De-SUMOylation of CTCF in Hypoxic Stress-induced Human Corneal Epithelial Cells

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Running Title: De-SUMOylation of CTCF by hypoxia

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Capsule

Background: CTCF plays important roles in the epigenetic control of cell fate.

Results: Hypoxic stress suppressed a higher MW form of CTCF by de-SUMOylation associated with lysine 74 and lysine 689 residues, resulting in significantly inhibited Pax6 expression.

Conclusion: Hypoxic stress induces de-SUMOylation of CTCF to functionally regulate CTCF activity.

Significance: CTCF plays important roles in growth factor/stress-regulated cell fates through post-translational modulation to control gene expression.

Epigenetic factor CTCF plays important roles in the genetic control of cell fate. Previous studies found that CTCF is positively and negatively regulated at the transcriptional level by epidermal growth factor (EGF) and ultraviolet (UV) stimulation, respectively. However, it is unknown whether other stresses modify CTCF protein. Here, we report that regulation of CTCF by de-SUMOylation is dependent upon hypoxic and oxidative stresses. We found that stimulation of human corneal epithelial cells with hypoxic stress suppressed a high molecular weight form of CTCF (150 kDa), but not a lower molecular weight form of CTCF (130 kDa). Further investigation revealed that the hypoxic stress-suppressed 150 kDa CTCF was a SUMOylated form of the protein. Hypoxic stress-induced de-SUMOylation of human CTCF was associated with lysine 74 and lysine 689 residues, but not to the phosphorylation of CTCF. Over-expression of SENP1 induced de-SUMOylation of CTCF. However, knockdown of SENP1 could not rescue hypoxic stress-induced CTCF de-SUMOylation. Over-expression of SUMO1 and SUMO2 increased SUMOylation of CTCF and partially blocked hypoxic stress-induced CTCF de-SUMOylation, suggesting that free cellular SUMO proteins play roles in regulating hypoxia-induced CTCF de-SUMOylation. In addition, hypoxic stress significantly inhibited Pax6 mRNA and protein expressions by suppression of Pax6 P0 promoter activity. The result was further supported by data showing that
knockdown of CTCF significantly enhanced expression of Pax6 and abolished hypoxia-induced suppression of Pax6. Thus, we conclude that hypoxic stress induces de-SUMOylation of CTCF to functionally regulate CTCF activity.

The corneal epithelial cell layer, which forms a barrier at the front of the eye to protect structures behind from biological, chemical and physical damage, undergoes a self-renewal process in 2-3 weeks. Growth factors and environmental stresses can alter corneal epithelial cell fates by affecting the corneal epithelial renewal process (1). It has been shown that hypoxic stress on the ocular surface leads to pathological conditions that result in corneal epithelial apoptosis, attenuation of re-epithelialization, and corneal neovascularization. For example, the extended wearing of contact lenses can induce hypoxic conditions, which increases susceptibility to corneal surface infections resulting in keratitis (2-4). Cellular responses to hypoxia in the corneal epithelium are complex and depend on degrees of altered oxygen tension (5). Hypoxic stress induces apoptosis in corneal epithelial cells through activations of JNK and Polo-like-kinase 3 (Plk3) that further activate the AP-1 transcription complex (4).

CCCTC binding factor (CTCF) is a zinc finger protein and epigenetic factor highly conserved across species. CTCF is characterized as both a negative and a positive regulator that binds to DNA motifs in promoter regions of different genes, including c-myc, chicken lysozyme, retinoblastoma (Rb), telomerase reverse transcriptase (TERT) and eye-specific Pax6 genes (6,7). More studies indicate that CTCF is a multifunctional nuclear protein that involves transcriptional activation/repression, gene insulation, DNA imprinting, and X chromosome inactivation. CTCF has been defined as an insulator protein because it can specifically bind to unique DNA sequences in both globin locus and h19/Igf2 genes (8). The interaction of CTCF with DNA targets blocks communication between adjacent regulatory DNA elements in a position-dependent manner, and serves as a barrier to buffer transgenes from position effects caused by spreading repressive heterochromatin from adjacent sequences (9,10). Recently a combination of computational methods and genome-wide screens revealed genome-wide CTCF-binding sites, indicating that CTCF is a universal “master weaver” of diverged genomes. CTCF is important for coordinating the organization and regulation of a whole range of distinct genomic functions in three dimensions (11-13).

We found that CTCF plays important roles in regulating proliferation, differentiation and apoptosis of these cells in response to growth factor and stress stimulation (1,14). Previous studies revealed that regulation of CTCF is dependent upon the altered ability of CTCF to interact with its partner proteins and to bind to target DNA sequences. Recent studies demonstrated that epidermal growth factor (EGF) and ultraviolet (UV) stress induce an increase and decrease in CTCF expression to mediate human corneal epithelial (HCE) cell proliferation and apoptosis, respectively (15,16). More interestingly, CTCF gene transcription is regulated by different subtypes of NF-κB in response to stimulation by EGF and UV stress (14). The latter results provide novel information indicating that CTCF is regulated at the transcriptional level. These findings
suggest that CTCF is capable of dynamically responding to stress stimulation. CTCF protein can also be modified by post-translational modifications, such as phosphorylation, poly(ADP-ribosyl)ation (PARylation) and SUMOylation (9). However, the molecular mechanisms underlying how CTCF protein is SUMOylated and de-SUMOylated in response to stimuli and whether CTCF de-SUMOylation affects its function are still largely unknown. In the present study, we focus on investigating hypoxic stress-induced regulation of CTCF de-SUMOylation. We explore the molecular mechanisms behind stress-induced effects on CTCF function by examining the effects of different stresses, including UV irradiation, hyperosmotic stress, hypoxia and hydrogen peroxide, on CTCF modification and function in corneal epithelial cells. Our results demonstrate that CTCF is modified by hypoxic conditions through a hypoxia-sensitive de-SUMOylation process to affect downstream CTCF-controlled gene activities.

EXPERIMENTAL PROCEDURES

Cell culture. Human corneal epithelial (HCE) cells were grown in Dulbecco’s modified Eagle’s Medium (DMEM)/F-12 (1:1) containing 10% fetal bovine serum and 5 µg/ml insulin. Mouse pancreatic alpha tumor cell 1, clone 6 (α-TC1-6) and HEK-293 cells were maintained in DMEM supplemented with 10% fetal bovine serum. Cells were maintained in an incubator supplied with 95% of air and 5% of CO₂ at 37 °C. The medium was replaced every 2 days, and cells were sub-cultured by treatment with 0.05% trypsin-EDTA. For hypoxia experiments, cells were incubated at 42°C for 4 h and treated with 500 µM hydrogen peroxide, respectively.

Antibodies and reagents. Antibody against CTCF (Cat. 07-729) was purchased from Millipore, Billerica, MA. Monoclonal anti-Flag M2 antibody (Cat. F3165) was purchased from Sigma, St. Louise, MO. Monoclonal antibody against HA (Cat. MMS-101R) was obtained from Covance, Princeton, NJ. Rabbit polyclonal antibody against Pax6 (Cat. 42-6600) was purchased from Invitrogen (Carlsbad, CA). Antibodies against SUMO1 (Cat. ab11672) and SUMO2/3 (Cat. ab3742) were from Abcam (Cambridge, MA). N-Ethylmaleimide (NEM, Cat. 128-53-0) was from Acros Organics (Fisher Scientifics). Proteinase inhibitors, including phenylmethylsulfonyl fluoride (PMSF), aprotinin and leupeptin, were purchased from Fisher Scientific. Sodium dodecyl sulfate (SDS) was obtained from Sigma (St. Louise, MS). Synthesized siRNA specific to human SENP1 (ON-TARGETplus SMARTpool SENP1, Cat. L-006357-00-0005) was purchased from Dharmacon RNAi Technologies (Lafayette, CO). Nonsilencing siRNA for the control was obtained from Qiagen (Valencia, CA).

Plasmid constructs. The expression plasmid for Flag-tagged CTCF were generated by subcloning human CTCF cDNA (NCBI Reference Sequence: NM_006565.3) from pCDNA4-CTCF into p-Flag –CMV-3 vector with 1% of O₂, 5% of CO₂ and 94% of N₂ at 37 °C for 0.5-4 h. For UV-irradiation experiments, HCE cells were exposed under UV-C light at an intensity of 45 µJ/cm². For hyperosmotic stimulation, cells were exposed to culture medium containing 600 mM sorbitol. For heat shock and oxidative stress stimulation, HCE cells were incubated at 42°C for 4 h and treated with 500 µM hydrogen peroxide, respectively.
(Sigma, St. Louise. MO) (16). The Pax6-P0 promoter luciferase reporter construct (pGL2-pax6-p0-Luc) was generated by subcloning a 4.2 kb DNA fragment located upstream of the Pax6 gene P0 promoter inserted in β-Gal-Pax6-P0 to a pGL2-Basic vector (Promega, Madison, WI) (17). SRa-HA-SUMO1 (plasmid 17359) and SRa-HA-SUMO2 (plasmid 17360) were obtained from Addgene (Cambridge, MA) (18). Expression vector of GFP-SENP1 was a generous gift from Dr. Thomas Pap (19). Plasmid of GFP-SENP3 was a gift from Dr. Jing Yi (20). Plasmid of GFP-SENP5 was kindly provided by Dr Mary Dasso (21). Construct of SEMP1-shRNA was received as a generous gift from Dr. Jinke Cheng (22). Mutants of human CTCF-K74R/K689R (it is in the site of K698 in its mouse counter part), and CTCF-ALA were generated by using a QuikChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Primers used for generating mutants of CTCF were: 

CTCF-K74R: 

5'- CACCCTTTCTCATGCAGATCAGCTGAAAGTAAATGGAGG-3'/reverse: 5'- CCTCCATTACTTTACGATCGGACTGAAGTTACTAGAGG-3’

CTCF-K689R: forward: 5'-GAACATTATAGTTGAAGTACGAAAAAGCCAGATTCGAG-3'/reverse: 5'-CTCAGCATCTGGCTCTTTGCCATCTCATTAGTAAATGGAGG-3’

CTCF-ALA: forward: 5'-AGATGCACCGTCAAGAAAGAAGATGCGGCTAGCAGCTGAAATGCT-3'/reverse: 5'-AGCATTTTCAGCGTCAGCGGCATCTTCTTTCTTAGCGCATCTT-3’.

**Lentiviral infection of shRNAs.** Lentiviral particles, containing shRNAs of SEMP1 or CTCF tagged with a turbo-GFP, were packaged in HEK-293T cells. The viral concentrations in the culture medium were titrated by PCR after co-transfecting cells with pCMV-VSV-G, psPAX2 and pGIPZ-shRNA-CTCF (or pGIPZ-shRNA-SENP1) fused to TurboGFP for 72 h (Open Biosystems Products, Huntsville, AL). The culture medium containing the lentivirus secreted from HEK-293T cell was added to HCE cells, and infected clones stably expressing the shRNAs were selected by selective culture in the presence of G418 (800 µg/ml). HCE cells infected with a pGIPZ-shRNA-control vector packed in lentivirus were served as the controls. In addition, expression of GFP from the pGIPZ-TurboGFP vector allowed to measure the efficiency of the viral infection, and to make distinguished green from none green cells.

**DNA transfection, siRNA and luciferase reporter assays.** HCE and HEK 293 cells were transfected by using Lipofectamine 2000 transfection reagent (Invitrogen). Transfection of SENP1-specific siRNA was done by adding 25 nM SENP1 specific siRNA and 12 µl of HiPerFect (Qiagen) in 100 µl of serum-free culture medium. Transfection mixture was incubated for 10 min at room temperature (RT). The mixture was evenly dropped onto cells. Transfected cells were cultured under normal growth conditions for 72 h prior to experiments. Control cells were transfected with nonsilencing (NS)-siRNA using the same method as described above. For luciferase reporter assays, HCE cells were plated to 12-well plate at a density of 7×10^4 per well 24 h prior to transfection experiments. A typical transfection experiment was carried out by adding 500 ng reporter plasmid of pGL2-Pax6-P0 or pGL2-CMV. Plasmid of pRL-TK containing a
Renilla-luciferase gene was used as a reading control for transfection efficiency. All assays were performed by using Dual-Luciferase® Reporter Assay System (Promega). Pax6 P0 promoter activity was analyzed by normalization of firefly luciferase activity with Renilla-luciferase activity.

**Immunoprecipitation and immunoblotting.** Immunoprecipitation (IP) experiments were performed as described in a previous publication (23). Briefly, cells were lysed in 200 µL of SDS buffer containing 62.5 mM Tris-Cl, 2% SDS, 10% glycerol, protease inhibitor cocktail (PIC, containing 1 mM PMSF, 1 µg/ml aprotinin and 1 µg/ml leupeptin) and 20 mM N-ethyl maleimide. Lysates were then sonicated 3× for 5 sec to reduce viscosity followed by centrifugation at 15,000 rpm for 15 min to remove cellular debris. Lysates were diluted 5-fold with a dilution buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% NP-40) and incubated with 1 µg of primary antibody at 4°C overnight. Immunocomplexes were recovered by incubation of the lysates with 40 µl of 25% protein A/G-sepharose (Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplex beads were washed with a wash-buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% NP-40), boiled in 2x SDS-PAGE loading buffer, and then subject to immunoblotting assays.

For immunoblotting assays, protein samples prepared in SDS-PAGE loading buffer (62.5 mM Tris-HCl, pH 6.8, 2%SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% bromphenol blue) were fractionated in SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a Trans Blot SD Transfer Cell (Bio-Rad). The membrane was blocked with 5% fat-free milk in Tris-buffered saline containing 0.5% Tween-20 (TBST) for 1 h at RT, and hybridized with respective primary antibodies at 4 °C overnight. Secondary antibodies conjugated with horseradish peroxidase (1:3000 in TBST with 5% milk, Santa Cruz Biotechnology) were used to probe positive signals, respectively. Positive protein bands were visualized using a Luminol reagent kit (Santa Cruz Biotechnology).

**RNA extraction, reverse transcription and real-time PCR.** Total RNAs extracted from cells were used in reverse transcription reactions. The cDNA samples were diluted 50 folds and subjected to real-time PCR assays using the Power SYBR® Green PCR Master Mix (Cat. 4368708, Applied Biosystems, Carlsbad, CA). Primers used in real-time PCR experiments were: 1) Human SENP1, forward: 5’-GCATTTCGCCTGACCATTACAC-3’/reverse: 5’-CCTTCTCTTTACTTCGCTCCA TCA-3’; 2) Human Pax6, forward: 5’-GAGTGCCCG TCCATCTTTG-3’/reverse: 5’-GTCTGCGCC CATCTGTTGCTTTC-3’; 3) Human GUSB, forward: 5’-CTCATTTGGAATTTTGCCGATT-3’/reverse: 5’-CCGAGTGAAGATCCCC TTGTTTA-3’; and 4) Human GAPDH, forward: 5’-TGGGGAAGGTGAAGGTCGG-3’/reverse: 5’-CTGGAAGATG GTGATGGGA-3’.

**RESULTS**

**Suppression of higher molecular weight CTCF by various stresses.** Previous studies found in corneal epithelial cells that CTCF expression is up-regulated and down-regulated by EGF and UV stress, respectively. To further study the role of CTCF in various environmental stress-induced cellular responses, we challenged corneal epithelial cells with
different stresses, including heat shock, UV irradiation, hyper-osmotic pressure, hydrogen peroxide (H$_2$O$_2$) and hypoxia (Fig. 1). Expression of CTCF protein was determined by Western analysis 4 h after stimulation. There were two forms of CTCF proteins visualized in a 6% SDS-PAGE gel: a primary form with a molecular weight (MW) of 130 kDa and a secondary form with a higher MW of 150 kDa. Interestingly, upon exposure of corneal epithelial cells to hypoxia and H$_2$O$_2$, expression of 150 kDa CTCF was diminished, while expression of 130 kDa CTCF was not affected (Fig. 1A). UV irradiation and hyper-osmotic stresses down-regulated expression of both forms of CTCF. However, there was no change in CTCF expression found in cells treated with heat shock. Thus, our study focuses on oxidative stress-induced effects on CTCF modification in corneal epithelial cells. Following a time course of treatments, hypoxic stress and H$_2$O$_2$ significantly diminished 150 kDa CTCF within 0.5 h without affecting 130 kDa CTCF (Fig. 1B&1C). The effect of hypoxic stress on CTCF expression was also examined in other cell types. We found that hypoxic stress also markedly suppressed the 150 kDa CTCF in HTCE (human telomerase-deficient immortalized corneal epithelial), HEK-393 (human embryonic kidney 293) and α–TC-1-6 (mouse pancreatic α tumor) cells (Fig. 1D). The results indicate that cellular CTCF activities are regulated by stimulation of various stresses. UV and hyper-osmotic stresses inhibited CTCF expression while hypoxic stress and H$_2$O$_2$ suppressed the higher MW 150 kDa CTCF, suggesting a novel mechanism in hypoxia-sensitive CTCF modification.

**Hypoxic stress induced de-SUMOylation of CTCF.** As mentioned previously, CTCF is modified by phosphorylation, PARylation and SUMOylation. However, which CTCF modifications are affected by hypoxic stress is unknown. We performed immunoprecipitation (IP) experiments with standard lysis buffer and found that anti-CTCF antibodies were able to pull down 130 kDa CTCF proteins, but not 150 kDa CTCF, suggesting that modification of 150 kDa CTCF might be altered by specific cellular enzymes that are active in the normal IP lysis buffer. We tested the effects of three components added to the standard IP lysis buffer, including NEM (inhibitor of cysteine peptidases), PIC (Proteinase Inhibitor Cocktail) and SDS (anionic surfactant), on preservation of the 150 kDa CTCF protein. The 150 kDa CTCF was preserved in the modified IP lysis buffer by adding NEM or SDS. However, 150 kDa CTCF was diminished in the presence of PIC in the lysis buffer (Fig. 2A). These results indicate that hypoxic stress-induced suppression of the 150 kDa CTCF could be resulted from loss of protein modification by SUMOylation or mono-ubiquitination. Furthermore, we performed a denaturing IP by using a lysis buffer containing both NEM and SDS. We found that the 150 kDa CTCF was present in the IP products pulled down by anti-CTCF antibody and recognized in Western analysis by anti-SUMO1 and anti-SUMO2/3 antibodies (Fig. 2B), but not by the anti-ubiquitin antibody (data not shown). The possibility of the ubiquitination of CTCF was further investigated by the addition of MG132, a proteasome inhibitor. De-SUMOylation of CTCF still occurred in the presence of MG132 following a time course (no ubiquitinated-CTCF was observed) (Fig. 2C). Both SUMO1 and SUMO2/3 conjugated CTCF proteins were suppressed by hypoxic stress in 4 h. The results also demonstrated that the 150 kDa CTCF was suppressed by hypoxic stress when CTCF proteins were pulled down by anti-SUMO2/3 antibodies and recognized by anti-CTCF antibody (Fig. 2D). In addition, the time course showed a consistent pattern in hypoxic stress-induced suppression of 150 kDa CTCF proteins within
0.5 h in whole cell lysates, detected by immunoblotting and immunoprecipitation with an anti-SUMO2/3 antibody (Fig. 2E). Accordingly, HEK-293 cells were co-transfected with Flag-tagged CTCF (Flag-CTCF) and HA-tagged SUMOs (HA-SUMO1 and HA-SUMO2) for 48 h, and then exposed to hypoxic stress. Expressed exogenous CTCF proteins were precipitated by an anti-Flag antibody and detected for HA-SUMO-conjugated CTCF by an antibody against HA tags (Fig. 2F). Consistent with data on endogenous CTCF modification, the exogenous CTCF proteins were modified by SUMO1 and SUMO2. Both of the SUMOylation modifications were markedly decreased following treatment of the hypoxic condition for 4 h. These results demonstrate hypoxic-stress-induced de-SUMOylation of CTCF proteins in the stimulated cells.

**Effect of altering SUMOylation on 150 kDa CTCF.** Hypoxic stress-induced changes in de-SUMOylation of 150 kDa CTCF were further verified. First, we tested whether the higher MW CTCF is sensitive to sentrin/SUMO-specific proteases (SEPNs). HEK-293 cells were transfected with GFP-vector only (control) and cDNAs encoding full-length of SENP1, 3 & 5. Expression of the 150 kDa form of CTCF was significantly decreased in cells over-expressing SENP1 compared with the control \( (p<0.05, \ n=4) \), but was not altered by over-expression of either SENP3 or SENP5 (Fig. 3A). Second, two SUMOylation sites in the human CTCF protein were mutated by replacing individual lysine 74 or lysine 689 residues with arginine \( \text{(K74R or K689R)} \), and by replacing both lysine 74 and lysine 689 residues with arginine residues \( \text{(K74R/K689R)} \) to examine whether 150 kDa CTCF was affected. Flag-tagged wildtype CTCF and CTCF mutants \( \text{(k74R, K689R and K74R/K689R)} \) were co-transfected with HA-tagged SUMO1 and SUMO2, respectively. Exogenous Flag-CTCF and CTCF proteins conjugated by HA-SUMO1 and HA-SUMO2 were immunoprecipitated and detected by anti-Flag and anti-HA antibodies, respectively. It showed that wildtype CTCF was modified by HA-SUMO1 and HA-SUMO2. Single site mutation of either K74R or K689R comprised the ability of CTCF to be modified by SUMO1 or SUMO2. However, SUMOylation of CTCF was completely prevented when both lysine 74 and lysine 689 were replaced by arginine residues \( \text{(K74R/K689R)} \) (Fig. 3B). In HEK-293 cells, over-expression of the wildtype and K74R/K689R mutant of CTCF were compared with cells transfected with the Flag-vector. In addition, the cells were co-transfected with HA-vector, HA-SUMO1 and HA-SUMO2. SUMOylation of CTCF was examined by Western analysis. Increases in SUMO levels resulted in an increase in the 150 kDa wildtype CTCF, but had no effect on the CTCF K74R/K689R mutant (Fig. 3C). Next, both wildtype and mutants of CTCF proteins expressed in transfected HCE cells were detected by immunostaining with an anti-Flag antibody. As shown in Fig. 3D, both exogenous wildtype CTCF and CTCF mutants demonstrated a similar pattern of sub-cellular distribution and remained in the nucleus. Taken together, these results provide direct evidence to support a mechanism in which regulation of 150 kDa CTCF by hypoxic stress is due to SUMOylation of CTCF.

**Mechanism involving hypoxic stress-induced de-SUMOylation of CTCF.** To verify the role of SENP1 in de-SUMOylation of CTCF in response to hypoxic stress, SENP1 mRNA was knocked down by two approaches in HCE cells: 1) a SENP1-specific siRNA was transiently transfected by lipofection; and 2) a SENP1-specific shRNA was stably expressed by infecting cells with SENP1-shRNA using a lentiviral delivery system, followed by culturing cells in a selection medium containing antibiotic G418. Results of
real-time PCR demonstrated that SENP1 siRNA transfection and shRNA infection significantly reduced the mRNA level of endogenous SENP1 compared with the nonsilencing (NS)-siRNA/shRNA controls (Fig. 4A & 4D). Knockdown of SENP1 mRNA also markedly suppressed SENP1 protein expression (Fig. 4B & 4E). However, knockdown of SENP1 did not block hypoxic stress-induced de-SUMOylation of CTCF, indicating that SENP1 is not involved in hypoxic stress-induced de-SUMOylation of CTCF (Fig. 4C & 4F). Furthermore, a human CTCF mutant (CTCF-ALA) was established by replacing four serine residues (S_{604}KKEDS_{608}S_{610}DS_{612}E) that consist of major phosphorylation sites with alanine residues in order to study the effect of CTCF phosphorylation on hypoxic stress-induced CTCF de-SUMOylation. In HEK-293 cells that were co-transfected with wildtype CTCF and CTCF-ALA mutant plus HA-SUMO1 and HA-SUMO2, wildtype and mutant CTCF, were equally modified by SUMO1 or SUMO2, suggesting that phosphorylation of CTCF occurring in these serine sites did not affect the SUMOylation of CTCF (Fig. 5A). Next studies were aimed towards answering the question of whether deficiency of free SUMO proteins contributes to hypoxic stress-induced de-SUMOylation of CTCF. Levels of free SUMO1 and SUMO2 were markedly increased in control and hypoxic stress-induced cells by over-expression of HA-SUMO1 and HA-SUMO2. Over-expression of HA-SUMO1 and HA-SUMO2 partially reversed hypoxia-induced de-SUMOylation of CTCF (Fig. 5B). The role of hypoxic stress in altering SUMOylation of cellular proteins was further examined by monitoring the global SUMO conjugation pattern in HCE cells and by Western analysis with anti-SUMO1 and anti-SUMO2/3 specific antibodies. Hypoxic stress decreased free SUMO1 and SUMO2/3 and altered the global patterns of SUMO-conjugations. In particular, rapid accumulation of the endogenous SUMO2/3 conjugates was observed (Fig. 5C). These results suggest that cellular free SUMO proteins (especially SUMO2) indeed play key roles in regulating hypoxia-induced de-SUMOylation of CTCF.

Effect of CTCF De-SUMOylation on downstream Pax6 gene activity. Previous studies found that CTCF controls eye development through regulation of the eye-specific Pax6 gene by binding to a repressor element between the EE enhancer and P0 promoter of the Pax6 gene (17). We explored the functional role of CTCF de-SUMOylation in HCE cells by comparing the effect of human wildtype CTCF and the CTCF-K74R/K689R mutant on the downstream Pax6 gene. We found that over-expression of wildtype CTCF inhibited Pax6-P0 promoter reporter activity, while over-expression of the CTCF-K74R/K689R mutant further enhanced the inhibitory effect on Pax6-P0 promoter reporter activity (Fig. 6A). Neither wildtype CTCF nor the CTCF-K74R/K689R mutant affected pGL2CMV activity in control experiments (Fig. 6B), indicating that the impact of CTCF de-SUMOylation on Pax6 P0 promoter is specific. Real time PCR experiments were performed on hypoxic stress-induced cells to investigate the effect of hypoxic stress that de-SUMOylates CTCF protein on Pax6 gene expression. We found that hypoxic stress induced significant decreases in Pax6 mRNA expression staring within 1 h, and that the Pax6 mRNA level continued decreasing to reach greater than a 50% reduction at 4 h. The mRNA level of GUSB (β-glucuronidase) was detected by real-time PCR in parallel to Pax6 mRNA measurements as a control (Fig. 6C). It has been shown that the mRNA level of GUSB is unchanged in hypoxic stress-induced cells (24). The effect of hypoxic stress on Pax6 protein expression was determined by Western analysis. Pax6
expression was significantly suppressed by hypoxic stress in 4 h in corneal epithelial cells (Fig. 6D). To further determine whether hypoxia affects Pax6 expression exclusively through CTCF de-SUMOylation, HCE cells were infected by lentiviral CTCF-specific shRNA to knock down the CTCF mRNA. The stable expression of CTCF-specific shRNA in the G418 selective medium resulted in a marked decrease in CTCF protein expression (Fig. 6E). Real time PCR and Western analysis data revealed that knockdown of CTCF effectively abolished hypoxia-induced suppression of Pax6 mRNA and protein expressions, respectively (Fig. 6F & 6G). These results indicate that hypoxic stress-induced suppression of Pax6 is mediated by CTCF and de-SUMOylation of CTCF enhanced CTCF capability to suppress Pax6 expression.

**DISCUSSION**

CTCF is a multifunctional transcription factor that plays important roles in epigenetic regulation of gene expression. Recently, it has been shown that CTCF is a leading candidate for mediating a network of local and long-range intra-chromosomal loops and inter-chromosomal contacts (25). Therefore, CTCF must be capable of dynamically responding to stresses and mediating stress-induced alteration of chromatin structures. Several stress-related proteins such as PUMA (p53 up-regulated modulator of apoptosis) have been reported to be regulated by CTCF (26). However, it is still unclear how CTCF is regulated in response to environmental stresses. We previously demonstrated that UV and hyper-osmotic stresses down-regulated CTCF by reducing its expression at gene transcription level. Here, we continue to investigate the response of CTCF to environmental stresses by challenging corneal epithelial cells with multiple stimuli including UV irradiation, hyper-osmotic pressure, heat shock, hydrogen peroxide (oxidative stress) and hypoxia. In addition to down-regulated CTCF expression induced by UV and hyper-osmotic stresses, the study was aimed at addressing the question of whether there is modification of CTCF protein in response to hypoxic and oxidative stresses. We found that a higher MW form of CTCF at 150 kDa disappeared after stimulation with hypoxic and oxidative stresses, indicating that there are certain types of environmental stresses that can alter CTCF activity through post-translational modifications at the protein level.

Three post-translational modifications of CTCF have been previously characterized, including phosphorylation (27,28), PARylation (29-31) and SUMOylation (23,32). CTCF phosphorylation that occurs in the carboxy terminus of CTCF is responsible for attenuation of CTCF activity towards c-myc promoter activity (27). PARylation in CTCF’s N-terminal domain can also regulate CTCF function. PARylation is essential for CTCF to function as an insulator for the Igf2/h19 imprinting gene and as a chromatin boundary upstream of p16 gene (10,30). Recently, it has reported that CTCF can be modified by SUMO (small ubiquitin-related modifier) proteins and the SUMOylation sites identified are at residues lysine 74 and lysine 698 of mouse CTCF protein (23). In the present study, we identified that lysine 74 and lysine 689 in human CTCF protein can be modified by SUMO, which is different from lysine 698 in mouse CTCF.

It is not known how CTCF protein is de-SUMOylated and whether de-SUMOylation of CTCF affects its function. In the present studies, we observed hypoxia-sensitive de-SUMOylation of CTCF. Our results demonstrate CTCF protein has two forms. A higher molecular weight form of CTCF is regulated by hypoxic stress, while
the major form of CTCF protein at 130 kDa is not sensitive to hypoxic stress. The difference between the two forms, as analyzed in a 6% SDS-PAGE gel, is in accordance with the size of a SUMOylated protein. More evidence revealed that hypoxic stress-induced de-SUMOylation of CTCF can be blocked in the presence of NEM, resulting in preservation of 150 kDa CTCF. NEM is commonly used as an inhibitor in protein de-SUMOylation or de-ubiquitination studies. In other reports, PARylated CTCF is characterized as a 180 kDa protein, as detected by Western analysis. However, we did not detect the PARylated 180 kDa CTCF in either immunoprecipitation or Western blot experiments in HCE cells. Instead, we found that there is a 150 kDa SUMOylated form of CTCF in HCE, HEK-293 and pancreatic islet α-cells. One possible explanation for this is that the methods of immunochemistry assays that we have used in the study were different from those reports (29,30). In this study, all cell lysis buffers for immunochemistry assays contained 2% SDS followed by a five-fold dilution with 1% triton X-100 buffer to optimize preservation of SUMOylated CTCF.

Most SUMO targets like CTCF itself are nuclear proteins, including transcription factors, transcription co-regulators, and chromosome remodeling regulators. SUMOylations of these proteins can affect their localization in different cellular compartments as well as their biological activities. SUMO-conjugation is dynamically catalyzed by SUMO-specific E1, E2 and E3 ligases and reversed by a family of the sentrin/SUMO-specific proteases (SEPNs) (33-35). Ubc9-deficient cells lacking SUMO conjugation enzymes showed severe defects in nuclear organization including chromosome condensation and segregation, nuclear envelope dysmorphism, and disruption of nucleoli and PML nuclear bodies. All these findings indicate a functional role of SUMOylation in regulating chromatin architecture (36). Thus, studies of CTCF SUMOylation are important in understanding CTCF-mediated DNA de-condensation (32). Although the detected 150 kDa CTCF is consistent in terms of size with a predicted SUMOylated CTCF, more supporting data are required to confirm this conclusion. In the present study, we provide the following evidence to directly prove that this 150 kDa form of CTCF is SUMOylated-CTCF: 1) hypoxia-induced suppression of 150 kDa CTCF following a time course found in Western blots could not be seen in IP experiments with un-denaturing lysis buffer (Fig. 2A); 2) over-expression of SENP1 as a major SUMO specific protease suppressed the 150 kDa CTCF detected by Western analysis (Figs. 3A); 3) the 150 kDa CTCF was regulated by increased free-SUMO levels in the cell (Fig. 5B); and 4) co-transfection of HA-SUMO1 and HA-SUMO2 increased the 150 kDa CTCF in cells transfected with wildtype CTCF, but not in cells transfected with CTCF K74R/K689R mutant that lacks SUMOylation sites (Fig. 3C).

In the present study, we explored the mechanisms involving de-SUMOylation of CTCF induced by hypoxic stresses, which is consistent with previous observations that SUMO conjugation and de-conjugation equilibrium is affected by various environmental stresses, including osmotic, hypoxic, heat, oxidative and genotoxic stresses. There are proposed mechanisms that explain stress-regulated protein SUMOylation through a machinery of conjugation by SUMO, E1, E2 and E3 ligases. However, de-conjugation of the target proteins is
through the effect of SENPs and the interaction between phosphorylation and SUMOylation of these proteins (37). We found that although SENP1 was able to reduce SUMOylated CTCF under normal conditions, it did not play an obvious role in hypoxic stress-induced de-SUMOylation of CTCF (Fig. 4). We also investigated the interaction between phosphorylation and SUMOylation of CTCF by establishing a human CTCF-ALA mutant in which four-serine residues within the highly conserved S604KKEDS609S610DS612E motif were changed. However, these mutations did not alter the status of CTCF SUMOylation (Fig. 4C). In addition, hypoxic stress can alter global protein SUMOylation from 6 to 24 h (38,39). In the present study, we demonstrated that hypoxic stress triggered a rapid accumulation of global SUMO2/3 conjugates accompanied by reduced levels of non-conjugated SUMO proteins, suggesting that hypoxia-induced de-SUMOylation of CTCF may be resulted from the reduction of free SUMO proteins. This is supported by our results showing that over-expression of HA-SUMO2 partially reversed hypoxic stress-induced de-SUMOylation of CTCF (Fig. 5). It is also consistent with the report that MG132, a proteasome inhibitor, increased global SUMO2 conjugates together with decreased non-conjugated SUMO2 proteins in corneal epithelial cells (data not shown). SUMO proteins are similar to ubiquitin. However, SUMOylation is not used to tag proteins for degradation. We did not observe an increase in the ubiquitin level after treating cells with MG132 that is a specific, potent and cell-permeable proteasome inhibitor. Instead, we found a reduction of SUMOylated-CTCF upon the MG132 treatment. The result is consistent to the previously published data, demonstrating that application of MG132 induces a significant decrease in CTCF SUMOylation (40). Thus, both hypoxic stress and proteasome inhibitor effects may share a common mechanism in regulation of CTCF SUMOylation.

It has been shown that SUMOylation regulates target protein activity by affecting their cellular localization, their ability to interact with targets, and their stability (34,35). So far, two publications have reported SUMOylation of CTCF. However, it is unknown whether SUMOylation regulates CTCF activity and function. One suggests that SUMOylation is required for CTCF to inhibit c-myc P2 promoter activity (23). The other indicates that SUMOylation of CTCF may inhibit transcriptional and chromatin opening abilities in the N-terminal of CTCF (32). It has shown that CTCF can interact with numerous protein partners, including cohesin, nucleophosmin, PARP1, Yy1 and RNA polymerase II (41-44). It has been shown that CTCF can function as a transcriptional insulator by blocking enhancer-promoter interaction at a few loci, where the cohesin subunit Rad21 has been involved in the interaction (45). We found that de-SUMOylation of CTCF by mutating both K74 and K689 sites altered the interaction between CTCF and Rad21 (Data not shown), which suggests that de-SUMOylation of CTCF can enhance interactions of CTCF with the cohesin complex in the nucleus. This may provide some insight into the interaction between CTCF and the Pax6 promoter in response to hypoxic stimulation.

We previously demonstrated that CTCF binds to a repressor upstream from the Pax6 P0 promoter resulting in inhibition of Pax6 transcription, affecting eye development (17,46,47). In the present study, we investigated whether hypoxic stress-induced de-SUMOylation of CTCF affects Pax6 gene transcription by measuring Pax6 P0 promoter activity (Fig. 5A). Consistently, there was a reduction of Pax6 gene expression under hypoxic conditions associated with hypoxic stress-induced de-SUMOylation of CTCF, indicating that hypoxia-induced
de-SUMOylation of CTCF contributes to its ability to inhibit Pax6 gene transcription. We believe it is very likely that under normoxic conditions, some CTCF is SUMOylated and restrained from serving its normal function as a genome organizer. Hypoxic stress triggers de-SUMOylation of CTCF to mobilize more CTCF proteins that can participate in regulation of Pax6 gene transcription. It is important to further investigate the mechanisms behind how SUMOylation affects CTCF activity and better understand the diversified functions of CTCF in relation to its numerous partner proteins, such as cohesin, nucleophosmin, PARP1, Yy1 and RNA polymerase II (48).

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FIGURE LEGENDS

Figure 1. Effects of stress stimulation on CTCF expression. (A) CTCF expression in HCE cells induced by UV irradiation, hyper-osmotic pressure, heat shock, H$_2$O$_2$ and hypoxic stress. (B) Time course of hypoxic stress-induced alteration of 150 kDa CTCF expression. (C) H$_2$O$_2$ oxidative stress-induced suppression of 150 kDa CTCF following a time course. (D) Hypoxic stress-induced alterations of 150 kDa CTCF in HTCE, HEK-293 and α-TC-6 cells. Cells were synchronized by serum starvation for 24 h prior to stress treatments at indicated time points. Endogenous CTCF was detected by Western analysis using anti-CTCF. Data were plotted as mean±SE. Symbol “*” indicates significant differences tested by ANOVA and Student’s t-test (p<0.05, n=3).

Figure 2. Effects of hypoxic stress on SUMOylation of CTCF. (A) Effects of NEM, PIC and SDS on SUMOylation of CTCF protein detected by Western analysis. Whole cell lysates were individually treated with 20 mM NEM (N-ethyl maleimide), PIC (protease inhibitor cocktail, containing 1 mM PMSF, 1 µg/ml aprotinin and 1 µg/ml leupeptin) or 2% SDS. (B) Detection of CTCF SUMOylation in control and hypoxic stress-induced HCE cells by immunoprecipitation (IP) using anti-CTCF antibody and immunoblotting (IB) with anti-SUMO1 and anti-SUMO2/3 antibodies. (C) Effect of MG132 on de-SUMOylation of CTCF following a time course in HCE cells. CTCF in whole cell lysate was detected by Western analysis using antibody against CTCF. (D) Detection of CTCF SUMOylation in control and hypoxic stress-induced HCE cells by immunoprecipitation using anti-SUMO2/3 antibody and immunoblotting with anti-CTCF antibody. (E) Comparison of hypoxic stress-induced CTCF de-SUMOylation detected by Western blots of whole-cell lysates and products immunoprecipitated with anti-SUMO2/3 antibody. (F) Effect of hypoxic stress on de-SUMOylation of CTCF by co-transfection of cells with Flag-CTCF and HA-SUMO1 or HA-SUMO2. Co-transfected HEK-293 cells were treated by hypoxic stress for 4 h prior harvesting. Tagged CTCF was pulled down by antibody against Flag and HA-SUMO groups were detected by anti-HA and anti-Flag. All of the data sets were repeated for three times from three independent experiments, and the results were very consistent.

Figure 3. Effects of overexpressing SENPs and CTCF-K74R/K689R mutant on CTCF SUMOylation. (A) Effects of overexpressing SENP1, SENP3 and SENP5 on CTCF SUMOylation. HEK-293 cells were transfected with GFP-SENP1, 3 and 5 for 48 h before they were harvested for Western analysis. Anti-CTCF was used to detect the endogenous CTCF. Anti-GFP was used to detect the expression level of exogenous SENPs. (B) Effect of
co-transfecting Flag-tagged wildtype and CTCF K74R/K689R mutant with HA-SUMO1/2 on SUMOylation of CTCF, respectively. Immunoprecipitation experiments were performed by using anti-Flag antibody and followed by immunoblotting using anti-HA and anti-Flag antibodies. (C) Effect of co-transfecting wildtype and K74R/K689R mutant of the human CTCF with HA-SUMO1 or HA-SUMO2 on 150 kDa CTCF band detected by Western analysis using antibody against CTCF. (D) Localization of wildtype CTCF and CTCF mutant in the nucleus. Exogenous CTCF and its mutant were localized in transfected 293T cells by immunostaining using anti-Flag antibody. Data were plotted as mean±SE. Symbol “*” indicates significant difference determined by ANOVA (p<0.05, n=3).

Figure 4. Effect of knocking down SENP1 mRNA on hypoxia-induced CTCF de-SUMOylation. (A) Detection of knocking down SENP1 mRNA with SENP1-specific siRNA by real-time PCR. (B) Effect of knocking down SENP1 mRNA with SENP1-specific siRNA on SENP1 protein expression. (C) Effect of knocking down SENP1 on hypoxic stress-induced de-SUMOylation of CTCF. HCE cells were transfected with siRNA against SENP1 or non-silencing siRNA for 72 h. (D) Knockdown of SENP1 mRNA with SENP1-specific shRNA detected by real-time PCR. (E) Knockdown of SENP1 mRNA with SENP1-specific shRNA suppressed SENP1 protein expression. (F) Effect of knocking down SENP1 with SENP1-specific shRNA on hypoxic stress-induced CTCF de-SUMOylation. HCE cells were infected with CTCF-specific shRNA or non-silencing shRNA in the lentiviral delivery system, and selected in the medium in the presence of 800 µg/ml G418 for two weeks. HCE cells were stimulated by hypoxic stress for 1 h before the cells were harvested for Western analysis to detect the SUMOylation level of CTCF. Real time PCR data were plotted as mean±SE and symbol “*” indicates significant difference determined by Student’s t test (p<0.05, n=3).

Figure 5. Regulation of CTCF de-SUMOylation by hypoxic stress. (A) Effect of mutating phosphorylation site of CTCF (CTCF-ALA mutant) on SUMOylation of CTCF. CTCF-ALA mutant was established by replacing four-serine residues with glycine residues. HEK 293 cells were co-transfected with Flag-wildtype CTCF and Flag-CTCF-ALA mutant with HA-SUMO1 or HA-SUMO2 for 48 h. (B) Effect of over-expressing SUMO1 or SUMO2 on hypoxic stress-induced de-SUMOylation of CTCF. HEK 293 cells were transfected with HA, HA-SUMO1 or HA-SUMO2 individually 48 h prior to exposure of cells to hypoxic stress for 2 h. Data were plotted as mean±SE. Symbol “*” indicates the significant difference between the marked groups determined by ANOVA (p<0.05, n=3). (C) Detection of global SUMOylation pattern in hypoxic stress-induced HCE cells. HCE cells were exposed to hypoxic stress for indicated time points, and samples were subjects to Western analysis by using antibodies against SUMO1 (the left panel) and SUMO2/3 (the right panel). Arrows indicate SUMO-conjugates and free-SUMO. Stars indicate non-specific bands.

Figure 6. Effect of CTCF de-SUMOylation on Pax6 activity in HCE cells. (A) Effect of CTCF de-SUMOylation on Pax6 P0 promoter activity. HCE cells were co-transfected with Flag-wildtype CTCF and CTCF-K74/689R mutant with pGL2-PAX6-P0 reporter, respectively. (B) Effect of CTCF desUMOylation on CMV-promoter reporter. For the control experiments, pGL2-CMV vector was co-transfected with Flag-wildtype CTCF and CTCF-K74/689R mutant in to HCE cells. (C) Effect of hypoxic stress on Pax6 mRNA expression. Expression of Pax6 mRNA was determined by real-time PCR and the level of GUSB (β-Glucuronidase) mRNA was also measured as an internal control. (D) Effect of hypoxic stress on Pax6 protein expression. (E)
Suppression of CTCF expression by knocking down CTCF mRNA with CTCF-specific shRNA. (F) Effect of knocking down CTCF on hypoxia-induced Pax6 mRNA expression. (G) Effect of knocking down CTCF on hypoxia-induced Pax6 protein expression. CTCF-specific shRNA was introduced into HCE cells by using the lentiviral delivery system to knock down CTCF mRNA and non-silencing shRNA was served as controls. The infected cells were cultured and selected in the medium containing 800 µg/ml G418 for two weeks. Expression of Pax6 protein was determined by Western analysis and the β-actin level was measured as a loading control. HCE cells were synchronized by serum-starvation for 24 h prior to hypoxic stimulation. Symbol “*” represents the statistical significance determined by ANOVA and Student’s t-test (p<0.05, n=3).
Figure 1

A

170

130

< 150kDa-CTCF

< 130kDa-CTCF

< β-actin

Ctrl  UV  Hyper-Osm  Heat shock  H_2O_2  Hypoxia

B

170

130

< 150kDa-CTCF

< 130kDa-CTCF

< β-actin

150kDa-CTCF/β-actin (%)

0 0.25 0.5 1 4 (h)

C

170

130

< 150kDa-CTCF

< 130kDa-CTCF

< β-actin

0 0.25 0.5 1 2 4 (h)

H_2O_2 (500µM)

D

HTCE  HEK-293  α-TC-1-6

< 150kDa-CTCF

< 130kDa-CTCF

< β-actin

Ctrl  Hypo  Ctrl  Hypo  Ctrl  Hypo

Figure 1
Figure 2
Figure 3

A

SUMO-CTCF/β-actin (%)

SUMO-CTCF
CTCF

Anti-GFP

β-actin

Vector
SENP1
SENP3
SENP5

B

HA-SUMO1

HA-SUMO2

Anti-HA

Anti-Flag

Wildtype
K74R
K689R
K74R/K689R
Wildtype
K74R
K689R
K74R/K689R

C

HA
SUMO1
SUMO2
HA
SUMO1
SUMO2
HA
SUMO1
SUMO2

Flag-
Vector
CTCF-WT
CTCF-K74R/K689R

D

HA
HA-SUMO1
HA-SUMO2

Flag-
CTCF-WT

Flag-
CTCF-K74R/K689R

Figure 3
Figure 4

A

Real-time PCR

SENPI/GAPDH (%)

NS-siRNA  SENP1-siRNA

B

SENP1  β-actin

NS-siRNA  SENP1-siRNA

C

SUMO  CTCF  CTCF

NS-siRNA  SENP1-siRNA

D

Real-time PCR

SENPI/GAPDH (%)

NS-shRNA  SENP1-shRNA

E

SENP1  β-actin

NS-shRNA  SENP1-shRNA

F

SUMO  CTCF  CTCF

NS-shRNA  SENP1-shRNA
Figure 5

A

SUMO-CTCF

CTCF

Wildtype  CTCF-ALA

B

SUMO-CTCF/β-actin (Relative density %)

C

Hypoxia (h)

0  0.5  1  2  4

Anti-HA

Anti-Flag

SUMO-CTCF

CTCF

Anti-SUMO1

Anti-SUMO2/3

SUMO conjugates

Free SUMO

Free SUMO2

β-actin

Hypoxia

Control

Figure 5
Figure 6
De-SUMOylation of CTCF in hypoxic stress-induced human corneal epithelial cells
Jie Wang, Yumei Wang and Luo Lu

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