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# KRIT1 loss-of-function induces a chronic Nrf2-mediated adaptive homeostasis that sensitizes cells to oxidative stress: Implication for Cerebral Cavernous Malformation disease

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## ABSTRACT

*KRIT1* (*CCM1*) is a disease gene responsible for Cerebral Cavernous Malformations (CCM), a major cerebrovascular disease of proven genetic origin affecting 0.3–0.5% of the population.

Previously, we demonstrated that KRIT1 loss-of-function is associated with altered redox homeostasis and abnormal activation of the redox-sensitive transcription factor c-Jun, which collectively result in pro-oxidative, pro-inflammatory and pro-angiogenic effects, suggesting a novel pathogenic mechanism for CCM disease and raising the possibility that KRIT1 loss-of-function exerts pleiotropic effects on multiple redox-sensitive mechanisms.

To address this possibility, we investigated major redox-sensitive pathways and enzymatic systems that play critical roles in fundamental cytoprotective mechanisms of adaptive responses to oxidative stress, including the master Nrf2 antioxidant defense pathway and its downstream target Glyoxalase 1 (Glo1), a pivotal stress-responsive defense enzyme involved in cellular protection against glycative and oxidative stress through the metabolism of methylglyoxal (MG). This is a potent post-translational protein modifier that may either contribute to increased oxidative molecular damage and cellular susceptibility to apoptosis, or enhance the activity of major apoptosis-protective proteins, including heat shock proteins (Hsps), promoting cell survival.

Experimental outcomes showed that KRIT1 loss-of-function induces a redox-sensitive sustained upregulation of Nrf2 and Glo1, and a drop in intracellular levels of MG-modified Hsp70 and Hsp27 proteins, leading to a chronic adaptive redox homeostasis that counteracts intrinsic oxidative stress but increases susceptibility to oxidative DNA damage and apoptosis, sensitizing cells to further oxidative challenges. While supporting and extending the pleiotropic functions of KRIT1, these findings shed new light on the mechanistic relationship between KRIT1 loss-of-function and enhanced cell predisposition to oxidative damage, thus providing valuable new insights into CCM pathogenesis and novel options for the development of preventive and therapeutic strategies.

*Abbreviations:* AGEs, advanced glycation end-products; ARE, antioxidant-response element; AP, argpyrimidine (Nδ-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-l-ornithine); BBB, blood-brain barrier; Casp-3, Caspase-3; CCM, Cerebral Cavernous Malformation; CNS, central nervous system; COX-2, cycloxygenase-2; Cyt c, cytochrome c; EndMT, endothelial-to-mesenchymal transition; Glo1, Glyoxalase 1; hBMEC, human brain microvascular endothelial cells; HO-1, Heme oxygenase-1; Hsp, heat-shock protein; ICH, intracerebral hemorrhage; JNK, c-Jun NH2-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; KRIT1, Krev interaction trapped 1; MEF, mouse embryonic fibroblast; MG, methylglyoxal; Nrf2, nuclear factor erythroid 2-related factor 2; NVU, neurovascular unit; PTM, post-translational modification; ROS, reactive oxygen species; SOD, superoxide dismutase; TUNEL, TdT-mediated dUTP nick-end labeling

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## 1. Introduction

Cerebral cavernous malformation (CCM), also known as cavernous angioma or cavernoma, is a major vascular dysplasia occurring mainly within the central nervous system (CNS) and consisting of closely clustered, abnormally dilated and leaky capillaries [1,2]. CCM lesions have a prevalence of 0.3–0.5% in the general population and can appear as single or multiple (up to hundreds) mulberry-like vascular sinusoids of varying size, lined by a thin endothelium devoid of normal vessel structural components, such as pericytes and astrocyte end-feet. They can remain asymptomatic throughout life or result in clinical symptoms of various type and severity at any age, including recurrent headaches, focal neurological deficits, seizures, stroke and intracerebral hemorrhage (ICH) [1–3].

This cerebrovascular disease is of proven genetic origin, and may arise sporadically or is inherited as autosomal dominant condition with incomplete penetrance and highly variable expressivity, including wide inter-individual differences in lesion number, size and susceptibility to ICH even among family members of similar ages carrying the same disease-associated genetic defect [4]. Genetic studies have identified three disease genes, *KRIT1* (also known as *CCM1*), *CCM2* and *PDCD10* (also known as *CCM3*) [5], whose functions need to be severely impaired for pathogenesis. Indeed, most of the more than one hundred distinct causative mutations identified so far in these genes are loss-of-function mutations. In particular, mutations of the *KRIT1* gene account for over 50% of familial cases (up to 70% in Hispanic Americans) [5]. This gene encodes for a 736 amino acid protein that has been implicated in the maintenance of endothelial cell-cell junction stability and blood-brain barrier (BBB) integrity through the regulation of major cell structures and signaling mechanisms, including cadherin-mediated cell-cell junctions [6], integrin-mediated cell-matrix adhesion [7,8], Rho GTPase-mediated cytoskeleton dynamics [9,10], and TGFβ-driven endothelial-to-mesenchymal transition (EndMT) [11]. In addition, in recent years it has become clear that KRIT1 plays an important role in controlling signaling pathways involved in cell responses to oxidative stress and inflammatory events [12–21]. In particular, original findings demonstrated that KRIT1 loss-of-function is associated with increased intracellular levels of reactive oxygen species (ROS) and enhanced cell susceptibility to oxidative stress-mediated molecular dysfunctions and oxidative damage [15]. Moreover, subsequent findings showed that KRIT1 may exert a protective role against oxidative stress by limiting c-Jun-dependent redox pathways [16] and defective autophagy [18–20]. Accordingly, recent evidence in animal models has suggested that oxidative stress is linked to the pathogenesis of CCM disease and may play an even more critical role than previously described due to systemic effects [14]. Furthermore, growing data in cellular and animal models indicate that limiting ROS accumulation and oxidative stress via distinct approaches may contribute significantly in preventing or reversing CCM disease phenotypes [14,16–18,20,22].

Despite the significant progress in understanding CCM pathogenesis, no direct therapeutic approaches for CCM disease exist so far other than the surgical removal of accessible lesions in patients with recurrent hemorrhage or intractable seizures [3]. Moreover, specific pharmacological strategies are also required for preventing the *de novo* formation of CCM lesions and counteracting disease progression and severity in susceptible individuals, including CCM gene mutation carriers. Indeed, while the great advances in knowledge of physiopathological functions of CCM proteins have led to an explosion of disease-relevant molecular information, they have also clearly indicated that loss-of-function of these proteins has potentially pleiotropic effects on several biological pathways, thus bringing new research challenges for a more comprehensive understanding [20,21]. In particular, further investigation into the emerging role of KRIT1 in redox-sensitive pathways and mechanisms is required to gain a better understanding of the likely complex signaling networks underlying the physiopathological functions of this important protein, thus facilitating the development of novel strategies for CCM disease prevention and treatment.

A fundamental mechanism that governs cellular adaptive defense against endogenous and exogenous oxidative stress is the activation of the redox-sensitive transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2), which controls constitutive and inducible expression of a plethora of antioxidant responsive element (ARE)-driven genes involved in detoxification of reactive oxidants and maintenance of cellular homeostasis [23–25]. Nrf2 is in fact the master regulator of cytoprotective responses to counteract oxidative and electrophilic stress through the coordinated induction of major antioxidant and phase II detoxification enzymes. These cytoprotective pathways may in turn prevent apoptosis and enhance cell survival by attenuating oxidative damage, mitochondrial dysfunction, and inflammation, and increasing cellular defense and repair mechanisms, thus playing a critical role in protection against various diseases, including vascular diseases [25,26]. In particular, activation of the essential Nrf2/ARE antioxidant defense pathway and its key downstream target heme oxygenase-1 (HO-1) within the neurovascular unit (NVU) has been shown to protect the cerebral vasculature against oxidative stress-mediated BBB breakdown and inflammation in stroke [27,28].

Besides HO-1, Glyoxalase 1 (Glo1) is emerging among the major downstream targets of Nrf2 transcriptional activity as a crucial stress-responsive defense protein for cellular protection against both dicarbonyl glycation and oxidative stress [29]. Glo1 is an ubiquitous glutathione-dependent enzyme that plays a critical cytoprotective role in limiting intracellular accumulation and toxicity of methylglyoxal (MG), a highly reactive dicarbonyl compound that is inevitably formed as a by-product of metabolic pathways, such as glycolysis [30]. MG readily reacts with lipids, nucleic acids and proteins (particularly with nucleophilic groups on side chains of Arg, Lys and Cys residues) to form the heterogeneous family of advanced glycation end-products (AGEs) [31,32]. MG-derived dicarbonyl adducts exert complex pleiotropic effects on normal and pathologic processes in cells, including modulation of protein biological activity [33] and stability [34], and generation of ROS and oxidative stress [35,36], which may culminate in distinct biological outcomes [36–41]. In particular, supra-physiological accumulation of argpyrimidine (AP), a major AGE formed by spontaneous reaction between MG and protein arginine residues [40], has been shown to induce oxidative DNA damage and apoptosis [39–42]. However, post-translational modifications (PTM) by MG-derived AP can also enhance the functionality of fundamental stress-inducible proteins implicated in cellular recovery after exposure to damaging stimuli and protection against apoptosis, including heat shock protein 27 (Hsp27), thus playing an important role in cell survival [43,44]. Furthermore, there is also evidence that some MG-derived AGEs, including AP, are endowed with antioxidant properties [45]. These apparently divergent functions imply that MG, like other reactive species, may exert different or even opposite biological effects, depending on its levels and the cellular context. Interestingly, Glo1 and MG-derived AGEs have been shown to play major and complex roles in vascular physiology and pathophysiology, including the pathogenesis of brain microvascular endothelial barrier dysfunctions [46–48].

The present study was designed to assess the putative involvement of major regulators of cellular responses to oxidative stress, including Nrf2 and Glo1, in the emerging relationship between KRIT1 loss-of-function and enhanced cell sensitivity to oxidative challenges [21].

## 2. Materials and methods

### 2.1. Cell culture and treatment

KRIT1$^{-/-}$ and KRIT1$^{+/+}$ mouse embryonic fibroblast (MEF) cell lines were established from KRIT1$^{-/-}$ and KRIT1$^{+/+}$ E8.5 mouse embryos, respectively, whereas KRIT1 9/6 MEFs were obtained by infecting KRIT1$^{-/-}$ cells with a lentiviral vector encoding KRIT1 [15]. Cells were cultured at 37 °C and 5% $CO_2$ in Dulbecco's modified Eagle's medium