The biphasic redox sensing of SENP3 accounts for the HIF-1 transcriptional activity shift by oxidative stress

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Aim: To investigate the mechanisms underlying the biphasic redox regulation of hypoxia-inducible factor-1 (HIF-1) transcriptional activity under different levels of oxidative stress caused by reactive oxidative species (ROS).

Methods: HeLa cells were exposed to different concentrations of H$_2$O$_2$, as a simple model for mild and severe oxidative stress. Luciferase reporter assay and/or quantitative real-time PCR were used to investigate the transcriptional activity. Immunoblot was used to detect protein expression. Chromatin immunoprecipitation assay was used to detect HIF-1/DNA binding. The interaction of p300 with HIF-1α or with SENP3, and the SUMO2/3 conjugation states of p300 were examined by coimmunoprecipitation.

Results: HIF-1 transcriptional activity in HeLa cells was enhanced by low doses (0.05–0.5 mmol/L) of H$_2$O$_2$, but suppressed by high doses (0.75–8.0 mmol/L) of H$_2$O$_2$. The amount of co-activator p300 bound to HIF-1α in HeLa cells was increased under mild oxidative stress, but decreased under severe oxidative stress. The ROS levels differentially modified cysteines 243 and 532 in the cysteine protease SENP3, regulating the interaction of SENP3 with p300 to cause different SUMOylation of p300, thus shifting HIF-1 transcriptional activity.

Conclusion: The shift of HIF-1 transactivation by ROS is correlated with and dependent on the biphasic redox sensing of SENP3 that leads to the differential SENP3/p300 interaction and the consequent fluctuation in the p300 SUMOylation status.

Keywords: ROS; redox; SENP3; HIF-1; p300; SUMOylation; HeLa cells
the prostate cancer cell line Du145 by the overwhelming production of ROS induced by a combined administration of the ROS producer emodin and the conventional chemotherapeutic agent cisplatin\[21\]. The capacity of HIF-1 to be activated by mild oxidative stress but inhibited by severe oxidative stress, ie, the mechanism for the biphasic regulation of HIF-1 transactivation, has thus become an intriguing question.

We have recently found that the SUMO2/3-specific protease SENP3 and its de-SUMOylating catalytic activity are required for the ROS-induced enhancement of HIF-1 transactivation, based on ROS-induced HIF-1α stabilization\[26\]. The SENP3-mediated deconjugation of SUMO2/3 from p300, the co-activator for HIF-1, is beneficial for HIF-1 transcriptional activity under both hypoxic and normoxic conditions. Being different with the other SENPs family members, SENP3 is specifically and sensitively responsive to low oxidative stress\[26, 27\]. SENP3 is stabilized upon exposure to hydrogen peroxide (H\(_2\)O\(_2\)) at very low doses, for instance, 0.05 mmol/L, and redistributes from the nucleolus to the nucleoplasm, which allows it to modulate various nuclear events, including the enhancement of HIF-1 transactivation. However, SENP3, along with all SENP family members, is a cysteine protease\[28–30\], meaning that its enzymatic activity relies on cysteine residues in the catalytic site. It has been demonstrated that highly oxidizing conditions (10 mmol/L H\(_2\)O\(_2\)) inhibit the deconjugation activity of SENP1 and SENP2\[26, 31\]. We therefore hypothesize that the oxidation of the catalytic cysteine in SENP3 under severe oxidative stress might inhibit its de-SUMOylating activity that is required for the physiological functions of various substrates, including p300, and HIF-1 transactivation might be thus suppressed.

To test this hypothesis, we used HeLa cells exposed to a series of concentrations of H\(_2\)O\(_2\) as a simple model of mild and severe oxidative stress, examined the steps of HIF-1 activation, and analyzed SENP3 activity and the SUMOylation status of p300. Our findings in the present study suggest that the shift of HIF-1 transactivation by ROS is correlated with and dependent on the differential SENP3/p300 interaction and consequent fluctuation in the SUMOylation status of p300. SENP3 protein is induced by mild oxidative stress but its catalytic activity is inactivated by severe oxidative stress, thus leading to opposite SUMOylation status of its substrate p300. This biphasic ROS effect on SENP3 is achieved through a sequential oxidative modification at two cysteine residues.

**Materials and methods**

**Cell culture and treatments**

HeLa cells were used for all experiments. Cells were cultured in Dulbecco’s modified Eagle’s medium (GibcoBRL, Gaithersburg, MD, USA). All media were supplemented with 100 U/mL penicillin, 100 mg/L streptomycin and 10% newborn calf serum (Biochrom AG, Germany). Cells were maintained at 37°C in a humidified atmosphere with 5% CO\(_2\).

To set a cell model of oxidative stress, cells were treated with hydrogen peroxide (H\(_2\)O\(_2\), Sigma-Aldrich, St Louis, MO, USA) at increasing doses. When needed, anti-oxidant N-ace-tylcysteine (NAC, Sigma-Aldrich, St Louis, MO, USA) was pre-incubated with cells for 4 h and protein reducing agent dithiothreitol (DTT, Sigma-Aldrich, St Louis, MO, USA) was pre-incubated for 2 h.

**Cell viability assay**

Cells were seeded at 1.5×10\(^4\)/mL cells per well in 96-microculture-well plates. After exposed to the various concentration of H\(_2\)O\(_2\) as indicated for 24 h, cell viability was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO, USA)\[22\].

**ROS detection**

2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich, St Louis, MO, USA) was used as ROS capturing reagent with the method described previously\[33, 34\].

**Luciferase reporter assay**

The constructs of the luciferase reporter specific for hypoxia response element (HRE) and the renilla control were transfected and the reporter assay were performed as described previously\[26\]. Briefly, cells were transfected with the double reporters, and 40 h post transfection, cells were exposed to H\(_2\)O\(_2\) at varied doses once for 6 h before relative luciferase activity was assayed.

**Quantitative real-time PCR**

Quantitative real-time PCR was carried out on the ABI Prism 7300 system (Applied Biosystems, Foster City, CA, USA) using SYBR Green following the manufacturer’s instructions. The primers for the vascular endothelial growth factor (VEGF) gene were 5’-CGGTATAAGTCTCTGGGCTGT-3’ and 5’-TCACCGGTCCTCGGTTGCA-3’. The primers for carbonic anhydrase 9 (CA9) were 5’-CTGCTACCTGCTGGCTGT-3’ and 5’-TCCTCTCAGGATAGCTC-3’. For real-time PCR, up to 1 µL of cDNA were used as template. Thermal cycling conditions were 95°C for 60 s, followed by 45 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 35 s. Primer efficiency of >90% was confirmed with a standard curve spanning four orders of magnitude. Following the reactions, the raw data were exported using the 7300 System Software v1.3.0 (Applied Biosystems, Foster City, CA, USA) and analyzed.

**Immunoblotting (IB)**

Cells were lysed in sample solution. Proteins were separated on 6%, 8%, or 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and bands were detected using various antibodies as indicated. The membranes were incubated with the primary antibodies at 4°C overnight and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at the room temperature (RT) before detection using an enhanced chemiluminescence (ECL) system (Pierce Biotechnology, Rockford, IL, USA).

**Immunofluorescence**

Cell monolayers were fixed with 4% paraformaldehyde, per-
meabilized with 0.2% Triton X-100, and were blocked with 5% BSA before incubation with the mouse monoclonal anti-HIF-1α at 4 °C overnight. Subsequently, the cells were incubated with fluorescent isothiocyanate (FITC)-conjugated anti-mouse antibody (SouthernBiotech, Birmingham, AL, USA) for 1.5 h at RT. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Cells were then examined under an Axiosplan 2 fluorescent microscope (Zeiss, Germany).

**Chromatin immunoprecipitation (ChIP) assay**

Cells were incubated with 150 μmol/L CoCl2 for 20 h to ensure the stabilization of HIF-1α, and then exposed to various concentration of H2O2 for 6 h. Protein-DNA were cross-linked in 1% formaldehyde for 10 min at 37 °C before the reaction was quenched by glycine (0.125 mol/L) for 5 min at RT. Cells were then harvested and the ChIP assay was performed using a kit (Upstate Biotechnology Inc, Lake Placid, NY, USA) according to the manufacturer’s instructions and as in our previous work[26]. The sonicated chromatin samples were precipitated with anti-HIF-1α antibody. The primer sequences for HRE were 5'-CTTITGTTTTTCCGAGA-3' and 5'-CAGTTTTGGGACGTGA-3'. The input fractions were used as the internal control.

**Co-immunoprecipitation (co-IP)**

Cells were washed once with cold PBS and lysed with cold 1×IP buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, pH 8.0, 0.2 mmol/L sodium ortho-vanadate, 1 mmol/L PMSF, 0.5% protease inhibitor cocktail, 0.5% IGEPA (CA-630) at 4 °C for 30 min. N-Ethylmaleimide (NEM 20 mmol/L) was included in the above buffer when SUMOylation of proteins needed to be examined. Cells were transiently co-transfected with various tagged constructs. At 48 h post-transfection, cells were exposed to various reagents as indicated before harvesting using the IP buffer. Cell lysates were passed several times through a 26-gauge needle and centrifuged at 14 000×g at 4 °C overnight. Subsequently, the cells were incubated with fluorescent isothiocyanate (FITC)-conjugated anti-mouse antibody (SouthernBiotech, Birmingham, AL, USA) for 1.5 h at RT. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI). Cells were then examined under an Axiosplan 2 fluorescent microscope (Zeiss, Germany).

**Antibodies**

The mouse monoclonal antibodies against HIF-1α for IB and immunofluorescence (BD Pharmingen, San Diego, CA, USA), HIF-1α for IP (ABR Affinity Bioreagents, Golden, CO, USA), RGS (Qiagen, Germany), HA, β-actin (Sigma-Aldrich), p300 for IB, p300 for IP (BD Bioscience, Franklin Lakes, NJ, USA) were purchased. The goat polyclonal anti-SUMO2/3 antibody and mouse monoclonal anti-SENP3 antibody were from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA).

** Constructs and the site-mutagenesis**

RGS tagged SENP3, HA tagged SUMO3, SENP3 mutants C243S and C532A were previously used in our work[35]. The mutant C532S was generated by QuikChange lightening Site-Directed Mutagenesis Kit (Agilent Technology, Santa Clara, CA, USA) with previously used method[36].

**siRNA**

siRNA specific for SENP3, SENP3 (3’UTR) and non-specific control (NC) siRNA were synthesized (RIBOBIO, China), and transfected using Lipofectamine 2000. The sequences of siRNA oligonucleotides for endogenous human SENP3 were: 5'-CAAUAAGGAGCUACUGCUAdTdT-3' and 5'-GUUAUCCUCGAGCAGAdTdT-3'. While those for 3'-UTR of SENP3 were: 5'-GATCCCTTGTGGATACGTA dTdT-3'.

**Transfection, co-transfection and rescue**

The constructs were transiently transfected or co-transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. HeLa cells were first transfected with non-specific siRNA control or siRNA for endogenous SENP3. After 24 h, cells were again transfected with RGS-SENP3 WT, the mutant C243S or the mutant C532S for another 48 h to rescue SENP3 functions.

**Statistical analysis**

Results were derived from at least three independent experiments and expressed as Mean±SD. The difference between groups were compared using Student’s t test.

**Results**

HIF-1α transcriptional activity is enhanced by low doses of H2O2 but suppressed by high doses of H2O2

Varied doses of H2O2 were administered to cultured HeLa cells and incubated for 24 h before the cell viability was assayed. The results showed that the cell viability was promoted at doses ranging from 0.05 mmol/L to 0.5 mmol/L with a peak at approximately 0.5 mmol/L, and was suppressed at doses higher than 0.75 mmol/L (Figure 1A). After screening the doses of H2O2 that resulted in opposite cellular effects, 0.5 mmol/L and 2 mmol/L were used as the doses to create the low and high oxidative stress conditions respectively. Truly, ROS increased to modest or dramatic levels following treatments with these doses of H2O2 (Figure 1B).
The transcriptional activity of HIF-1 was assessed using luciferase reporters after cells were exposed to H\(_2\)O\(_2\) for 6 h. The results showed that the transcriptional activity of HIF-1 was enhanced by 0.5 mmol/L of H\(_2\)O\(_2\) but suppressed by 2 mmol/L of H\(_2\)O\(_2\) (Figure 1C). The antioxidant NAC and the reducing agent DTT could reverse the alteration of HIF-1 transactivation caused by ROS regardless of whether transactivation was enhanced or suppressed, indicating that this effect was under redox control.

As 2 mmol/L of H\(_2\)O\(_2\) led to the repression of viability or mitotic senescence at later time points, it was necessary to distinguish a specific inhibition toward HIF-1 transactivation from a general transcriptional suppression. The luciferase reporter activity for the control (renilla) at this level of oxidative stress was compared with the activity at the lower level, and the result excluded a general transcriptional suppression (data not shown). A reporter assay for p53 showed that the transcriptional activity of HIF-1 was suppressed by 2 mmol/L of H\(_2\)O\(_2\) (Figure 1C). The antioxidant NAC and the reducing agent DTT could reverse the alterations in the mRNA levels of HIF-1 target genes caused by ROS, regardless of whether they were enhanced or suppressed (Figure 1D).

**Differential HIF-1 transcriptional activity under mild or severe oxidative stress results from altered HIF-1α/p300 binding**

Because HIF-1 activation is a multistep process involving the stabilization of the HIF-1α protein, the dimerization of the HIF-α and -β subunits, translocation to the nucleus, binding to HIF-1 responsive elements HRE, and the formation of active transcriptional complexes with the co-activators p300/CBP[36-40], we examined these processes during mild and severe oxidative stress. The result of the immunoblot showed that, although HIF-1α was stabilized by treatment with a very low dose of H\(_2\)O\(_2\), it remained unchanged under increasing doses of H\(_2\)O\(_2\) (Figure 2A). In addition, the nuclear accumulation of HIF-1α remained stable in the presence of increasing doses of H\(_2\)O\(_2\) as shown by immunofluorescence (Figure 2B). However, a ChIP assay showed that the amount of HIF-1α bound to HRE was remarkably increased by 0.5 mmol/L H\(_2\)O\(_2\) but was decreased by 2 mmol/L H\(_2\)O\(_2\) (Figure 2C). Likewise, a co-IP assay showed that the amount of p300 bound to HIF-1α increased at low doses of H\(_2\)O\(_2\) but decreased at high doses (Figure 2D). These data clearly demonstrated that the biphasic regulation of HIF-1 transactivation by different levels of ROS was not attributable to the altered expression or nuclear accumulation of HIF-1α but rather to an alteration in HIF-1/p300 binding.
binding and a concomitant alteration in HIF/DNA binding.

The ROS level regulates the SENP3-p300 interaction to cause differential SUMOylation of p300

As we had previously found that the SUMO protease SENP3 accumulated in response to a mild increase in ROS and could specifically deconjugate SUMO2/3 from p300 thus promoting p300 binding with HIF-1α, we hypothesized that the unchanged SUMOylation status of p300 under high stress might be caused by the inactivation of the enzymatic activity of SENP3, given that the SENP3 protein level was unchanged. To clarify this, RGS-tagged SENP3 was ectopically expressed in cells, and co-IP for RGS-SENP3 was then used to detect the interaction of SENP3 with p300. The results showed that the SENP3 interaction with p300 was enhanced under low stress condition. However, this interaction was markedly blocked under high stress and was even weaker than that under non-stress condition. This result indicated that the inactivation of SENP3 catalytic capability might occur under severe oxidative stress, and this inactivation could be manifested as the loss of its ability to bind to its substrates.

SENP3 is a cysteine protease, and its enzymatic activity relies on a specific cysteine, C532. As the mutant that substitutes C532 with alanine, C532A, is typically used as a dominant-negative SENP3, we used this mutant to examine SENP3-p300 binding under low and high stress conditions. The results showed that this mutant could not bind the substrate under any conditions, clearly confirming that C532, which is responsible for enzymatic activity, is required for substrate binding. Hence, SENP3-p300 binding capacity could be used as a readout for the enzymatic activity of SENP3 in the following experiments.

The ROS level regulates the SENP3-p300 interaction by affecting different cysteines

Our previous studies have shown that the decrease in the SUMO2/3 modification of p300 during mild stress is due to an accumulation of SENP3. We also found that this accumulation following a blockage of ubiquitination can be attributed to the oxidation of cysteines 243 or 274 within the redox sensing domain of SENP3; the mutants (C243S or C274S) in which these cysteines were replaced by serine, an amino acid residue non-responsive to ROS, failed to accumulate under stress. Given that the SENP3 protein level was unchanged upon high stress as compared with low stress and that the enzymatic activity of SENP3, as represented by its substrate binding capacity, was lost only under high stress, we hypothesized that, whereas mild stress oxidizes cysteines 243 or 274 to induce SENP3 stabilization, severe stress might oxidize another cysteine and inactivate the enzymatic activity of SENP3, i.e., its capacity to bind with its substrates. We then generated another mutant in which C532 was replaced with serine. The SENP3-p300 binding was then analyzed by co-IP in cells transfected with the wild-type SENP3 or the mutants C243S or C532S.

The results showed that the C243S mutant could not be stabilized in response to either high or low oxidative stress conditions. However, the level of the SENP3 protein remained stable in cells undergoing low or high stress (Figure 3A).

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ing a similar response as the wild-type SENP3. Interestingly, the C532S mutant was sensitively responsive to ROS-induced accumulation, regardless the degree of stress (Figure 4B, compare the blot of the input RGS of C532S with the wild-type). Furthermore, the co-IP results showed that it retained the capacity to bind with p300 under both low and high stress, being not responsive to ROS-induced inactivation (Figure 4B). These data indicate that the oxidation of C243 is responsible for SENP3 accumulation under both low and high stress conditions, while the oxidation of C532 is responsible for the blockage of the SENP3-substrate interaction under high stress condition.

Oxidative modification of the individual cysteines in SENP3 regulates p300 SUMOylation and HIF-1 transactivation

To elucidate the biochemical consequences of SENP3 regulation by ROS in a biphasic manner, we investigated whether C243S and C532S truly affected p300 SUMOylation. Endogenous SENP3 was knocked down by siRNA that did not target exogenous SENP3, and the SENP3 functions were then rescued by transfection with the wild-type or the C243S or C532S mutants before the co-IP experiments were conducted. The results showed that siRNA effectively abolished basal and the ROS-enhanced SENP3 accumulation and that the conjugation of p300 with SUMO2/3 was unchanged upon stress conditions. The wild-type SENP3 could rescue the ROS-enhanced SENP3 accumulation and p300 de-SUMOylation under low stress, but SENP3 accumulation could not mediate p300 de-SUMOylation under high stress conditions. The mutant C243S could not rescue SENP3 accumulation or p300 de-SUMOylation under high stress conditions.
both stress conditions. Remarkably, the mutant C532S could de-SUMOylate p300 under both mild and severe stress conditions, because SENP3 could accumulate under both stress conditions, and the enzyme remained catalytically active under severe stress (Figure 5A).

Taken together, these data suggested that the two redox-sensing cysteines have different functions. C243 is responsible for sensing the increase in ROS production and in turn blocking ubiquitination. After SENP3 becomes stabilized upon mild stress, C532 is responsible for the interaction of SENP3 with its substrates, but this site is inactivated during a further increased ROS generation. The sequential oxidation of these two cysteines leads to changes in SENP3 protein abundance and its catalytic activity during the increase in ROS level, which eventually results in a fluctuation in the SUMOylation status of p300.

We then examined the HIF-1 transcriptional activity by measuring the mRNA level of the HIF-1 specific target gene VEGF in cells with SENP3 knockdown and rescue. The results showed that the HIF-1 target gene expression was also differently affected by the two SENP3 mutants, which was consistent with the SUMOylation statuses of p300 (Figure 5B).

To exclude the possibility that 2 mmol/L H2O2 might cause oxidative damage to SENP3, we finally performed an intermolecular cross-linkage assay. This experiment showed that SENP3 cross-linking only occurred after treatment with 10 mmol/L of H2O2 (supplementary Figure 2). This result suggested that SENP3 underwent a selective oxidative modifica-

![Figure 5](image-url)

**Figure 5.** Oxidative modification of the individual cysteines in SENP3 regulates p300 SUMOylation and HIF-1 transactivation. (A) The SUMOylation of p300 was detected by co-IP. HeLa cells were transfected with non-specific siRNA control or siRNA for endogenous SENP3 for 24 h, and then transfected with RGS-SENP3 WT or the mutants C243S or C532S to rescue for another 48 h. HA-SUMO3 was simultaneously co-transfected. Cells were treated with H2O2 for 1 h. The proteins were co-immunoprecipitated using anti-p300 and detected by IB using the antibodies against p300 and HA. 🟢Actin as a control. (B) HeLa cells were transfected with non-specific siRNA control or SENP3 siRNA for 24 h, and then transfected with RGS-SENP3 WT, the mutants C243S or C532S to rescue for another 48 h. Cells were treated with H2O2 for 6 h. The VEGF mRNA levels were evaluated by real-time PCR and shown as folds of control. The results were shown as the Mean±SD of three independent experiments, and samples were duplicated in each experiment. Data were represented as Mean±SD. ³P<0.05 vs siRNA SENP3 rescue WT H2O2 0 mmol/L. ³P<0.05 vs siRNA SENP3 rescue WT H2O2 0.5 mmol/L. ³P<0.05 vs siRNA SENP3 rescue C532S H2O2 0 mmol/L.

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Discussion

The dual or biphasic regulatory effects of ROS have been reported for many years\(^{[2, 6, 41-43]}\). For instance, ROS can mediate phenotypes ranging from survival and growth to apoptosis in vascular endothelial and smooth muscle cells, which may be considered both physiological and pathophysiological\(^{[41]}\). It has been concluded that the specific response elicited by ROS is determined by their specific intracellular target(s), which, in turn, depends on the species of oxidant(s), the source and sub-cellular localization of the oxidant(s), the kinetics of production, and the quantities produced\(^{[45]}\). For example, in some cell types, low levels of ROS (usually submicromolar concentrations) induce growth, but higher concentrations (usually >10 micromolar) induce apoptosis or necrosis\(^{[44]}\). These studies, although using the words dual or biphasic, actually intended to highlight the fact that ROS may cause opposing effects at different doses.

It has been observed that cellular signaling pathways are generally subjected to dual redox regulation in which ROS have opposing effects on upstream signaling systems and downstream transcription factors\(^{[21, 26, 32, 45, 46]}\). Our previous research has shown that, when increased by chemical compounds or genetic manipulation, ROS may enhance the transcriptional activities of NF-κB, AP-1 and HIF-1 at lower doses but suppress them at higher doses\(^{[21, 26, 32, 45, 48]}\). For instance, when ROS increase modestly, they activate NF-κB by triggering the degradation of IκBα and other events in the cytoplasm, which usually lead to prosurvival signaling events. However, as the ROS levels become further excessive, the nuclear environment is shifted from reductive to oxidative, which inhibits the activation of NF-κB and other transcription factors by preventing them from binding to DNA. As a consequence, the prosurvival transcriptional activity is abolished. This finding shows how ROS can shift the activation/inactivation control of the NF-κB pathway\(^{[45]}\).

Based on findings in the literature and our own observations, we propose that the opposing effects caused by low vs high levels of ROS may be mediated by the biphasic redox sensing of proteins, ie, the ability of proteins to sense redox changes through the oxidation of their cysteine residues by different levels of ROS. Oxidative modification of protein thiols is now considered as an emerging role in cell signaling. It has been well recognized that the thiol oxidation state of reactive cysteine residues in proteins controls the function of the proteins and the pathways that they are part of\(^{[49-52]}\). We hypothesize that, during the biphasic redox sensing, the different cysteines of a given protein molecule might be sequentially oxidized by different levels of ROS, thus leading to different conformational characteristics and functional statuses of the protein.

HIF-1 is one of the most well known redox-sensitive transcription factors. An increasing body of evidence shows that ROS induce the stabilization of HIF-1α under hypoxic or normoxic conditions\(^{[14, 26, 55, 56]}\). Our previous study proposed an additional mechanism in which mild oxidative stress induced by low doses of H\(_2\)O\(_2\) can rapidly stabilize SENP3, in turn promoting the transcriptional activity of HIF-1 through the deconjugation of SUMO2/3 from the co-activator p300. This activating mechanism functions independently of HIF-1α stabilization, and is required for ROS-mediated HIF-1 transcriptional activity under both hypoxia and normoxia\(^{[53]}\). To establish why the transcriptional activity of HIF-1 can be suppressed by a further dramatic increase of ROS, we hypothesized that SENP3 is a biphasic redox sensor that mediates the biphasic redox regulation of HIF-1. We used a variety of SENP3 mutants to study the responsiveness of SENP3 to increasing levels of ROS. The results of the present study demonstrated that the oxidation of C243 is required for ROS-induced SENP3 accumulation and consequent HIF-1 transactivation, while in contrast, the oxidation of C532 is responsible for ROS-induced SENP3 inactivation and the consequent suppression of the transcriptional activity of HIF-1. Therefore, we suggest that the biphasic redox sensing of SENP3 mediates, at least in part, the bidirectional ROS regulation of HIF-1. To our knowledge, the present study has demonstrated for the first time that the different cysteines in a redox-sensitive protein can sense different ROS levels, and thus mediate a shift in protein function and the related signaling activity.

The cell model of the biphasic redox effects in this study is produced by treating HeLa cells with 0.5 and 2 mmol/L of H\(_2\)O\(_2\). This dose range of H\(_2\)O\(_2\) used in cancerous or transformed cells is considered to be appropriate in mimicking oxidative stress conditions\(^{[31, 57-59]}\). Bossis et al reported that ROS at low concentrations, 1 mmol/L, result in the rapid disappearance of most SUMO conjugates, which is due to direct and reversible inhibition of SUMO conjugating enzymes through the formation of (a) disulfide bond(s) involving the catalytic cysteines of the SUMO E1 subunit Uba2 and the E2-conjugating enzyme Ubc9. And they also observed the same phenomenon in a physiological scenario of endogenous ROS production, the respiratory burst in macrophages\(^{[31]}\). Xu et al showed a reversible oxidative modification of SUMO protease SENP1 by 10 mmol/L of H\(_2\)O\(_2\) that serves as a protective mechanism for the enzyme\(^{[59]}\). Thus, the cell model of low vs high stress in this study may represent ROS fluctuation that is correlated with protein oxidation.

The findings in the present study will shed light on biphasic redox effects in cells and increase our understanding of how the generation of ROS may be relevant to both cancer therapy and to cancer genesis and progression. A typical example of this rationale is arsenic, which is classically recognized as a ROS-dependent environmental carcinogen but becomes an anticancer agent by inducing the apoptosis of leukemic cells\(^{[60, 61]}\).

In summary, SENP3 is a biphasic redox sensor. The increase in intracellular ROS generation could induce SENP3 stabilization through the oxidation of the cysteines 243 or 274 that in turn blocks its ubiquitin-mediated degradation. But, when an overwhelming ROS are generated, the cysteine 532 that is required for the substrate interaction undergoes oxidation
and consequent inactivation. This biphasic redox sensing of SENP3 leads to a fluctuation in the SUMOylation status of its substrate p300, a co-activator of HIF-1, thus making a shift of HIF-1 transcriptional activity from activation to inactivation (Figure 6).

![Figure 6](image_url)

**Figure 6.** The schematic illustration for the biphasic redox sensing of SENP3 and its role in mediating the shift of HIF-1 transcriptional activity by ROS

**Notes:** "↑", to lead to a result; "+ ↓", to enhance; "↓ –", to suppress; [from low to high; from high to low.

**Abbreviation**

ROS, reactive oxygen species; SUMO, small ubiquitin-like modifier; NAC, N-acetylcysteine; DTT, dithiothreitol; RT-PCR, real-time polymerase chain reaction; DCFH-DA, 2, 7-dichlorodihydrofluorescein diacetate; HRE, hypoxia response element; IB, immunoblotting; co-IP, co-immunoprecipitation; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; RT, room temperature.

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**Author contribution**

Ying Wang, Jie Yang, Xin-zhi Huang, and Jing Yi designed the experiments and analyzed the data; Ying Wang, Kai Yang, Hui Cang, and Hui Li performed the experiments; Ying Wang, Jie Yang, and Jing Yi prepared the manuscript.

**Supplementary information**

Supplementary figures are available at website of Acta Pharmacologica Sinica on NPG.

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