293T cells treated with or without HDACi (5 μ M TSA and 10 mM NAM) for 8 h. (B) Western analysis of Ubc9 acetylation level in 293T cells transfected with indicated constructs and treated with 5 μ M TSA and 10 mM NAM for the indicated periods of time. Arrow: acetylated Ubc9. (C) Coomassie blue staining and immunoblotting of recombinant WT and K65-acetylated Ubc9. (D–G) Western blots of WT or K65-acetylated Ubc9 protein pulled down by recombinant GST-Elk-1_{201–261} (D), GST-CBP_{900–1174} (E), GST-Daxx_{501–740} (F) or GST-RanGAP1_{481–587} (G). Coomassie blue staining of GST fusion proteins used for each binding reaction. Source data for this figure is available on the online supplementary information page.

SIRT1 downregulates Ubc9 K65 acetylation

We next explored which HDAC family proteins could regulate Ubc9 acetylation levels. Immunoprecipitation and western analysis of endogenous Ubc9 showed that Ubc9 acetylation levels were higher in NAM- versus TSA-treated cells (Figure 4A), implicating HDAC class III family members in the regulation of Ubc9 acetylation. *In vitro* deacetylation assays of Ubc9 K65Ac protein showed that recombinant SIRT1 but not SIRT2 reduced Ubc9 K65 acetylation levels (Figure 4B), suggesting a potential role for SIRT1 in the regulation of Ubc9 K65 acetylation in cells.

Ubc9 acetylation levels were then investigated in SIRT1 knockdown cells. Transient transfection of different shRNAs

against SIRT1 expression increased Ubc9 acetylation (Figure 4C), and increased Ubc9 acetylation levels inversely correlated with SIRT1 knockdown levels. Treatment with SIRT1 activator resveratrol or SIRT1720 reduced Ubc9 acetylation (Figure 4D, lane 2 versus 3 and 5). The effect of SIRT1 activator treatment was abrogated by concomitant treatment of cells with shSIRT1 (lanes 4 and 6), implicating SIRT1 in the downregulation of Ubc9 acetylation. Similarly, treatment with SIRT1 inhibitor sirtinol increased Ubc9 acetylation (Figure 4E, lane 3), which was blunted in shSIRT1-treated cells (lanes 4 and 5), further suggesting that upregulation of Ubc9 acetylation by sirtinol was SIRT1 dependent. We also found that acetylation levels in the Ubc9 K65R mutant were

Figure 3 Ubc9 K65 acetylation reduces its potential for NDSM substrate sumoylation. (A–D) Western blots of *in vitro* sumoylation reactions of recombinant GST-Elk-1₂₀₁₋₂₆₀ (A), GST-CBP₉₀₀₋₁₇₇₄ (B), GST-Daxx₅₀₁₋₇₄₀ (C) or GST-RanGAP₄₈₁₋₅₈₇ (D) with WT or K65-acetylated Ubc9. Asterisk or bracket and arrow: SUMO-1-modified and -unmodified proteins, respectively. The intensity of SUMO-modified and -unmodified bands was quantified by densitometry, and the ratio of modified to unmodified protein is indicated after normalization to the sample with His-tagged Ubc9 WT protein in sumoylation reaction for 120 min. (E–H) Western blotting of sumoylation of 293T cells expressing Flag-Elk-1 (E), Gal-HA-CBP₉₀₀₋₁₁₀₀ (F), HA-Daxx (G) or Gal-TCF4 (H) with EGFP-SUMO-1 and WT or K65Q Ubc9. Asterisk and arrow: SUMO-1-modified and -unmodified proteins, respectively. The intensity of SUMO-modified and -unmodified bands was quantified by densitometry, and the ratio of modified to unmodified protein is indicated after normalization to the sample expressing Flag-Ubc9 WT. Source data for this figure is available on the online supplementary information page.

insensitive to resveratrol treatment (Figure 4F, lanes 5 and 6). Our data indicate that SIRT1 downregulates Ubc9 K65 acetylation in cells.

SIRT1 modulates NDSM substrate sumoylation

Our data showing SIRT1 downregulation of Ubc9 K65 acetylation suggested that SIRT1 knockdown or SIRT1 activity

Figure 4 SIRT1 modulates Ubc9 K65 acetylation and Elk-1 sumoylation. (A) Immunoblotting of endogenous Ubc9 acetylation in 293T cells treated with or without 5 μ M TSA and/or 10 mM NAM for 5 h. The ratio of Ubc9 acetylation in cells is indicated after normalization to Ubc9 acetylation in untreated cells. (B) Western analysis of recombinant K65-acetylated Ubc9 incubated with recombinant SIRT proteins. (C) Western blots show endogenous Ubc9 acetylation in 293T cells treated with distinct SIRT1 shRNAs. (D, E) Western blotting of 293T cells expressing EGFP-Ubc9 treated with resveratrol (5 μ M, 4 h), SIRT1720 (20 μ M, 4 h) (D) or sirtinol (30 μ M, 4 h) (E) with or without shSIRT1. The ratio of Ubc9 acetylation in cells is indicated after normalization to Ubc9 acetylation in untreated cells. (F) Immunoblotting of 293T cells expressing indicated EGFP-Ubc9 and treated with resveratrol (5 μ M, 4 h). The ratio of Ubc9 acetylation in cells is indicated after normalization to the Ubc9 WT acetylation in untreated cells. (G) Immunoblotting of SENP1 isopeptidase cleavage of sumoylated Elk-1 protein prepared from 293T nuclear extracts. (H) Western blots of endogenous Elk-1 from the nuclear extract of 293T cells treated with sirtinol (30 μ M, 4 h), shSIRT1 or SIRT1720 (20 μ M, 4 h). Asterisk and arrow: SUMO-modified and -unmodified Elk-1, respectively. (I) Western analysis of Elk-1 sumoylation in shUbc9-treated 293T cells expressing shUbc9-resistant (SiRe) Ubc9 WT or K65R with EGFP-SUMO-1 and treated with SIRT1720 (20 μ M, 4 h). Asterisk and arrow: EGFP-SUMO-1-modified and unmodified Elk-1, respectively. Arrowhead and unfilled arrowhead: endogenous and shUbc9-resistant Ubc9, respectively. Source data for this figure is available on the online supplementary information page.

inhibition or activation could modulate NDSM substrate sumoylation. 293T cells were treated with shSIRT1 or SIRT1 activator/inhibitor for Elk-1 sumoylation analysis. Due to the anti-Elk-1 antibody inefficient to precipitate endogenous Elk-1 proteins, nuclear extracts of 293T cells were used for such analysis. We observed two Elk-1 bands slowly migrating around molecular weight marker 95 and 130 kDa, which were sensitive to SUMO-deconjugase SENP1 cleavage

in vitro (Figure 4G). These results suggest that these two slowly migrating bands are endogenous sumoylated Elk-1 proteins. This notion was further supported by the finding that two slowly migrating bands of endogenous Elk-1, similar to the pattern obtained from 293T nuclear extracts, were detected from HeLa cell line stably expressing His-tagged SUMO-1 by nickel-NTA agarose beads to pull down sumoylated factors (Supplementary Figure S4A). Notably, SIRT1

knockdown or sirtinol treatment resulted in a reduction in endogenous Elk-1 sumoylated bands (Figure 4H, left panel, indicated by asterisk). In contrast, 293T cells treated with SRT1720 showed increased Elk-1 sumoylation (Figure 4H, right panel). Furthermore, shSIRT1 treatment also attenuated Elk-1 sumoylation induced by SRT1720 treatment (right panel, lane 3), consistent with our data showing shSIRT1 treatment blunted SRT1720-reduced Ubc9 acetylation. Such upregulation and downregulation of Elk-1 sumoylation was specific, as treatment of SIRT1 activator, inhibitor or shSIRT1, did not significantly alter global sumoylation levels (Supplementary Figure S4B). In addition, sumoylation levels of Elk-1 acidic residue mutant E3A were not significantly altered by SRT1720 or sirtinol treatment, compared to WT (Supplementary Figure S4C and D, lanes 2–5). These results suggest that SIRT1 modulates NDSM substrate Elk-1 sumoylation, likely via Ubc9 K65 acetylation.

To further substantiate that SIRT1-regulated Elk-1 sumoylation occurs in an Ubc9 K65 acetylation-dependent manner, endogenous Ubc9 in 293T cells was depleted by shUbc9 prior to transfection with shUbc9-resistant Ubc9 WT or K65R vector. Notably, treatment of SRT1720 failed to enhance Elk-1 sumoylation in Ubc9 K65R-transfected cells, compared to Ubc9 WT-transfected cells (Figure 4I, *asterisk*). These results suggest that SIRT1-regulated Elk-1 sumoylation is Ubc9 K65 acetylation dependent.

Hypoxia regulates Ubc9 acetylation and the sumoylation of Elk-1 and CBP via SIRT1

Because SIRT1 activity and/or expression is upregulated by various stimuli including hypoxia (Yang *et al*, 2007; Chen *et al*, 2011; Gerhart-Hines *et al*, 2011), we investigated whether the effects of hypoxia on sumoylation of NDSM substrates occurs via the SIRT1/Ubc9 regulatory axis. Western analysis revealed that endogenous Ubc9 acetylation levels in HCT116 cells declined at 2 h exposed to hypoxia, reached to the trough at 4 h, and then returned close to normal around 8 h of hypoxia (Figure 5A, lanes 2–6). Conversely, endogenous Elk-1 sumoylation levels increased at 2 h of hypoxia, peaked at 6 h and then declined close to normal at 8 h of hypoxia (Figure 5B, lanes 2–6, *asterisk*). Similar observations were made with endogenous CBP sumoylation (Figure 5C). In contrast, sumoylation of endogenous Daxx and RanGAP1 was not significantly altered in cells exposed to hypoxia (Supplementary Figure S5A and B). These data implicate hypoxia in the modulation of Ubc9 acetylation and the sumoylation of NDSM substrates Elk-1 and CBP. The results that the peak of Ubc9 deacetylation appeared earlier than that of Elk-1 and CBP sumoylation further suggest a sequentially regulatory cascade of SIRT1/Ubc9 in controlling NDSM substrate sumoylation. The peak interval between these sequential modifications was within 2 h, which may have resulted from sumoylation processes such as the thioester formation of deacetylated Ubc9 with SUMO-1 and the binding of deacetylated Ubc9 to NDSM substrates.

We further tested whether hypoxia-regulated Ubc9 acetylation and NDSM substrate sumoylation occurs via SIRT1. HCT116 cells were treated with either shSIRT1 or sirtinol, followed by 4 h exposure to hypoxia. Western blot data showed that hypoxia-induced Ubc9 deacetylation was abrogated by shSIRT1 or sirtinol treatment (Figure 5D, lanes 3–6), suggesting that hypoxia-induced Ubc9 deacetylation was

SIRT1 dependent. In line with these observations, hypoxia-induced sumoylation of endogenous Elk-1 and CBP was attenuated by sirtinol treatment (Figure 5E and F). Similar observations were made with Ubc9-depleted 293T cells with Ubc9 WT rescue and EGFP-SUMO-1 expression (Figure 5G, the 2nd and 3rd panels, lanes 2–4). These results suggested that hypoxia-induced sumoylation of NDSM substrate Elk-1 and CBP occurred via SIRT1. Moreover, 293T cells with Ubc9 K65R rescue failed to enhance Elk-1 and CBP sumoylation following hypoxia stimulation (Figure 5G, lanes 5 and 6). These results suggest that hypoxia-enhanced Elk-1 and CBP sumoylation occurs *via* SIRT1-mediated Ubc9 K65 deacetylation.

SIRT1/Ubc9 regulatory axis modulates hypoxia-activated promoter activity and gene expression

CBP and Elk-1 were reported to activate hypoxia responsive gene expression such as VEGF and CITED2 (Aprelikova *et al*, 2006; Loboda *et al*, 2010), respectively. Furthermore, sumoylation downregulates the transcriptional potential of CBP and Elk-1 (Yang and Sharrocks, 2004; Kuo *et al*, 2005). If Ubc9 K65 acetylation/deacetylation during hypoxia is responsible for fine-tuning CBP and Elk-1 sumoylation, then it is conceivable that the Ubc9 acetylation-mimic K65Q and the acetylation-deficient mutant K65R could upregulate and downregulate the transcriptional activity of CBP and Elk-1, respectively, in the promoter activity and expression of VEGF and CITED2 in response to hypoxia. To test this possibility, HCT116 cells were depleted of endogenous Ubc9, rescued with Ubc9 WT, K65Q or K65R, exposed to hypoxic conditions and subsequently analysed for VEGF and CITED2 gene reporter activity. The data showed that hypoxia-induced reporter activity of VEGF and CITED2 was elevated at 8 h and further enhanced at 16 h (Figure 6A). Reporter activity was not significantly induced at the 4-h time point, which may have been due to the lag time required for effective promoter activation and/or limited reporter protein production. Notably, Ubc9 WT and K65R mutant conferred similar reporter activity to that observed at 8 h, but gave distinct reporter activity at 16 h. These results correlated with those observed in the deacetylation and re-acetylation of Ubc9 and the resulting increase and decrease in Elk-1 and CBP sumoylation at early and later time points of hypoxia treatment, respectively (Figure 5A–C). In contrast to the hypoxia-induced deacetylation in Ubc9 WT, the K65Q mutant yielded greater VEGF and CITED2 reporter activity compared to WT (Figure 6A). These data implicated Ubc9 K65Q and K65R in the upregulation and downregulation of VEGF and CITED2 reporter activity via altering CBP and Elk-1 sumoylation, respectively. In line with this notion, we observed that sumoylation-deficient mutants CBP 3KR and Elk-1 2KR were insensitive to Ubc9 K65Q or K65R in terms of VEGF and CITED2 reporter activity regulated by hypoxia (Figure 6B and C).

We further investigated the role of SIRT1 in modulating VEGF and CITED2 reporter activity via Ubc9 K65 acetylation regulation. HCT116 cells were depleted of Ubc9 and SIRT1 and rescued with Ubc9 WT or K65Q, with or without SIRT1 re-expression. The 16-h hypoxia time point was used, given our data showing the greatest effects of Ubc9 WT and K65Q on activation of VEGF and CITED2 reporter activity at this time point. shSIRT1 treatment enhanced hypoxia induction of VEGF and CITED2 reporter activity in Ubc9 WT cells (Figure 6D and E, lanes 7 and 8), which was attenuated by

Figure 5 Hypoxia modulates Ubc9 acetylation and NDSM substrate sumoylation via SIRT1. (A, D) Western analysis of the acetylation levels of Ubc9 immunoprecipitated from HCT116 cells exposed to hypoxia for indicated time periods (A) with or without shSIRT1 or sirtinol (10 and 30 μ M) treatment (D). The ratio of Ubc9 acetylation after normalization to Ubc9 acetylation in untreated cells is indicated. (B, C, E, F) Western blots of sumoylation levels of endogenous Elk-1 from nuclear extracts (B, E) or CBP from immunoprecipitation (C, F) of HCT116 cells exposed to hypoxia for indicated time periods, with or without 30 μ M sirtinol treatment. HA-SEN1 transfection reduced Elk-1 sumoylation bands (B). Asterisk and arrow: SUMO-modified and -unmodified Elk-1 or CBP, respectively. (G) Western blots of sumoylation of endogenous Elk-1 and CBP in shUbc9-treated 293T cells expressing shUbc9-resistant WT or K65R Ubc9 with EGFP-SUMO-1 under hypoxic conditions (4 h), with or without 30 μ M sirtinol treatment, as indicated. Asterisk, arrowhead and unfilled arrowhead: EGFP-SUMO-1-modified CBP or Elk-1 proteins, endogenous and shUbc9-resistant Ubc9, respectively. Source data for this figure is available on the online supplementary information page.

SIRT1 re-expression (lane 9). More importantly, neither SIRT1 depletion nor re-expression significantly affected VEGF and CITED2 reporter activity in cells expressing Ubc9 K65Q (lanes 10–12). These results demonstrate that hypoxia-induced SIRT1 regulation of VEGF and CITED2 promoter activity occurs via modulation of Ubc9 K65 acetylation.

Reporter gene data led us to further examine whether VEGF and CITED2 gene expression was regulated by the

SIRT1/Ubc9 pathway. Similarly to the results of reporter gene assays, re-expression of Ubc9 K65Q and K65R in shUbc9-treated HCT116 cells resulted in upregulation and downregulation, respectively, of hypoxia-induced VEGF and CITED2 mRNA, compared to cells with re-expression of Ubc9 WT (Figure 7A). Similar observations were also made with another CBP and Elk-1 target genes, GLUT-1 and IGFBP3 (Figure 7A), respectively. The 12-h hypoxia treatment time

Figure 6 The SIRT1/Ubc9 regulatory axis modulates hypoxia-activated promoter activity. (A) Time-responsive curves show the reporter activities of shUbc9-treated HCT116 cells transfected with indicated reporter constructs along with constructs expressing shUbc9-resistant Ubc9 WT, K65R or K65Q for 24 h then stimulated with hypoxia for indicated periods of time. Error bars: standard deviation; $n = 3$, in triplicate; asterisk indicates significant differences of K65Q and K65R versus WT Ubc9 ($*P < 0.05$). Statistical significance was ascertained with a Student's *t*-test. (B, C) Reporter gene analysis of HCT116 cells transfected with expression vectors of Flag-Ubc9 WT or K65 mutants and Flag-CBP WT or sumoylation-deficient mutant 3KR and VEGF-Luc reporter (B) or Flag-Elk-1 WT or sumoylation-deficient mutant 2KR and CITED2-Luc reporter (C) and cultured in normoxic or hypoxic condition (16 h). Error bars: standard deviation; $n = 3$, in triplicate. Statistical significance was ascertained with a Student's *t*-test. (D, E) Reporter gene analysis of HCT116 cells with indicated reporter constructs and Ubc9 and/or SIRT1 knockdown with rescue of Ubc9 WT, K65Q and SIRT1, exposed to 16 h hypoxia treatment. Error bars: standard deviation; $n = 3$, in triplicate. Statistical significance was ascertained with a Student's *t*-test. Arrowhead and unfilled arrowhead: endogenous and shUbc9-resistant Ubc9, respectively. Source data for this figure is available on the online supplementary information page.

point was used to assess the effects of SIRT1 on these four gene expression, given the effects of hypoxia-induced gene expression between Ubc9 WT and K65Q observed at this time point was optimal (Figure 7A). HCT116 cells were depleted with Ubc9 and SIRT1 and rescued by Ubc9 WT or K65Q, with or without SIRT1 re-expression. Similarly to our reporter

activity data, shSIRT1 treatment resulted in increased mRNA levels of VEGF, CITED2, GLUT-1 and IGFBP3 in HCT116 cells expressing Ubc9 WT, an effect that was abrogated by the re-introduction of SIRT1 (Figure 7B, each panel, lanes 7–9). Moreover, depletion or re-expression of SIRT1 resulted in a slight change of the mRNA levels of these four

ascertained with a Student's *t*-test. Source data for this figure is available on the online supplementary information page.

genes in cells expressing the Ubc9 K65Q mutant (lanes 10–12). These data implicated that the SIRT1/Ubc9 regulatory axis modulates the expression of these hypoxia responsive genes.

SIRT1/Ubc9 regulatory axis modulates cell survival of hypoxic HCT116 cells

VEGF acts as a survival factor not only for endothelial cells but also for cancer cells, protecting them from apoptosis, particularly under hypoxic stress (Bachelder *et al*, 2001; Barr *et al*, 2008; Calvani *et al*, 2008). Previous reports showed that hypoxia-dependent autocrine production of VEGF mediates survival of several cancer cells including HCT116 (Barr *et al*, 2008; Calvani *et al*, 2008). Because the SIRT1/Ubc9 regulatory axis modulates VEGF expression in hypoxic HCT116 cells, it is conceivable that this regulatory axis could modulate HCT116 cell survival under hypoxic conditions. To this end, Ubc9-depleted HCT116 cells were transfected with EGFP-Ubc9 WT, K65R or K65Q, then serum starved for 24 h, followed by hypoxia or normoxia treatment for an additional 16 h. Under normoxia, serum starvation induced cell apoptosis (~28%) and hypoxia stimulation reduced cell apoptosis to ~16% in cells expressing Ubc9 WT (Supplementary Figure S6A), representing a 43% relative cell survival rate modulated by hypoxia (Figure 7C, lane 1). Cells expressing Ubc9 K65R and K65Q showed a 19 and 66% relative cell survival rate, respectively, under hypoxic conditions (Figure 7C, lanes 3 and 5), correlating with the potential of these two mutants to downregulate and upregulate hypoxia-induced VEGF gene expression, respectively. More importantly, sirtinol treatment enhanced the relative cell survival rate of cells harbouring the Ubc9 WT protein, with no effects observed in cells expressing Ubc9 K65R and K65Q proteins (Figure 7C). These data further support the role of SIRT1/Ubc9 axis in modulating the hypoxia response.

Discussion

Acetylation has been linked to the regulation of protein sumoylation. Previous studies reported that the same lysine residue within a protein substrate can be competitively targeted by acetylation and sumoylation, such as MEF2D, p53, PEA3 and HIC-1 (Zhao *et al*, 2005; Stankovic-Valentin *et al*, 2007; Wu and Chiang, 2009; Guo *et al*, 2011), causing distinct functional outcomes. Such acetylation-regulated sumoylation mainly occurs at the substrate level. In this study, we show that acetylation-regulated sumoylation occurs at the level of sumoylation machinery Ubc9, rendering a selective modulation on sumoylation of protein substrates with an NDSM motif. Compared to substrate acetylation to alter individual protein sumoylation, Ubc9 acetylation may provide a wider ranging control on substrate sumoylation.

We demonstrated that K65 acetylation downregulated Ubc9 binding to NDSM substrates but not to substrates associated with an SIM- or a consensus motif ψ -K-X-E/D-dependent sumoylation. This correlates with K65-Ac Ubc9 protein in conferring these substrate sumoylation. No significant change in sumoylation of the latter substrates by K65-acetylated Ubc9 was expected, as SIM-mediated substrate sumoylation depends on its interaction with SUMO rather than Ubc9, and the consensus motif ψ -K-X-E/D mainly

contacts Ubc9 residues I125 and Y134 for hydrophobic interaction and S89, T91 and K74 for hydrogen bonding interactions (Bernier-Villamor *et al*, 2002), which are unrelated to K65. In contrast, the effect of K65 acetylation on NDSM substrate sumoylation was initially not anticipated, given a previous report indicating that a basic patch on Ubc9, such as K59 and R61, required for the interaction with Elk-1 NDSM acidic residues, is important for Elk-1 sumoylation (Yang *et al*, 2006). However, Lima's group pointed out that glutamate substitution for both K59 and R61 simultaneously in Sharrocks' group study might significantly change Ubc9 intra-molecular interactions, thereby altering Ubc9 configuration in Elk-1 interaction and sumoylation (Mohideen *et al*, 2009). Although Ubc9 residues involvement in Elk-1 NDSM acidic patch recognition is not clear and requires further investigation, our study using recombinant acetylated Ubc9 did avoid possible artificial effects derived from amino-acid substitution and clearly demonstrated the effect of K65 acetylation in the downregulation of Ubc9 in NDSM substrate interaction.

We encountered two possible scenarios that K65 acetylation attenuates Ubc9 binding to NDSM substrates. In one scenario, K65 directly contacts NDSM acidic residues and acetylation dampens the K65 charge interactions with NDSM acidic residues. In the second scenario, acetylation alters Ubc9 configuration, resulting in a reduction in Ubc9 residues accessible for NDSM acidic residue interaction. Our NMR chemical shift perturbation study revealed that NDSM peptides failed to induce significant perturbation at the Ubc9 K65 residue (unpublished data), which excluded the first scenario. Conversely, we observed that acetylation at K65 induced chemical shift perturbations at Ubc9 residues F64, D67, S70 and K74 and altered NDSM peptide binding, but not RanGAP1 peptide interaction (unpublished data), supporting the latter scenario. The exact nature of how this conformational change in affects the NDSM substrate interaction is subject to further structural analysis.

Interestingly, Lima's group reported that a basic patch surface on Ubc9 formed by K65, K74 and K76 residues was important for PDSM discrimination of MEF2A and HSF1 (Mohideen *et al*, 2009). Since K65 is within this basic surface, it is conceivable that K65 acetylation would remodel this basic surface by affecting PDSM substrate interaction and sumoylation. Indeed, we observed that K65-Ac Ubc9 significantly reduced its interaction with phospho-PDSM peptides derived from MEF2A and HSF1 (unpublished data). In addition, acetylation-mimic mutant Ubc9 K65Q also attenuated the sumoylation of PDSM substrate MEF2D in cells (Supplementary Figure S7). Given that the Ser phosphorylation in PDSM provides a negative charge C-terminal to the ψ -K-X-E/D consensus site (Hietakangas *et al*, 2006; Mohideen *et al*, 2009), similar to the acidic residues within NDSM, it is not surprising that Ubc9 K65 acetylation also affected PDSM discrimination, in addition to NDSM.

Previous studies showed that HIF-1 α sumoylation can be induced under hypoxia (Carbia-Nagashima *et al*, 2007; Cheng *et al*, 2007). Since we observed HIF-1 α target genes such as VEGF and GLUT-1 upregulated by SIRT1/Ubc9 regulatory axis, this raised a possibility that HIF-1 α sumoylation is also modulated by SIRT1/Ubc9 regulatory axis under hypoxic condition. We found that the sumoylation levels of HIF-1 α were similar in cells exposed to hypoxia for 4 and 8 h

(unpublished data), distinct from the pattern of CBP and Elk-1 sumoylation regulated by hypoxia. Furthermore, SIRT1 inhibitor sirtinol treatment did not alter hypoxia-induced HIF-1 α sumoylation in HCT116 cells or HeLa cell line stably expressing SUMO-1 (unpublished data). Lastly, *in vitro* sumoylation assays revealed that both WT and K65-acetylated Ubc9 proteins conferred similar levels of sumoylated HIF-1 α recombinant proteins (unpublished data). These observations exclude the possibility of HIF-1 α sumoylation regulated by SIRT1/Ubc9 axis under hypoxia. It should be noted that RSUME, a small RWD-containing protein, can be induced by hypoxia and enhance global substrate sumoylation including HIF-1 α during hypoxia in part via Ubc9 activity regulation (Carbia-Nagashima *et al*, 2007). Because SIRT1/Ubc9 regulatory axis selectively controls NDSM substrate sumoylation, unlike RSUME in regulating global sumoylation, under hypoxia, our findings of Ubc9 deacetylation by SIRT1 uncover a novel molecular mechanism in hypoxia-induced protein sumoylation.

Our data that SIRT1 knockdown or inhibitor treatment affected hypoxia regulation of Elk-1 and CBP sumoylation, reporter activity, expression levels of VEGF, CITED2, GLUT-1 and IGFBP3, and cell survival in a Ubc9 K65 acetylation-dependent manner (Figures 5–7) clearly demonstrated a role of SIRT1 in the control of Ubc9 K65 acetylation under hypoxic conditions. SIRT1 activity and/or expression regulated by hypoxia might serve as a switch for Ubc9 acetylation. Previous studies showed that SIRT1 expression was high during the acute phase of hypoxic exposure and began to decline with continued hypoxic exposure beyond 8–12 h (Chen *et al*, 2011; Lim *et al*, 2010). We observed that SIRT1 protein levels in HCT116 cells remained insignificant changed up to 12 h of hypoxic exposure and declined ~50% at 16 h time point (Supplementary Figure S6E). The Ubc9 deacetylation peak observed at 4 h of hypoxic exposure (Figure 5A) implicates hypoxia activated SIRT1 activity in Ubc9 deacetylation during early phase of hypoxia. Thus, changes in Ubc9 acetylation provide a control for CBP and Elk-1 sumoylation levels, which limits hypoxia response gene activation during the acute phase of hypoxia (Figures 5B, C, 6A, and 7A). During prolonged hypoxia, reduced SIRT1 activity and/or expression results in reduced CBP and Elk-1 sumoylation, thereby enhancing hypoxia responsive gene activation. In this scenario, the dynamic control of Ubc9 deacetylation/acetylation, regulated by SIRT1, serves as an important switch to fine-tune hypoxia responsive gene expression levels in cells when adapting to acute or prolonged hypoxia. In line with this notion, alteration of SIRT1 activity and/or expression level would change the time point of cellular adaptive response to hypoxia. Indeed, we found that increasing SIRT1 expression significantly slowed down HCT116 cellular adaptive response to hypoxia in VEGF expression and cell survival (Supplementary Figure S6B–D).

In studying Elk-1 target genes, we have additionally tested the expression levels of two well-characterized Elk-1 targets c-Fos and Egr-1 under hypoxia. Consistent with previous reports (Muller *et al*, 1997; Yan *et al*, 1999), c-Fos and Egr-1 expression was rapidly elevated in HCT116 cells exposed to hypoxic condition for 30 min and declined to basal level at 2 h (unpublished data). Such an immediate response is different from the regulation profiles of CITED2 and IGFBP3. Although

the expression levels of both c-Fos and Egr-1 were also upregulated again in HCT116 cells for 8–12 h hypoxic exposure, SIRT1 knockdown or Ubc9 K65Q expression could not enhance the expression levels of c-Fos and Egr-1 (unpublished data). While both c-Fos and Egr-1 expression have been reported to be upregulated by Elk-1 phosphorylation in hypoxia (Muller *et al*, 1997; Yan *et al*, 1999), our results suggest that both CITED2 and IGFBP3 expression are modulated by SIRT1/Ubc9 regulatory axis in part via Elk-1 sumoylation during hypoxia. It is possible that differential post-translational modifications (phosphorylation and sumoylation) of Elk-1 induced by hypoxia may contribute to distinct profile of Elk-1 target gene expression. Alternatively, since Elk-1 interacts with CBP (Janknecht and Nordheim, 1996), it is equally plausible that the regulatory effect of hypoxia on CITED2 and IGFBP3 expression might be due to CBP sumoylation during hypoxia.

Previous studies reported that SIRT1 plays an important role for hypoxia-induced radioresistance in hepatoma cells (Xie *et al*, 2012a,b). SIRT1 overexpression caused HepG2 cells to become more resistance to irradiation damage under hypoxia in part via downregulating the levels of c-Myc protein and its acetylation (Xie *et al*, 2012b). However, our findings that SIRT1 expression and/or activity suppress VEGF expression *via* Ubc9 deacetylation correlated with a reduction in hypoxic HCT116 cell survival in serum starvation condition. These findings implicate that SIRT1 in regulation of distinct substrates/pathways may contribute to differential cellular milieu in response to various stress stimuli, leading to diverse cellular outcomes.

In summary, our findings provide a novel paradigm for selective regulation of protein sumoylation via Ubc9 acetylation/deacetylation and also report a previously undescribed SIRT1/Ubc9 regulatory pathway in modulation of hypoxia response.

Materials and methods

Plasmid constructs, antibodies and inhibitors

The cDNAs coding for SUMO-1, Daxx^{501–740}, Ubc9, CBP^{900–1100}, CBP^{900–1774}, calpain-2, TCF4, SIRT1 and SIRT2 were amplified by PCR and subcloned into indicated tag vectors including pEGFP-C1 (EGFP), pCMV-Tag 2A (Flag), pcDNA3-HA (HA), pcDNA3.1-myc-His (Myc), pCMX (Gal), pGEX4T-1 (GST) and pCDF PylT-1 (His). The bacterial and mammalian constructs expressing each mutant of Ubc9, CBP^{900–1100} and calpain-2 were created by site-directed mutagenesis (QuikChange, Stratagene). The pAcKRS-3 and pCDF PylT-1 vectors kindly provided by Dr Jason W Chin were used to create recombinant site-specific acetylated protein in bacteria, as described previously (Neumann *et al*, 2009). The constructs expressing Flag-Elk-1 WT and E3A, GST-Elk-1^{201–260} and GST-RanGAP1^{481–587} were kindly provided by Drs Shen-Hsi Yang and Andrew D Sharrocks (Yang *et al*, 2006). CITED2 promoter (–2186 to +65) luciferase reporter was kindly provided by Dr Olga Aprelikova (Aprelikova *et al*, 2006). The VEGF promoter (–2273 to +379) luciferase reporter was kindly provided by Dr Lee-Young Chau (Academia Sinica). The lentivirus constructs expressing shUbc9 and shSIRT1 were generated by inserting oligonucleotides corresponding to human Ubc9 sequence ³⁹⁷GCCTACACGATTTAC TGCCAA⁴¹⁷ and human SIRT1 #1 'GCAAGCCTTTCTGAATCTAT' (3'UTR) and #2 ⁶⁴⁴CCTCGAACAATCTTAAAGAT⁶⁶⁴, into pLKO.1 vector, respectively. pLKO.1-Luc was obtained from the RNAi consortium at Academia Sinica. The shUbc9-resistant constructs of Flag-Ubc9 and EGFP-Ubc9 were generated by two rounds of site-directed mutagenesis to introduce silent mutations (5'-GCAGAGGCC TACACGATTTACTGCCAAAACAGA-3' to 5'-GCAGAGGCCTATACAAT CTATTGCCAAAACAGA-3'). The following primary antibodies were used: anti-HA (Convance); anti-TCF4, anti-Daxx (D7810), anti-Flag

(F3165) (Sigma-Aldrich); anti-GST (Upstate Inc.); anti-acetyl-Lysine (Cell Signaling); anti-Gal, anti-RanGAP-1, anti-SIRT1, anti-GFP (Santa Cruz Biotechnology Inc.); anti-SUMO-1 and anti-Ubc9 for endogenous Ubc9 immunoprecipitation were generated by LTK BioLaboratories; anti-acetyl-Histone H4 (Millipore); anti-Ubc9, anti-CBP, anti-HIF-1 α (Epitomics); anti-Elk-1 (SignalChem). Acetyl-lysine, trichostatin A (TSA), nicotinamide (NAM), resveratrol and sirtinol were from Sigma. SRT1720 was from Selleckchem.

Preparation of K65-acetylated Ubc9, in vitro sumoylation, and deacetylation assays

K65-acetylated Ubc9 was generated by a strategy described previously (Neumann *et al*, 2009). Briefly, BL21 DE3 (C41) transformed with pAcKRS-3 and pCDF PylT-1-Ubc9 with amber codon at K65 were grown in LB supplemented with kanamycin (50 μ g/ml) and spectinomycin (50 μ g/ml) at 37°C till OD₆₀₀ ~ 0.7, then further supplemented with 20 mM NAM and 10 mM acetyl-lysine (30 min) followed by addition of IPTG (0.5 mM; 3 h) for protein induction. The induced K65-acetylated Ubc9 protein was purified by Ni²⁺-NTA column then dialyzed (4°C) in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5% glycerol, protease inhibitors and 20 mM NAM. *In vitro* sumoylation was performed as described previously (Chang *et al*, 2011) with indicated substrates for indicated time; one-quarter of each sample was subjected to western analysis. For *in vitro* deacetylation assay, 50 μ M K65-Ac Ubc9 recombinant protein was incubated with purified GST-SIRT1 or GST-SIRT2 proteins (8 unit determined by HDAC activity kit; Millipore) in 30 μ l reaction mixture (2 mM NAD⁺, 50 mM Tris, pH 8.0, 137 mM NaCl, 1 mM MgCl₂, 2.7 mM KCl, 2.5% glycerol) at 37°C for 2 h followed by western analysis.

Cell culture, transfection, and reporter gene assays, and cell survival

293T, HCT116 and HeLa stably expressing His-Tagged SUMO-1 cells were maintained in DME supplemented with 10% FBS as described (Huang and Shih, 2009). Lipofectamine (Invitrogen) was used for DNA transfection into 293T cells while polyjet (SignaGen Laboratories) was used for DNA transfection into HCT116 cells and HeLa His-SUMO-1 stable line. For Ubc9 knockdown and re-constitution experiments in 293T cells, 2 $\times$ 10⁶ 293T cells were infected with a lentivirus expressing shUbc9 for 16 h and then selected for 3 days with puromycin (2 μ g/ml). The Ubc9 depletion cells were expanded and selected by puromycin for additional 3 days and then 1 $\times$ 10⁶ cells were seeded on 60 mm dish for transfection with 50 ng Ubc9 shRNA-resistant expression construct and 1 μ g shUbc9 plasmid. For reporter gene assays, 4 $\times$ 10⁴ HCT116 cells transfected in 24-well plates with 500 ng DNA, including indicated constructs, VEGF or CITED2 luciferase reporter, with or without shSIRT1 or Myc-tagged SIRT1 vector and pTK-RL (Rellina luciferase) for normalization of transfection efficiency. Hypoxia treatment was carried out in an incubator chamber (1% oxygen, 94% nitrogen and 5% CO₂) for indicated periods of time. For cell survival measurement of hypoxic HCT116 cells, 1 $\times$ 10⁷ HCT116 cells infected with lentivirus expressing shUbc9 for 24 h, followed by transfection of shUbc9-resistant EGFP-tagged Ubc9 WT or K65 mutants for another 24 h. The resulting cells were serum starved for 24 h under normoxia then subjected to hypoxia or normoxia for an additional 16 h, with or without sirtinol treatment. Apoptosis was assessed by propidium iodide (BD Pharmingen) and Annexin-V-Cy5 (BioVision). GFP-positive cells were gated and analysed by flow cytometry (BD FACSCanto). Relative survival rate of indicated HCT116 cells was calculated by subtracting the apoptosis percentage of cells under hypoxia from cells in normoxia, then normalizing to cell apoptosis values under normoxic conditions.

Immunoprecipitation, western analyses and GST pull-down assays

Cells with or without transfection or infection were lysed in lysis buffer containing 20 mM sodium phosphate (pH 7.0), 0.15 M KCl, 5 mM EDTA, 0.1% Nonidet P-40, 20 mM N-ethylmaleimide (NEM), and protease inhibitor mixture (Complete; Roche Applied Science) without or with TSA and/or NAM or SRT1720 or sirtinol, as indicated. Immunoprecipitation and western analyses were performed as described (Chang *et al*, 2011; Lin *et al*, 2006) to analyse

the levels of acetylated Ubc9 and indicated sumoylated factors in lysates. For GST pull-down assays, purified GST or indicated GST fusion proteins (5 μ g) were incubated with recombinant WT or K65-Ac Ubc9 (5 μ g) in a binding buffer (10 mM HEPES pH 7.5, 50 mM NaCl, 0.1% NP-40 and 0.5 mM EDTA) for 30 min followed by quadruple washing with binding buffer prior to western analysis.

RNAi knockdown

293T cells were transduced with lentivirus containing indicated shRNA in pLKO.1 vector as described previously (Chang *et al*, 2011). In brief, the lentivirus pLKO.1 construct (5 μ g) expressing indicated shRNA was cotransfected with packaging pCMV R8.91 (5 μ g) and envelope VSV-G pMD.G (0.5 μ g) into 6 $\times$ 10⁶ 293T cells. Lentivirus was harvested at 48 h post transfection and 293T or HCT116 cells were infected using polybrene (10 μ g/ml).

Quantification of VEGF, CITED2, GLUT-1 and IGFBP3 expression

For the total cellular RNAs from HCT116 cells with Ubc9 knockdown and re-constitution experiments with or without SIRT1 knockdown and re-constitution, 2 $\times$ 10⁶ HCT116 cells were transfected with shUbc9 construct (3 μ g) for 16 h and the resulting cells were further transfected with shUbc9 construct (3 μ g) and Ubc9 shRNA-resistant construct (0.5 μ g) with or without shSIRT1 construct (3 μ g) and Myc-SIRT1 construct (0.5 μ g) for additional 6 h and then selected by puromycin (0.5 μ g/ml) for 2 days. The resulting cells were treated with or without hypoxia for indicated time periods and then total RNAs were extracted using the TRIzol (Invitrogen). RNA of each sample (2 μ g) was then reverse transcribed using ThermoScript reverse transcription-PCR system (Invitrogen) in 20 μ l of reaction mix. The reverse transcription reaction product (250 ng) was used for real-time quantitative PCR with primers of VEGFA, CITED2, GLUT-1, IGFBP3 and 18S rRNA and KAPA SYBR FAST ABI Prism 2 $\times$ qPCR master mix utilized StepOne Plus Real-Time PCR system (Applied Biosystems), according to manufacturer's protocol. The following primers were used in this study: VEGFA forward primer 5'-GTACTTGCAGATGTGACAAGC-3' and reverse primer 5'-GGATTAAGGACTGTTCTGTCG-3'; CITED2 forward primer 5'-ATCGACGAGGAAGTTCT-3' and reverse primer 5'-ACACGAAGTCCGTCAT-3'; IGFBP3 forward primer 5'-GGTGTCGATCCCAAGTTCC-3' and reverse primer 5'-CGGAGGAGAAGTTCTGGTA-3'; GLUT-1 forward primer 5'-CTTTCTGTGGGGGCATGAT-3' and reverse primer 5'-CCGCAGTACACACCGATGAT-3'; 18S rRNA forward primer 5'-ACAGGTCTGTGATGCC-3' and reverse primer 5'-ATCGGTAGTAGCGACG-3'.

For each sample, average threshold (Ct) value assays were repeated in triplicate, and the Δ Ct value was determined by subtracting the average 18S rRNA Ct value from the average VEGF, CITED2, GLUT-1 or IGFBP3 Ct value.

Supplementary data

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

Acknowledgements

We thank Drs Jason Chin, Shen-Hsi Yang, Andrew Sharrocks, Olga Aprelikova and Lee-Young Chau for pAcKRS-3, pCDF PylT-1, Flag-Elk-1 WT and E3A, GST-Elk-1₂₀₁₋₂₆₀, GST-RanGAP₄₈₁₋₅₈₇, CITED2-Luc and VEGF-Luc reporter constructs; the RNAi for providing lentivirus constructs for shRNAs of Ubc9, SIRT1 and Luc. The work was supported by grants NSC101-2321-B-001-029, NSC100-2321-B-001-004 and NSC100-3112-B-001-004 from National Science Council and an Academia Sinica Investigator Award to H-M Shih.

Author contributions: Y-LH, H-YK and H-MS designed the study; Y-LH, H-YK, MTN, C-CC, P-HL, C-CH, T-CH, J-CJ and P-HH performed the research; Y-LH, H-YK, M-DT, T-HH and H-MS analysed the data; Y-LH and H-MS wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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