Cezanne Regulates Inflammatory Responses to Hypoxia in Endothelial Cells by Targeting TRAF6 for Deubiquitination

Le A. Luong, Maria Fragiadaki, Jennifer Smith, Joseph Boyle, Jens Lutz, Jonathan L.E. Dean, Sarah Harten, Margaret Ashcroft, Sarah R. Walmsley, Dorian O. Haskard, Patrick H. Maxwell, Henning Walczak, Charles Pusey, Paul C. Evans

Rationale: Hypoxia followed by reoxygenation promotes inflammation by activating nuclear factor κB transcription factors in endothelial cells (ECs). This process involves modification of the signaling intermediary tumor necrosis factor receptor–associated factor 6 with polyubiquitin chains. Thus, cellular mechanisms that suppress tumor necrosis factor receptor–associated factor 6 ubiquitination are potential therapeutic targets to reduce inflammation in hypoxic tissues.

Objective: In this study, we tested the hypothesis that endothelial activation in response to hypoxia-reoxygenation can be influenced by Cezanne, a deubiquitinating enzyme that cleaves ubiquitin from specific modified proteins.

Methods and Results: Studies of cultured ECs demonstrated that hypoxia (1% oxygen) induced Cezanne via p38 mitogen–activated protein kinase–dependent transcriptional and post-transcriptional mechanisms. Hypoxia-reoxygenation had minimal effects on proinflammatory signaling in unmanipulated ECs but significantly enhanced Lys63 polyubiquitination of tumor necrosis factor receptor–associated factor 6, activation of nuclear factor κB, and expression of inflammatory genes after silencing of Cezanne. Thus, although hypoxia primed cells for inflammatory activation, it simultaneously induced Cezanne, which impeded signaling to nuclear factor κB by suppressing tumor necrosis factor receptor–associated factor 6 ubiquitination. Similarly, ischemia induced Cezanne in the murine kidney in vascular ECs, glomerular ECs, podocytes, and epithelial cells, and genetic deletion of Cezanne enhanced renal inflammation and injury in murine kidneys exposed to ischemia followed by reperfusion.

Conclusions: We conclude that inflammatory responses to ischemia are controlled by a balance between ubiquitination and deubiquitination, and that Cezanne is a key regulator of this process. Our observations have important implications for therapeutic targeting of inflammation and injury during ischemia-reperfusion. (Circ Res. 2013;112:1583-1591.)

Key Words: endothelium ■ hypoxia ■ inflammatory activation

Tissues are exposed to hypoxia followed by reoxygenation during ischemia-reperfusion, which occurs in several clinical settings, including organ transplantation, percutaneous coronary intervention, and bypass grafting. Hypoxia-reoxygenation promotes inflammation by activating nuclear factor κB (NF-κB) transcription factors, which induce adhesion proteins (eg, vascular cell adhesion molecule-1 [VCAM-1], E-selectin) and other inflammatory molecules. NF-κB transcription factors are regulated by an intricate signaling network that governs their intracellular localization and transcriptional activity. In the basal state, NF-κB dimers are inactivated by binding to inhibitor of κB (IκB) proteins, which sequester NF-κB in the cytoplasm by masking its nuclear localization sequence. IκB kinase promotes NF-κB activation by phosphorylating IκBα, a modification that targets it for ubiquitin-mediated degradation, and by phosphorylating RelA NF-κB subunits to enhance DNA binding and transcriptional activation.

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Recent studies have shed light on the signaling events that control NF-κB activation by hypoxia. In normoxic conditions, IκB kinase proteins are targeted for hydroxylation by prolyl hydroxylase domain 1, a modification that leads to their...
ubiquitination and degradation. Hypoxia can promote nuclear localization of NF-κB by preventing prolyl hydroxylase domain 1–mediated repression of IκB kinase, and by inducing the expression of several NF-κB subunits. The mechanism of NF-κB activation by hypoxia also involves modification of tumor necrosis factor receptor–associated factor 6 (TRAF6) proteins with a noncanonical form of polyubiquitin (linked through Lys63) that is known to activate IκB kinase. Despite these insights, the mechanisms for NF-κB activation by hypoxia are understood incompletely and may vary according to particular cell types.

We previously discovered a regulator of NF-κB called Cezanne (Cellular Zinc-finger Anti–NF-κB; also known as OTUD7B) and cloned full-length cDNA from endothelial cells. Our subsequent studies revealed that Cezanne can be an inhibitor of NF-κB activation. The molecular mechanism of the expression of several NF-κB subunits. The mechanism of NF-κB activation by hypoxia also involves modification of tumor necrosis factor receptor–associated factor 6 (TRAF6) proteins with a noncanonical form of polyubiquitin (linked through Lys63) that is known to activate IκB kinase. Despite these insights, the mechanisms for NF-κB activation by hypoxia are understood incompletely and may vary according to particular cell types.

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Cezanne was confirmed by reverse transcriptase PCR (Online Figure I) and was obtained from the Canadian Mouse Mutant Repository. Conditional Mouse Mutagenesis Project revealed that CezanneGT/GT mice were normal in terms of gross appearance, histopathology of multiple tissues, weight gain, glucose tolerance, and clinical chemistry analysis. Male Fisher rats (F344, RT1 lvl; 170–210 g) were obtained commercially (Charles River, Sulzfeld, Germany). Transgenic gene-trapped (GT) mice,22 in which the Cezanne gene is disrupted by removal of exons 4 to 7 and insertion of a splice acceptor site, LacZ open reading frame, neomycin resistance gene (Neo), and polyA tail in the third intron (B6-Otud7btm1(NCOM)Cmhd; CezanneGT/GT; C57BL/6 background) were obtained from the Canadian Mouse Mutant Repository. Splicing of the LacZ-Neo-polyA sequences to the third exon of Cezanne was confirmed by reverse transcriptase PCR (Online Figure I) and is predicted to generate a fusion protein comprising the peptide MTLDMDAVLSDFVSTGAEPLARDLLE (encoded by exon 3 of Cezanne) linked to LacZ. Baseline phenotyping analysis performed at the Toronto Center for Phenogenomics as part of the North American Mouse Mutagenesis Project revealed that CezanneGT/GT mice are normal in terms of gross appearance, histopathology of multiple tissues, weight gain, glucose tolerance, and clinical chemistry (http://www.europhenome.org).

Antibodies. Beads were then washed extensively using lysis buffer. Precipitated material or lysates were analyzed by Western blotting.

Animals
Male Fisher rats (F344, RT1 lvl; 170–210 g) were obtained commercially (Charles River, Sulzfeld, Germany). Transgenic gene-trapped (GT) mice,22 in which the Cezanne gene is disrupted by removal of exons 4 to 7 and insertion of a splice acceptor site, LacZ open reading frame, neomycin resistance gene (Neo), and polyA tail in the third intron (B6-Otud7btm1(NCOM)Cmhd; CezanneGT/GT; C57BL/6 background) were obtained from the Canadian Mouse Mutant Repository. Splicing of the LacZ-Neo-polyA sequences to the third exon of Cezanne was confirmed by reverse transcriptase PCR (Online Figure I) and is predicted to generate a fusion protein comprising the peptide MTLDMDAVLSDFVSTGAEPLARDLLE (encoded by exon 3 of Cezanne) linked to LacZ. Baseline phenotyping analysis performed at the Toronto Center for Phenogenomics as part of the North American Mouse Mutagenesis Project revealed that CezanneGT/GT mice are normal in terms of gross appearance, histopathology of multiple tissues, weight gain, glucose tolerance, and clinical chemistry (http://www.europhenome.org).

Renal Ischemia/Reperfusion
For the induction of renal ischemia/reperfusion in rats, the abdomen was opened under inhalation anesthesia using isoflurane. The left kidney was clamped with an atraumatic vascular clamp for 45 minutes. During this time, the right kidney was removed. The left kidney was reperfused for 2 to 72 hours. Renal ischemia/reperfusion in mice was performed under inhalation anesthesia using isoflurane. In wild-type and Cezanne GT/GT mice, the left renal artery was clamped with an atraumatic vascular clamp for 45 minutes and subsequently reperfused for 6 hours. Ischemia and reperfusion in rats was performed under inhalation anesthesia using isoflurane. In wild-type and Cezanne GT/GT mice, the left renal artery was clamped with an atraumatic vascular clamp for 45 minutes and subsequently reperfused for 6 hours. Ischemia and subsequent reperfusion were confirmed at a macroscopic level by monitoring changes in tissue appearance. Principles of NIH Guide for the Care and Use of Laboratory Animals as well as the German Law on the Protection of Animals and UK Home Office regulations were followed.

Immunohistochemistry and Morphological Studies
Sections made from formalin-fixed, paraffin-embedded tissues were incubated in xylene for 5 minutes, hydrated by sequential exposure to decreasing concentrations of ethanol (100% to 50%) and water. Heat-mediated antigen retrieval was performed in trisodium citrate in a
Hypoxia Induces Cezanne via Transcriptional and Posttranscriptional Mechanisms

Studies of cultured HUVECs revealed that Cezanne mRNA and protein levels were enhanced by exposure to 1% O₂ and remained elevated for 24 hours after reoxygenation (Figure 1A and 1B). This level of hypoxia (1% O₂) also induced Cezanne in cultured microvascular ECs at both the mRNA (Figure 1C) and protein levels (Online Figure II). By contrast, exposure of ECs to 2% or 5% O₂ had little or no effect on Cezanne expression in HUVECs or human coronary microvascular ECs (Figure 1D). Thus, cellular responses to hypoxia were studied using 1% O₂ conditions in all subsequent experiments.

We studied the molecular mechanism for Cezanne induction and focused on the role of p38 mitogen–activated protein kinase and the transcription factor HIF1α, which are known to be activated by hypoxia. Activation of HIF1α in response to dimethyloxallyl glycine, desferrioxamine, or CoCl₂ did not influence Cezanne expression (Online Figure III), and silencing of HIF1α or HIF2α did not suppress Cezanne expression in hypoxic ECs (Online Figure IV), indicating that induction of Cezanne by hypoxia is HIF independent. By contrast, pharmacological inhibition of p38 using either CT8730 or SB202190 (Figure 1E and 1F) or gene silencing of p38α (Figure 1G) suppressed the induction of Cezanne by hypoxia, indicating that p38 positively regulates Cezanne expression. We examined the mechanism of Cezanne induction by p38, which is known to influence gene expression via members of the ATF family and also by enhancing mRNA stability. We observed that ATF2 was phosphorylated in response to hypoxia in both HUVECs and microvascular ECs (Figure 2A). Thus, gene silencing studies were performed to assess the function of ATF2 in hypoxic ECs. We observed that silencing of ATF2 using 2 different siRNA sequences suppressed the induction of Cezanne by hypoxia (Figure 2B). By contrast, transfection using nontargeting scrambled sequences or using sequences that target an irrelevant mRNA (Src homology 2-containing protein tyrosine phosphatase) demonstrated that transfection and mRNA processing, per se, did not alter Cezanne induction by hypoxia (Figure 2C). Finally, Cezanne expression was reduced by 2 different siRNAs designed to target Cezanne, thus, validating the quantitative real-time PCR readout (Figure 2B). Thus, we conclude that ATF2 positively regulates Cezanne expression in hypoxic ECs. p38 also enhanced the stability of Cezanne transcripts in hypoxic ECs because pharmacological inhibition of p38 using SB202190 destabilized Cezanne mRNA in actinomycin D chase experiments (Online Figure IV).
hypoxia-reoxygenation (compare 5 and 6, and 7 and 8). By
comparing 1 and 2) B to examine whether Cezanne regulates proin-
flammatory responses to hypoxia-reoxygenation. Silencing
of Cezanne using a pool of siRNA sequences from an alternative
source also significantly enhanced the induction of E-selectin,
VCAM-1, and intercellular adhesion molecule-1 in ECs
exposed to hypoxia-reoxygenation (Online Figure VI).

Because E-selectin, VCAM-1, and intercellular adhesion
molecule-1 are induced by NF-κB, we examined whether
Cezanne influences the activation of this transcription factor
in response to hypoxia. Exposure of ECs to hypoxia-reoxygenation
enhanced Ser536 phosphorylation of RelA NF-κB subunits but
did not influence IkBα stability or nuclear localization of NF-
κB (Figure 3B). Thus, hypoxia-reoxygenation activates NF-
κB in ECs through a noncanonical pathway that involves RelA
phosphorylation. Although silencing of Cezanne led to a modest
reduction in IkBα expression (Figure 3B, top), the biological
significance is questionable because there was no noticeable
effect of Cezanne siRNA on NF-κB nuclear localization
(Figure 3B, bottom). By contrast, silencing of Cezanne led to
a pronounced elevation in Ser536 phosphorylation of RelA in
cells exposed to hypoxia-reoxygenation (Figure 3B, middle),
indicating that endogenous Cezanne negatively regulates NF-
κB activation and proinflammatory transcriptional responses
under these conditions.

Cezanne Reduces Inflammation and Injury in
Response to Ischemia-Reperfusion
Our finding that Cezanne can be induced by hypoxia in vitro led
us to examine the expression of Cezanne in tissues exposed
to ischemia in vivo. We observed by quantitative reverse transcrip-
tase PCR (Figure 5A and 5C) and Western blotting (Figure 5B)
that Cezanne was induced in rat or murine kidneys exposed to
ischemia-reperfusion. Immunohistochemistry was performed
to assess the cellular localization of Cezanne expression in
ischemic tissues. This was performed exclusively using the
murine model, so that tissues from Cezanne<sup>GT/GT</sup> mice could be
used to control for staining specificity. Immunohistochemistry
using anti-Cezanne antibodies revealed staining in vascular
contrast, silencing of Cezanne enhanced the expression of in-
tercellular adhesion molecule-1 in ECs exposed to normoxia
or hypoxia-reoxygenation (Figure 3A, bottom). Silencing of
Cezanne using a pool of siRNA sequences from an alternative
source also significantly enhanced the induction of E-selectin,
VCAM-1, and intercellular adhesion molecule-1 in ECs
exposed to hypoxia-reoxygenation.

Cezanne Regulates NF-κB Activation by Hypoxia

We used a previously validated RNA interference method<sup>13</sup>
(Figure 2B) to examine whether Cezanne regulates proin-
flammatory responses to hypoxia-reoxygenation. Silencing
of Cezanne in HUVECs had no effect on basal levels of
E-selectin or VCAM-1 in unstimulated cells (Figure 3A,
compare 1 and 2) but enhanced their subsequent induction by
hypoxia-reoxygenation (compare 5 and 6, and 7 and 8). By

V). Thus, we conclude that p38 induces Cezanne through
both transcriptional and post-transcriptional mechanisms.

Cezanne Suppresses Inflammatory Activation in
Response to Hypoxia-Reoxygenation by Inhibiting
RelA Phosphorylation

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compare 1 and 2) but enhanced their subsequent induction by
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ECs, glomerular ECs, podocytes, and tubular epithelial cells in wild-type mice exposed to ischemia-reperfusion, whereas tissues from Cezanne<sup>GT/GT</sup> mice were negative (Figure 5D). Thus, we conclude that ischemia-reperfusion induces Cezanne in multiple cell types in the kidney, including ECs.

The influence of Cezanne on NF-κB activation, inflammation, and injury was assessed by comparing responses to ischemia-reperfusion in wild-type and Cezanne<sup>GT/GT</sup> mice. In the first instance, we demonstrated by quantitative reverse transcriptase PCR that Cezanne-reperfusion induces Cezanne in multiple cell types in the kidney, including ECs.

Inflammatory signaling activates multiple delayed negative feedback loops that precisely control the kinetics of NF-κB activation, NF-κB-dependent inflammatory activation in response to hypoxia-reoxygenation by reducing Lys63-polyubiquitin. To our knowledge, Cezanne is the first example of a deubiquitinating enzyme that controls inflammatory responses to hypoxia.

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Discussion

Recent studies have shown that the assembly of polyubiquitin chains on specific signaling intermediaries promotes signaling to NF-κB in response to several stimuli, including cytokines, microbial products, and hypoxia. Our previous studies revealed that Cezanne is a deubiquitinating enzyme that suppresses NF-κB activation in response to TNFα or interleukin-1 by removing polyubiquitin chains from signaling intermediaries. Here, we demonstrate that Cezanne can also inhibit NF-κB-dependent inflammatory activation in response to hypoxia-reoxygenation by reducing Lys63-polyubiquitination of TRAF6. To our knowledge, Cezanne is the first example of a deubiquitinating enzyme that controls inflammatory responses to hypoxia.
usually relies on additional factors that suppress NF-κB (e.g., viral infection). By analogy, although hypoxia can prime ECs for inflammatory activation, the expression and activity of Cezanne determines whether inflammatory signaling proceeds in response to reoxygenation. Given that Cezanne can be modulated by shear stress, TNFα, interleukin-1, and reactive oxygen species, future studies should address whether these factors influence inflammatory responses to hypoxiareoxygenation by altering the expression and activity of Cezanne.

We translated our in vitro findings to a murine model by demonstrating that renal inflammation and injury in response to ischemia/reperfusion is enhanced by genetic deletion of Cezanne. Although several articles have studied the function of Cezanne in vivo, we further characterized its expression and activity in response to ischemia/reperfusion.

**Figure 5. Cezanne was induced by renal ischemia in vivo.** A and B, Expression levels of Cezanne were assessed in left kidneys of male Fisher rats (n=4) that were exposed to ischemia followed by reperfusion. Basal levels of Cezanne were measured in the uninjured right kidney of each animal. A, Cezanne transcript levels were quantified in kidneys exposed to ischemia-reperfusion (6 hours) and uninjured kidneys by real-time polymerase chain reaction (PCR) and normalized by quantifying β-actin transcripts. B, Lysates from kidneys exposed to ischemia-reperfusion (6 hours) or uninjured kidneys were tested by Western blotting using anti-Cezanne antibodies or by using anti-α-tubulin antibodies to assess total protein levels. A representative blot and results from densitometry analysis are shown. C and D, Left kidneys of wild-type or CezanneGT/ GT mice (n=6 per group) were exposed to ischemia followed by reperfusion for 6 hours, whereas contralateral kidneys were uninjured. C, Cezanne transcript levels were quantified in kidneys exposed to ischemia-reperfusion and uninjured kidneys by real-time PCR, and normalized by quantifying β-actin transcripts. D, Cezanne expression was assessed by immunohistochemistry in kidneys exposed to ischemia-reperfusion. Positive staining was observed in wild-type tissues in vascular and glomerular ECs (arrows), in podocytes (arrowhead), and in epithelial cells.

**Figure 6. Cezanne reduced inflammation and injury in response to ischemia-reperfusion.** Left kidneys of wild-type or CezanneGT/ GT mice (n=6 per group) were exposed to ischemia followed by reperfusion for 6 hours, whereas contralateral kidneys were uninjured. A, Granulocytes were identified by naphthol AS-D chlorocacetate esterase staining. Representative images are shown, and positive cells are indicated (arrow heads; top). Granulocytes were counted manually, and data were pooled from multiple animals per group (bottom). B, Tissue sections were stained with hematoxylin and eosin before histological assessment. Representative images are shown with areas of acute tubular necrosis (arrows) and hemorrhage (arrow heads) indicated (top). Quantitative assessment of tissue injury was performed by 2 experienced renal researchers, and average scores are shown (bottom). 0 indicates healthy; 1, mild injury; 2, moderate injury; and 3, severe injury.
of Cezanne in cultured cells, this is the first demonstration that Cezanne protects against inflammation and injury in vivo. Because Cezanne was shown to be expressed in renal tissues, this is the first demonstration that Cezanne protects against inflammation and injury in vivo. However, we cannot rule out the possibility of systemic effects entirely because a recent study revealed that Cezanne influences B cell function. Immunohistochemistry revealed that Cezanne was expressed in vascular and glomerular ECs in ischemic kidneys as well as in epithelial cells. Thus, the function of Cezanne identified in ischemic kidneys may be attributable, in part, to its induction in ECs in response to hypoxia. The expression of Cezanne in ischemic tissues may also be related to inflammatory cytokines, which are known to induce Cezanne in cultured cells. Future studies using tissue-specific conditional knockout mice should now be performed to define the specific cell populations that are regulated by Cezanne during ischemia. Further studies are also required to examine whether Cezanne is a pleiotropic molecule that possesses divergent functions because baseline phenotyping studies revealed that Cezanne knockout mice have a reduced startle response and lower serum cholesterol levels (http://www.europhenome.org).

Our observation that endogenous Cezanne can reduce inflammation and injury in response to ischemia-reperfusion has important implications for therapeutic targeting of inflammation during transplantation of vascularized organs, bypass grafting, percutaneous coronary intervention of arteries, and other procedures that involve reperfusion. Future studies should examine the potential role of Cezanne induction in ischemic preconditioning, a procedure that is known to protect tissues from subsequent inflammation and injury in response to ischemia-reperfusion. In addition, strategies to enhance Cezanne expression (eg, pharmacologically) in ischemic tissues may inform the development of novel therapies to reduce ischemia-reperfusion injury.

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**Disclosures**

None.

**References**


**Figure 7.** Cezanne suppressed NF-kappaB activation and adhesion molecule expression in response to ischemia-reperfusion. Left kidneys of wild-type or CezanneGT/GT mice (n=6 per group) were exposed to ischemia followed by reperfusion for 6 hours, whereas contralateral kidneys were uninjured. A, Levels of VCAM-1 or E-selectin transcripts were quantified by real-time polymerase chain reaction. B, Lysates were tested by Western blotting using anti-RelA or anti-Ser536 phosphorylated RelA antibodies or by using anti-GAPDH antibodies to assess total protein levels. Representative blots and results from densitometry analysis are shown.
Although hypoxia is known to influence vascular inflammation, the molecular mechanisms that control this process are not well understood. Herein, we report that the ubiquitin-editing enzyme Cezanne controls inflammatory responses to hypoxia/reoxygenation in endothelial cells by forming a feed-forward inhibition pathway. Our studies reveal that Cezanne suppresses nuclear factor-κB signaling in response to ischemia/reperfusion by removing polyubiquitin chains from the signaling intermediary tumor necrosis factor alpha receptor–associated factor 6. In mice, inflammation and injury in response to ischemia/reperfusion were significantly enhanced by genetic deletion of Cezanne. These findings indicate a novel signaling pathway that could be targeted therapeutically to suppress inflammation in vessels exposed to ischemia/reperfusion and identify Cezanne as the first example of a deubiquitinating enzyme that regulates inflammatory responses to hypoxia/reoxygenation.
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Expanded methods

RNA interference.

Human coronary microvascular EC (HCMEC) and human dermal microvascular EC (HDMEC) were obtained commercially (Promocell). Human umbilical vein endothelial cells (HUVEC) were collected using collagenase. EC were cultured as described previously (1). RNA interference was carried out using small interfering (si)RNAs that specifically target Cezanne (5'-GAAUCUAUCUGCCUUUGGA-3'; Dharmacon14 or SMARTpool ON-TARGETplus Human Otud7b siRNA), ATF2 (Dharmacon or SMARTpool ON-TARGETplus Human ATF2 siRNA) or p38α (Dharmacon). Alternatively, they were transfected with non-targeting scrambled controls (Silencer Negative control # 1 siRNA; Ambion). To control for the possible effects of mRNA processing, cells were transfected with siRNA targeting SHP2 (Dharmacon) which is unlikely to be involved in TRAF signalling. Cell cultures that were 80% to 90% confluent were transfected with siRNA (1-5 mM final concentration) using the Neon transfection system (Life Technologies) following the manufacturer’s instructions and then incubated in growth medium without antibiotics for 48h before analysis.

Comparative real time PCR

Extraction and reverse transcription of total RNA and real-time PCR were carried out as described previously (1, 2). Transcript levels were quantified by comparative real-time PCR using PerfeCTa SYBR Green Supermix (Quanta Biosciences) and the CFX96 Real-Time PCR Detection System (BioRad) and gene-specific primers (see main body for details of primer sequences). Reactions were performed in triplicate. Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product. Adjustment for the amount of input RNA was achieved by measuring b-actin mRNA levels as we described previously (1, 2).
Supplemental References


Online Figure I

Analysis of Cezanne^{GT/GT} mice. (A) The murine Cezanne gene contains 13 exons. In Cezanne^{GT/GT} mice, exons 4-7 were replaced with a cassette that included a splice acceptor site, LacZ open reading frame, neomycin resistance gene (Neo) and polyA tail (pA). The expected product of transcription is depicted. (B) Splicing of the LacZ-Neo-pA cassette was investigated. RNA was extracted from kidneys exposed to ischemia (45min)-reperfusion (6h) from wild-type or Cezanne^{GT/GT} mice and converted to cDNA. Samples were amplified by PCR using a forward primer specific for exon 3 and reverse primers specific for either the LacZ-Neo-pA cassette (left panel) or exon 13 (right panel) and products were analysed by agarose gel electrophoresis. These data confirm that exon 3 is spliced to the LacZ-Neo-pA cassette in Cezanne^{GT/GT} mice.
Online Figure II

Hypoxia induced Cezanne protein in human microvascular EC

HMEC were exposed to hypoxia (4 h) or hypoxia followed by reoxygenation (1 h) or remained untreated. Cytosolic lysates were tested by Western blotting using anti-Cezanne antibodies and by using anti-α-tubulin antibodies to assess total protein levels. Data are representative of those obtained from 3 independent experiments.
Online Figure III

Cezanne expression was not induced by HIF agonists.
HUVEC were treated with varying concentrations of dimethyloxallyl glycine (DMOG), desferrioxamine (DFO) or CoCl$_2$ for 6 h. Cytosolic lysates were tested by Western blotting using anti-Cezanne or anti-HIF1 antibodies or by using anti-$\alpha$-tubulin antibodies to assess total protein levels. Data shown are representative of three independent experiments.
Online Figure IV

Induction of Cezanne by hypoxia did not require HIF.
HUVEC were treated with siRNA sequences that target HIF1α or HIF2α or with a scrambled, non-targeting sequence as a control. They were then exposed to hypoxia (4 h) or remained untreated (normoxia). Levels of HIF1α, HIF2α or Cezanne transcripts were quantified by real-time PCR. Data were pooled from 3 independent experiments.
Online Figure V

Hypoxia elevates Cezanne mRNA stability via p38.
HUVEC were exposed to hypoxia for 4 h and then treated with an inhibitor of transcription (actinomycin D) in the presence of a p38 inhibitor (SB202190 1 mM) or DMSO vehicle alone (Control). Cezanne mRNA levels were measured at various times by real-time PCR. Data shown are representative of 5 independent experiments.
Online Figure vi

Cezanne silencing enhanced inflammatory activation in response to hypoxia-reoxygenation.

HUVEC were treated with Cezanne-specific siRNA (si-Cezanne; SMARTpool) or with a scrambled, non-targeting sequence and were then exposed to hypoxia (4 h) or hypoxia (4 h) followed by reoxygenation (4-20 h) or remained untreated. Levels of E-selectin, VCAM-1 or ICAM-1 transcripts were quantified by real-time PCR. Data were pooled from three independent experiments.
Online Figure vii

Kinetics of TRAF6 modification in response to hypoxia/reoxygenation
HUVEC were treated with Cezanne-specific siRNA or with a scrambled, non-targeting sequence and were then exposed to hypoxia (4 h) or hypoxia (4 h) followed by reoxygenation (2-24 h) or remained untreated. TRAF6 immunoprecipitates were tested by Western blotting using antibodies that recognise Lys63-polyubiquitin. Data are representative of three independent experiments that gave closely similar results.
Online Figure viii

Baseline characterization of Cezanne expression in a panel of murine tissues.
Cezanne mRNA levels were assessed by real-time PCR in tissues and in whole blood. Data from four mice were pooled and mean values +/- standard deviations are shown.
Online Figure IX

Genetic deletion of Cezanne promoted injury and generated enhanced expression of kidney injury marker-1 in ischemic tissues. Left kidneys of wild-type or Cezanne$^{GT/GT}$ mice (n=6 per group) were exposed to ischemia followed by reperfusion for 6 h whereas contralateral kidneys were uninjured. (A) Tissue sections were stained with hematoxylin and eosin prior to histological assessment. Representative high magnification images are shown with areas of acute tubular necrosis (arrows) and haemorrhage (arrow heads) indicated. (B) Levels of kidney injury marker-1 were assessed by immunohistochemistry. Representative images are shown.