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REVIEW

Ubiquitous regulation of cerebrovascular diseases by ubiquitin-modifying enzymes

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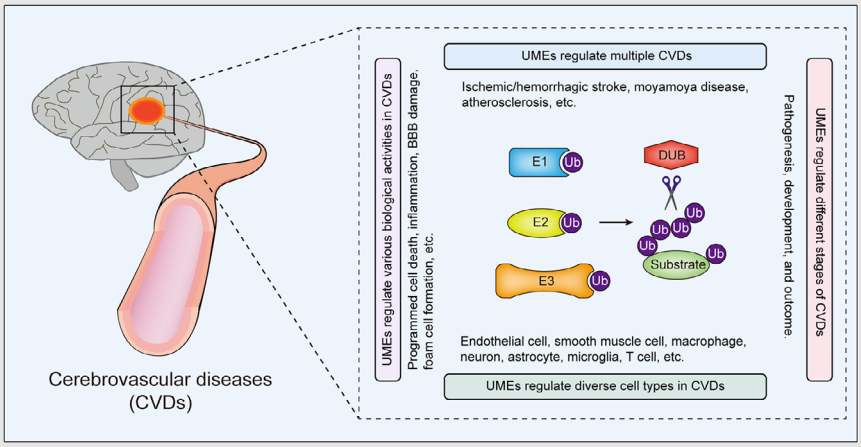
Graphical Abstract



1. Ubiquitin-modifying enzyme (UMEs), comprising E1, E2, E3 ubiquitinating enzymes and deubiquitinating enzymes, orchestrate ubiquitination and thereby critically regulate the pathophysiology of cerebrovascular diseases (CVDs).
2. Alteration in the abundance or activity of UMEs affects the outcome of CVDs.
3. UME-targeting therapy and therapeutic techniques applying UMEs may be beneficial for the treatment of CVDs.

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Abstract

Cerebrovascular diseases (CVDs) are a major threat to global health. Elucidation of the molecular mechanisms underlying the pathology of CVDs is critical for the development of efficacious preventative and therapeutic approaches. Accumulating studies have highlighted the significance of ubiquitin-modifying enzymes (UMEs) in the regulation of CVDs. UMEs are a group of enzymes that orchestrate ubiquitination, a post-translational modification tightly involved in CVDs. Functionally, UMEs regulate multiple pathological processes in ischemic and hemorrhagic stroke, moyamoya disease, and atherosclerosis. Considering the important roles of UMEs in CVDs, they may become novel druggable targets for these diseases. Besides, techniques applying UMEs, such as proteolysis-targeting chimera and deubiquitinase-targeting chimera, may also revolutionize the therapy of CVDs in the future.

KEYWORDS
cerebrovascular disease, deubiquitinating enzyme, disease mechanism, therapeutic target, ubiquitinating enzyme, ubiquitination

1 | INTRODUCTION

As the "central processing unit" of the body, the brain needs a relatively large amount of energy to maintain its sophisticated functionality. Although its weight accounts for only 2% of the body weight, the brain consumes about 20% of the human body's oxygen and glucose supply.[1,2] Unlike other major energy consumers of the body, such as the liver, the brain has barely any reservation of energy materials. Therefore, the cerebrovascular system is the only important source of glucose and oxygen for the brain. Cerebral blood flow, which is mainly supplied by internal carotid arteries and vertebral arteries, accounts for up to 20% of total cardiac output.[3,4] Given the importance of the cerebrovascular system in brain energy supply, interruption of the cerebral blood supply leads to termination of cerebral electrical activity and irreversible brain damage within minutes. Chronic cerebrovascular diseases (CVDs), such as moyamoya disease (MMD) and intracranial atherosclerotic disease, cause insufficient blood supply to the brain, inciting symptoms including headache, visual disturbance, paresthesia, motor nerve dysfunction, mental abnormality, and cognitive impairment.[5–9] These chronic CVDs and other factors, for example, hypertension, can also trigger life-threatening acute cerebrovascular accidents such as ischemic and hemorrhagic stroke.[5,6,8] CVDs have become the main cause of disability and mortality in both developing and developed countries.[10]

Accumulative studies have shown that the pathological processes of CVDs are closely regulated by ubiquitination, a post-translational modification (PTM) that is widely involved in signal transduction, inflammatory responses, metabolism, and cell fate determination.[11–17] In the process of ubiquitination, ubiquitin, a 76-amino acid small protein, is covalently conjugated to a lysine residue of the substrate protein under the sequential catalysis of ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s)[18] (Figure 1A). First, an E1 hydrolyzes ATP and forms a thioester bond between the sulfhydryl group of its active cysteine and the carboxyl group of the ubiquitin C-terminal glycine. Second, the activated ubiquitin is attached to an active cysteine of the E2 through a new E2-ubiquitin thioester bond. Then, the E2-ubiquitin complex interacts with an E3, which recognizes the protein substrate to mediate the final ubiquitin transfer.[18] E3s containing the really interesting new gene (RING) domain catalyze the direct transfer of ubiquitin from the E2 to the substrate. In contrast, E3s belonging to the RING-between-RING (RBR) and homologous to the E6AP carboxyl terminus (HECT) subfamilies form a ubiquitin-E3 thioester intermediate before transferring the ubiquitin to the substrate[18,19] (Figure 1A). According to the type of ubiquitin attachment, ubiquitination can be classified as polyubiquitination, monoubiquitination, and multi-monoubiquitination (Figure 1A). In polyubiquitin chains, ubiquitin monomers are linked together via isopeptide bonds between the C-terminal glycine carboxyl group of one ubiquitin and the amino group of the N-terminal methionine residue (M1) or any one of the internal lysine residues (K6, K11, K27, K29, K33, K48, K63) in the next ubiquitin.[20] Of note, polyubiquitin chains can be added to substrates by "sequential addition" or "en bloc transfer." In the sequential addition, individual ubiquitin monomers are transferred stepwise to the end of a growing polyubiquitin chain. By contrast, in the en bloc mechanism, a pre-assembled polyubiquitin chain is transferred as a whole to a substrate.[21] Functionally, ubiquitination controls the localization, activity, stability, or binding partners of protein substrates. As a reversible PTM, ubiquitination is antagonized by deubiquitinating enzymes (DUBs), which are divided into seven subfamilies based on sequence and domain conservation: ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), ubiquitin C-terminal hydrolases (UCHs), Machado–Josephin domain-containing proteases (MJDs), JAB1/MPN/MOV34 family (JAMMs), motif interacting with ubiquitin-containing novel DUB family (MINDYs), and zinc finger with UFM1-specific peptidase domain protein (ZUFSP/ZUP1).[22,23] Mostly, DUBs directly remove ubiquitin molecules from protein substrates (Figure 1B). However, OTUB1, a DUB of the OTU subfamily, possesses a non-canonical function that enables it to obstruct the ubiquitination of protein targets by blocking ubiquitin transfer from E2s[24–26] (Figure 1C). Of note, the removed ubiquitin molecules from substrates will be recycled for new ubiquitination processes. Besides, DUBs can also enrich the free ubiquitin pool by cleaving ubiquitin precursors encoded by *UBA52*, *RPS27A*, *UBB*, and *UBC* to release free ubiquitin molecules[27] (Figure 1D).

Ubiquitin-modifying enzymes (UMEs) comprising E1s, E2s, E3s, and DUBs orchestrate the diverse and exquisite ubiquitination of target proteins to precisely control their cellular localization, protein interaction, enzymatic activity, and degradation, thereby affecting the initiation and/or outcome of brain diseases. For example, loss-of-function mutations in the *TRIM37* gene, which encodes the E3 ligase TRIM37, cause the Mulibrey (muscle–liver–brain–eye) nanism.[28,29] In the scope of CVDs, the gene encoding the E3 ligase RNF213 has been identified as the most prominent susceptibility gene for MMD.[30–34] In this review, we elaborate on the functional roles and molecular mechanisms of UMEs in CVDs and discuss the possibility of exploring UMEs as therapeutic targets for these diseases.

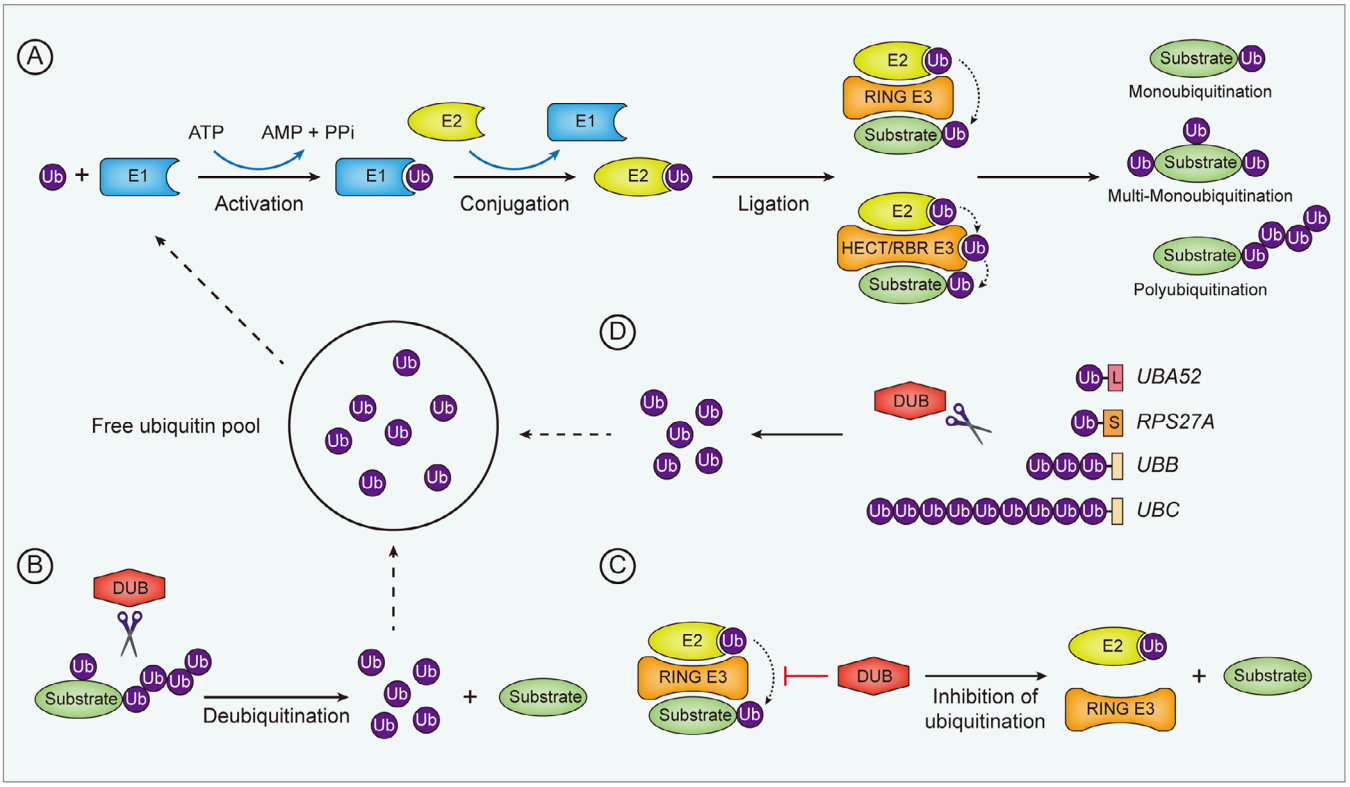

FIGURE 1 Overview of the process of ubiquitination and deubiquitination. (A) Ubiquitination is an enzymatic cascade catalyzed sequentially by E1s, E2s, and E3s. Ubiquitin is first activated by an E1 and subsequently transferred to an E2. An E3 mediates the final attachment of the ubiquitin molecule to a protein substrate. In contrast to HECT and RBR E3s, which receive ubiquitin molecules from E2s before transferring to substrates, RING E3s mediate the transfer of ubiquitins directly from E2s to substrates. (B,C) Ubiquitination is inhibited by DUBs, which either directly remove ubiquitins from substrates (B) or block the ubiquitination process. (D) DUBs cleave ubiquitin precursor proteins to release free ubiquitin molecules, which are further used for ubiquitination.

1.1 | UMEs regulate cerebral injury after ischemic stroke

Stroke is the second leading cause of death and disability worldwide. Around the world, there are more than 12 million new stroke cases per year and approximately 100 million people are living with the aftermath of a stroke.[10] Stroke is caused by infarction or rupture of a blood vessel in the brain, and accordingly can be classified into ischemic stroke and hemorrhagic stroke, with the former accounting for nearly 80% of all stroke cases.[35,36] Thrombolytic therapy with tissue plasminogen activator (tPA) and mechanical thrombectomy are two common ways to restore blood supply after ischemic stroke.[37] However, blood reperfusion can cause secondary brain damage known as cerebral ischemia/reperfusion (CI/R) injury. Multiple mechanisms, such as calcium overload, excitotoxicity, mitochondrial damage, oxidative stress, blood-brain barrier (BBB) disruption, and inflammation, are involved in CI/R injury, culminating in neuronal cell death and neurological deficits.[38] The middle cerebral artery occlusion (MCAO) model, an animal model phenocopying key features of human ischemic stroke, is widely used to study the pathophysiology and treatment of ischemic stroke. With the MCAO model, regulatory functions of UMEs in ischemic stroke injury have been revealed. Recent studies have shown that UMEs affect the injury in ischemic stroke predominantly by regulating neuronal death, axonal function, neuroinflammation, BBB integrity, and mitochondrial dysfunction.

1.1.1 | UMEs regulate neuronal death after ischemic stroke

Neuronal cell death is the basic pathophysiology of stroke, and the ischemic insult sequentially induces two major forms of programmed cell death, that is, necroptosis and apoptosis[39] (Figure 2). Necroptosis is a regulated necrosis that is rapidly induced in neurons shortly after cerebral ischemia.[39] In response to necroptosis-inducing stimuli, receptor-interacting protein kinase 1 (RIPK1) undergoes a conformational change and recruits RIPK3 to form a RIPK1/RIPK3 oligomer. In this kinase complex, RIPK3 is

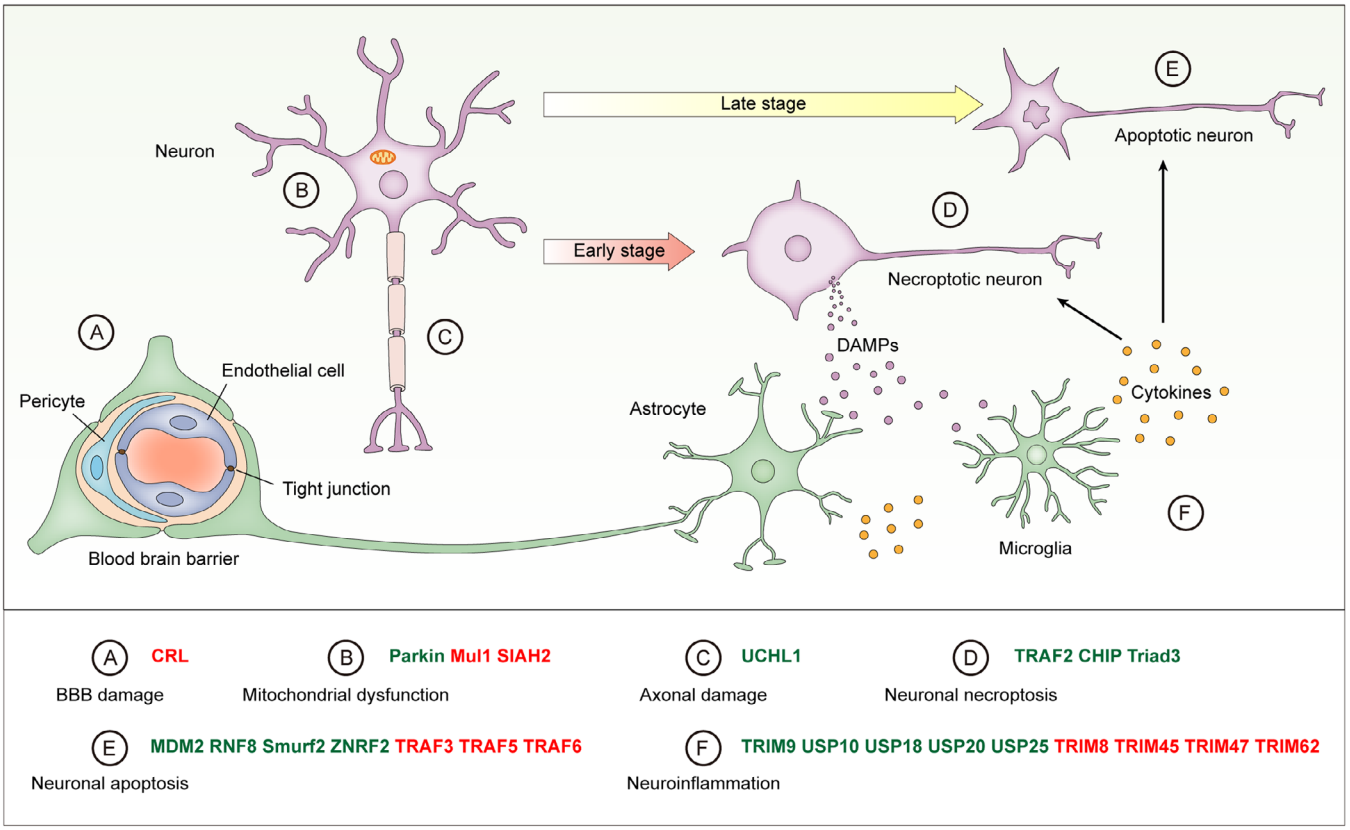

FIGURE 2 The role of UMEs in ischemic stroke. Ischemic stroke induces BBB damage, mitochondrial dysfunction, axonal damage, and neuronal death. Shortly after cerebral ischemia/reperfusion, neurons undergo necroptosis, leading to the release of DAMPs including S100A8/A9 and HMGB1. These DAMPs stimulate adjacent astrocytes and microglia to produce pro-inflammatory cytokines. Neuroinflammation in turn promotes neuronal necroptosis in a positive feedback loop and instigates neuronal apoptosis in the late stage. UMEs influence ischemic stroke injury by regulating BBB damage (A), mitochondrial dysfunction (B), axonal damage (C), neuronal necroptosis (D), neuronal apoptosis (E), and neuroinflammation (F). UMEs inhibiting ischemic stroke injury are in green and UMEs promoting ischemic stroke injury are in red.

activated and then phosphorylates the effector molecule mixed lineage kinase domain-like protein (MLKL).[40] Phosphorylated MLKL forms oligomers that translocate to the plasma membrane. At the membrane, MLKL undergoes conformational changes, leading to rapid breakage of the cell membrane and cell death.[41,42] Due to membrane permeabilization, necroptotic cells leak intracellular contents comprising damage-associated molecular patterns (DAMPs), which further activate innate immune responses to incite inflammation[43] (Figure 2). Inhibition of necroptosis has been shown to be neuroprotective in mice subjected to MCAO.[44,45] Necroptosis is inhibited by E3 ligases TRAF2, CHIP, and Triad3, and they can mitigate cerebral ischemic injury by attenuating necroptosis and neuroinflammation.[46–48] Consistently, viral vector-mediated overexpression of CHIP has been shown to prevent neuronal cell death after cerebral ischemia.[49]

In mice, MCAO-induced neuronal cell death undergoes the transition from necroptosis to apoptosis over time, and apoptosis becomes the main type of neuronal cell death following the initial necroptosis[39] (Figure 2). Ischemia-induced neuronal apoptosis is inhibited by UMEs such as MDM2, RNF8, Smurf2 and ZNRF2, and enhanced by TRAF3, TRAF5, and TRAF6.[11,50–55] Cerebral ischemia induces the upregulation of MDM2, an E3 ligase that negatively regulates p53 via both repressing p53 target gene transcription and ubiquitinating p53 for degradation.[50] Consistent with studies showing that p53 promotes stroke-induced apoptosis and affects functional recovery after stroke,[56,57] the single-nucleotide polymorphism of the *MDM2* gene (SNP309T > G), which enhances MDM2 expression, is associated with better functional outcomes in patients with ischemic or hemorrhagic stroke.[50] RNF8 is an E3 ligase that is involved in DNA damage repair via histone ubiquitination, and ablation of RNF8 leads to DNA damage accumulation and neuronal apoptosis.[58,59] In mice subjected to MCAO, RNF8 plays a neuroprotective role by inducing the ubiquitination and degradation of HDAC2, which enhances oxygen-glucose deprivation (OGD)-induced neuronal apoptosis via regulating

GSK3β activation.[51] Smurf2 is another E3 ligase that can inhibit neuronal apoptosis induced by cerebral ischemia and OGD, and overexpression of Smurf2 reduces brain injury in mice subjected to MCAO.[11] Smurf2 ubiquitinates Yin Yang 1 (YY1) for proteasome-dependent degradation, thereby suppressing apoptosis via inactivating the apoptosis-inducing YY1/HIF1α/DDIT4 axis.[11] CI/R-induced neuronal apoptosis can also be inhibited by the E3 ligase ZNRF2, which inhibits apoptosis by preventing excessive autophagy, and overexpression of ZNRF2 attenuates cerebral injury in rats after MCAO.[52] In sharp contrast to the aforementioned apoptosis-inhibiting E3 ligases, several E3 ligases of the tumor necrosis factor receptor-associated factor (TRAF) family, including TRAF3/5/6 can enhance CI/R-induced neuronal apoptosis.[53–55] For example, TRAF6 potentiates CI/R-induced neuronal apoptosis by K63 ubiquitinating and activating Rac1.[54]

1.1.2 | UME regulates axonal function after ischemic stroke

In addition to grey matter, white matter can also be injured by ischemic stroke.[60,61] UCHL1 is a neuron-specific DUB that is essential for axonal function.[14] After cerebral ischemia, UCHL1 is deactivated by reactive lipids, which bind to the C152 residue of UCHL1, leading to an impaired ubiquitin-proteasome pathway. However, the UCHL1 C152A mutant preserves the ubiquitin hydrolase activity in the presence of reactive lipids.[62] As compared with wild-type controls, the UCHL1 C152A knock-in mice show decreased accumulation of ubiquitinated proteins and axonal injury after MCAO, suggesting that UCHL1 plays a critical role in maintaining axonal function after ischemic stroke.[14]

1.1.3 | UMEs regulate neuroinflammation after ischemic stroke

Neuroinflammation is an indispensable component of the pathological machinery in ischemic stroke.[63] Shortly after ischemic stroke, DAMPs such as S100A8/A9 and HMGB1 are released from necroptotic cells. These DAMPs are recognized by microglia and astrocytes, two innate immune cell populations in the brain, through pattern recognition receptors, resulting in the production of pro-inflammatory cytokines and chemokines[39,64] (Figure 2). The post-stroke neuroinflammation is driven by various pro-inflammatory signaling pathways, in particular the nuclear factor-kappa B (NF-κB) pathway, which is tightly regulated by ubiquitination and UMEs.[63,65–68] Noteworthy, in response to pro-inflammatory stimuli, polyubiquitin chains catalyzed by UMEs provide large scaffolds to induce multi-protein structures comprising IκB kinases (IKKs), which serve as an upstream organizing center regulating NF-κB activation.[69,70] As such, multiple UMEs have been shown to influence ischemic stroke injury by regulating neuroinflammation (Figure 3). Ischemic stroke-induced neuroinflammation has been shown to be promoted by TRIM8,[71] TRIM45,[72] TRIM47[73] and TRIM62,[74] and inhibited by TRIM9,[13] USP10,[75] USP18,[76] USP20,[77] and USP25.[78] For example, after CI/R injury, microglia-mediated neuroinflammation and neurological deficit are enhanced by the E3 ligase TRIM45.[72] After OGD/R, TRIM45 catalyzes K63-specific polyubiquitination on TAB2, which is crucial for the phosphorylation of TAK1 and the subsequent activation of NF-κB signaling. Moreover, microglia-specific knockdown of TRIM45 significantly mitigates neurological deficit following CI/R injury in mice.[72] In sharp contrast to TRIM45, the DUB USP25 inhibits CI/R-induced K63 ubiquitination of TAB2 in microglia.[78] In both mice and humans, microglial expression of USP25 is upregulated in the ischemic penumbra.[78] USP25 physically interacts with TAB2 through the UIM2 domain and cleaves K63 polyubiquitin chains on TAB2. In mice, ablation of USP25 significantly exacerbated MCAO-induced cerebral deficits by enhancing neuroinflammation.[78] Ubiquitination and degradation of IκBα, the inhibitor that retains NF-κB in the cytoplasm in resting cells, is essential for the activation of NF-κB signaling. Of note, the ubiquitination of IκBα is induced by an E3 ligase complex comprising β-TrCP.[79] As a counter-regulating mechanism, the degradation of IκBα is inhibited by TRIM9, which competes with IκBα for β-TrCP interaction and thereby inhibits the ubiquitination of IκBα.[80] Upon ischemic stroke, TRIM9 inhibits NF-κB-mediated neuroinflammation by stabilizing IκBα, resulting in alleviated cerebral damage.[13]

1.1.4 | UMEs regulate BBB integrity after ischemic stroke

BBB disruption, characterized by loss of BBB junctional proteins and enhanced permeability, is another pathological process associated with cerebral ischemic stroke. Disrupted BBB subsequently leads to cerebral edema and neuronal cell death.[81] After ischemic stroke, BBB damage is promoted by the E3 ubiquitin ligase CRL, which induces the degradation of the protective protein neurofibromatosis 1 (NF1).[82] The activity of CRL is inhibited by the small-molecular inhibitor MLN4924, and treatment of mice with MLN4924 ameliorates ischemic brain injury by inducing the accumulation of NF1.[82] In addition to neuronal apoptosis, BBB disruption is also mitigated in TRAF5

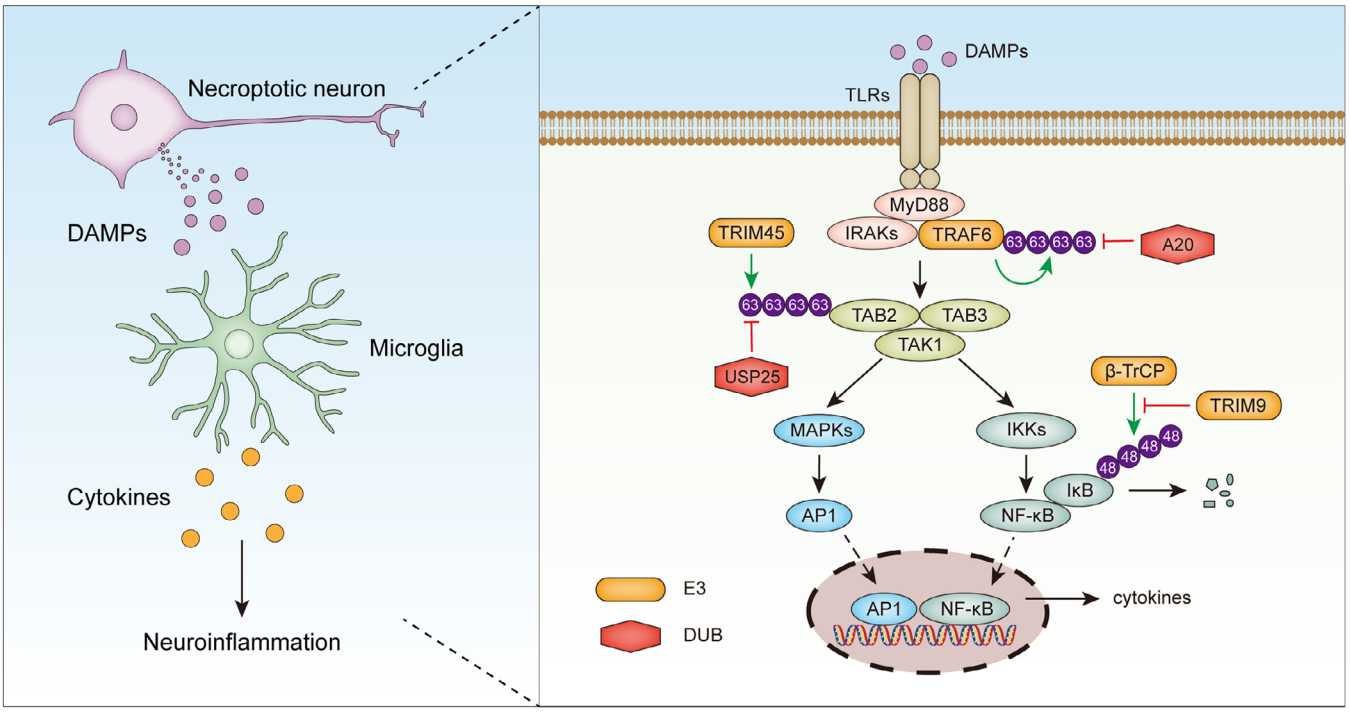

FIGURE 3 UMEs regulate neuroinflammatory signal transduction after ischemic stroke. DAMPs released from necroptotic neurons induce the production of pro-inflammatory cytokines in glial cells mainly by activating NF-κB and MAPK signaling pathways. E3s and DUBs tightly control the activity of these signaling pathways, thereby affecting neuroinflammation and ischemic stroke outcomes.

knockout mice after CI/R, indicating a role of TRAF5 in regulating BBB damage.[53]

1.1.5 | UMEs regulate mitochondrial dysfunction after ischemic stroke

Mitochondrial dysfunction is a key mechanism contributing to brain injury in ischemic stroke.[83] Mul1 is a mitochondrial membrane protein with dual E3 ligase functions in both ubiquitination and sumoylation, a ubiquitination-like PTM.[84,85] Mul1 is upregulated in the rat brain after MCAO, and it aggravates mitochondrial dysfunction by regulating the protein abundance of the mitochondrial fission protein Drp1 and the mitochondrial fusion protein Mfn2 through sumoylation and ubiquitination, respectively.[84] Knockdown of Mul1 ameliorates MCAO-induced brain injury by restoring protein abundance of Drp1 and Mfn2.[84] In response to OGD, the E3 ligase SIAH2 is activated in neurons, and it induces the ubiquitination and degradation of mitochondrial NCX3, a protein essential for mitochondrial integrity and neuronal survival during hypoxia. As compared with control neurons, SIAH2-deficient neurons show improved mitochondrial function under OGD conditions due to elevated NCX3 levels.[86] Another study demonstrated that SIAH2 could also aggravate ischemia-induced mitochondrial damage by inducing the ubiquitination and proteasomal degradation of AKAP121, a mitochondrial scaffold protein essential for mitochondria activity.[87] Therefore, the two studies jointly show that SIAH2 contributes to mitochondrial damage upon ischemic stress.[86,87] Selective mitochondrial autophagy, known as mitophagy, serves as a key mechanism in clearing damaged mitochondria and it is activated in ischemic brains.[88] Upon ischemic injury, the E3 ligase Parkin is recruited to the damaged mitochondria and ubiquitinates mitochondrial membrane proteins to trigger mitophagy.[89] In mice, Parkin-mediated mitophagy has been shown to be a key protective mechanism in CI/R injury.[88,90]

In aggregate, these studies show that, after ischemic stroke, UMEs impinge on cerebral injury by regulating a broad range of biological activities, implying that UMEs may serve as potential therapeutic targets for ischemic stroke.

1.2 | UMEs regulate cerebral injury after hemorrhagic stroke

Nearly 20% of stroke cases are hemorrhagic, with intracerebral hemorrhage (ICH) accounting for about 10–15%.[91,92] ICH causes neuronal cell death and neurological deficits by hematoma-associated mechanical damage and sec-

ondary injury mechanisms such as oxidative stress, mitochondrial dysfunction, neuronal excitotoxicity, calcium overload, neuroinflammation, and free radical production.[93] UMEs have been shown to affect the outcome of ICH by regulating many of these pathological processes.[94–98] The DUB A20, encoded by the *TNFAIP3* gene, serves as a brake on inflammatory responses via suppressing multiple pro-inflammatory signaling pathways, such as NF-κB and JAK-STAT signaling[66,99] (Figure 3). Mutations in or close to the *TNFAIP3* gene are associated with various autoimmune diseases including systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, and colitis.[66,100,101] ICH-induced inflammatory injury is also inhibited by A20, and overexpression of A20 ameliorates brain damage after ICH.[94] Moreover, in humans, A20 mRNA levels in peripheral blood mononuclear cells are negatively correlated with neurological deficits after ICH, indicating that A20 is a key suppressor for ICH injury.[94] Mitochondrial dysfunction and oxidative stress are inhibited by PGC-1α and enhanced by RNF34, an E3 ligase inducing the ubiquitination-mediated degradation of PGC-1α.[95,102] Overexpression of RNF34 exacerbates ICH-induced brain injury by promoting PGC-1α protein degradation and increasing oxidative stress and mitochondrial dysfunction.[95] Necroptosis is an important mechanism causing brain injury after ICH and it can be regulated by CHIP, which ubiquitinates the key component of necroptosis, RIPK3, for lysosomal degradation.[103–105] Overexpression of CHIP inhibits neuronal necroptosis and neuroinflammation in rats after ICH, resulting in reduced hemorrhagic lesions. Concordantly, CHIP deficiency leads to aggravated brain injury after ICH.[96] In addition to necroptosis, apoptosis is also induced by ICH, and it can be enhanced by the DUBs USP4 and USP11.[97,98]

Subarachnoid hemorrhage (SAH) is another subtype of hemorrhagic stroke, accounting for nearly 5—10% of acute stroke.[92,106] The E3 ligase RNF216, also known as Triad3A, has been shown to modulate synaptic plasticity in glutamatergic neurons by inducing the ubiquitination and degradation of Arc.[107] Upon SAH, RNF216 increases oxyhemoglobin-induced intracellular Ca^{2+} accumulation in neurons by restraining the Arc-AMPAR pathway, thereby promoting cytotoxicity and neuronal apoptosis. Moreover, the downregulation of RNF216 ameliorates brain injury following SAH.[108] In addition to RNF216, neuronal apoptosis induced by SAH is also enhanced by the E3 ligase TRAF3, which enhances SAH-induced NF-κB and MAPK signaling by activating TAK1.[109] Inflammation is a key pathological process contributing to early brain injury (EBI) following SAH. After experimental SAH, microglia upregulate the expression of Peli1, an E3 ligase that positively regulates neuroinflammation by promoting c-IAP2 ubiquitination and downstream inflammatory signaling in microglia.[65,110] Consistently, the knockdown of Peli1 reduces neuroinflammation and improves neurological outcomes during EBI after SAH.[110]

1.3 | UMEs regulate moyamoya disease (MMD)

MMD is an idiopathic cerebral vasculopathy characterized by progressive narrowing of the intracranial portion of the internal carotid artery and its main branches including the middle cerebral and anterior cerebral arteries.[7,111] In MMD, a hazy network of collateral arteries named moyamoya vessels develops around the occlusive region to compensate for the blood flow. MMD usually causes cerebral ischemia in pediatric and adult patients, but half of adult patients can also develop intracranial bleeding.[112] Therefore, MMD poses a key risk factor for both hemorrhagic and ischemic stroke.[111,112]

The annual incidence of MMD is as high as 0.5–1.5/100 000 in East Asian countries including China, Japan, and Korea, but as low as 0.1/100 000 in other parts of the world.[7] The difference in MMD prevalence is largely due to genetic susceptibility factors in East Asian populations. Indeed, *RNF213*, which encodes the E3 ligase RNF213, was identified as the principal susceptibility gene for MMD.[7,30,31] The heterozygous p.Arg4810Lys variant of *RNF213* has been identified as a founder mutant present in East Asian MMD patients.[30,32–34] Around 1.5% of the population of South Korea and Japan carry this variant, but it is rarely found in Caucasians, which may explain, at least partly, the nearly tenfold higher frequency of MMD in East Asian countries than in other regions.

RNF213 is the biggest E3 ligase in the human proteome with a mass of 591 kD, consisting of an N-terminal stalk, a dynein-like ATPase core, and a C-terminal multidomain E3 module.[113] Since most of the pathological MMD variants map to the E3 module of RNF213, these MMD-related variants may disturb the E3 ligase activity of RNF213.[113] Consistently, a recent study found that proteins encoded by MMD-associated *RNF213* variants, including the most prevalent Arg4810Lys variant, had reduced ubiquitination activity, suggesting that decreased E3 ligase activity of RNF213 contributes to the pathogenesis of MMD.[114] A recent study found that ablation of RNF213 disrupted the barrier function of human cerebral endothelium in vitro, which could be a potential pathogenic mechanism causing MMD.[15] However, the exact biological functions and molecular mechanisms of RNF213 in MMD remain largely unclear. In the future, studies on macromolecular interactions, conformational dynamics, and biochemical functions of RNF213 may reveal the role and mechanism of

action of this E3 in MMD and accelerate the development of RNF213-targeting therapies for MMD.

1.4 | UMEs regulate atherosclerosis

Atherosclerosis is a chronic vascular disease resulting from the complex interplay between lipid metabolism and immune responses. Besides, atherosclerosis can also contribute to the development of other diseases of the circulation system, such as coronary artery disease, peripheral artery disease, and stroke.[6,115,116] The chronic build-up of atherosclerotic plaques in the sub-endothelial intimal layer of medium- and large-sized arteries causes stenosis and restricts blood flow to critical organs, particularly the brain. In addition, rupture of the atherosclerotic plaque leads to acute thrombo-occlusive events, including ischemic stroke.[117] The p.Arg4810Lys variant of *RNF213*, which represents the most prevalent genetic abnormality in East Asian MMD patients, is also closely associated with intracranial atherosclerosis and ischemic stroke.[118–121] Besides, this *RNF213* variant predisposes patients with symptomatic intracranial atherosclerosis to stroke recurrence.[122]

Atherosclerosis is a cholesterol-related disease caused by the deposition of lipoproteins, especially low-density lipoproteins (LDLs), in the intimal space of arteries (Figure 4). In the intima, LDLs are oxidized by free radicals to form oxidized LDLs (OxLDLs), which can be taken up primarily by macrophages through scavenger receptors (SRs).[123,124] Macrophages and vascular smooth muscle cells (VSMCs) engulfing excessive OxLDLs differentiate into foam cells, and the accumulation of foam cells contributes to the development of atherosclerotic fatty streaks and plaques.[125,126] In addition to driving the transition of macrophages and VSMCs to foam cells, OxLDLs as a group of metabolism-associated molecular patterns (MAMPs) can also promote atherosclerosis by triggering inflammation, which is a key pathological process underlying the pathogenesis and progression of atherosclerosis[123] (Figures 4 and 5). UMEs have emerged as key regulators of atherosclerosis and they affect the onset and progression of atherosclerosis by modulating endothelial cell function, foam cell formation, and vascular inflammation.

1.4.1 | UMEs regulate endothelial cell function in atherosclerosis

The atherosclerotic process begins with the accumulation of LDLs in the sub-endothelial space of arteries[127] (Figure 4A). Endothelial cell dysfunction, such as altered permeability and apoptosis, is the critical initial step in atherogenesis, and this process is tightly regulated by E3 ligases HRD1, MDM2, and WWP2.[128–130] The binding of OxLDLs with lectin-like oxidized LDL receptor-1 (LOX-1), the specific scavenger receptor for OxLDLs on endothelial cells, induces endothelial dysfunction and OxLDL uptake.[131] LOX-1 can be ubiquitinated by the E3 ligase HRD1 for degradation.[128] HRD1 expression is downregulated in human atherosclerotic intima and its overexpression attenuates OxLDL-induced apoptosis of endothelial cells by reducing LOX-1 abundance, indicating that decreased HRD1 expression induces endothelial dysfunction in atherosclerosis.[128] Oxidative stress is a primary driving factor in endothelial dysfunction. The E3 ligase MDM2 promotes OxLDL-induced mitochondrial damage and oxidative stress in endothelial cells.[129] MDM2 induces the UPS-dependent degradation of retinoid X receptor beta (RXRβ), a protein that plays a protective role in endothelial cells upon OxLDL stimulation. In LDLr$^{-/-}$ mice, pharmacological inhibition of MDM2 increases the protein abundance of RXRβ in the aorta and decreases the formation of atherosclerotic lesions.[129] In sharp contrast, the E3 ligase WWP2 can inhibit OxLDL-induced endothelial cell injury by antagonizing oxidative stress. Mechanistically, WWP2 ubiquitinates PDCD4 for degradation, thereby activating the antioxidant HO-1 pathway in endothelial cells. In ApoE$^{-/-}$ mice, overexpression of WWP2 ameliorates atherosclerosis by reducing oxidative stress and inflammation.[130]

1.4.2 | UMEs regulate foam cell formation in atherosclerosis

Foam cell formation is a hallmark of atherosclerosis (Figure 4B). A majority of foam cells are derived from macrophages, which ingest OxLDLs through scavenger receptors SR-A1 and SR-B2 (CD36)[124,132] (Figure 5A). Upon binding with OxLDLs, SR-A1 is K63 polyubiquitinated at the K27 residue, and this PTM facilitates SR-A1 internalization, OxLDL uptake, and foam cell formation.[133] The K63-linked polyubiquitination of SR-A1 is counter-regulated by the DUB USP9X (Figure 5A). Pharmacological or genetic inhibition of USP9X increases OxLDL-induced SR-A1 ubiquitination and internalization in macrophages. Furthermore, disrupting the interaction between SR-A1 and USP9X with a cell-penetrating peptide exacerbates atherosclerosis by increasing foam cell formation, showing that USP9X is an important beneficial regulator of atherosclerosis.[133] The other key scavenger receptor CD36 can also be ubiquitinated, and the ubiquitination of CD36 leads to its proteasomal degradation.[134,135] Of note, the ubiquitination and degradation of CD36 is inhibited by DUBs including USP10, USP14, and UCHL1[136–138]

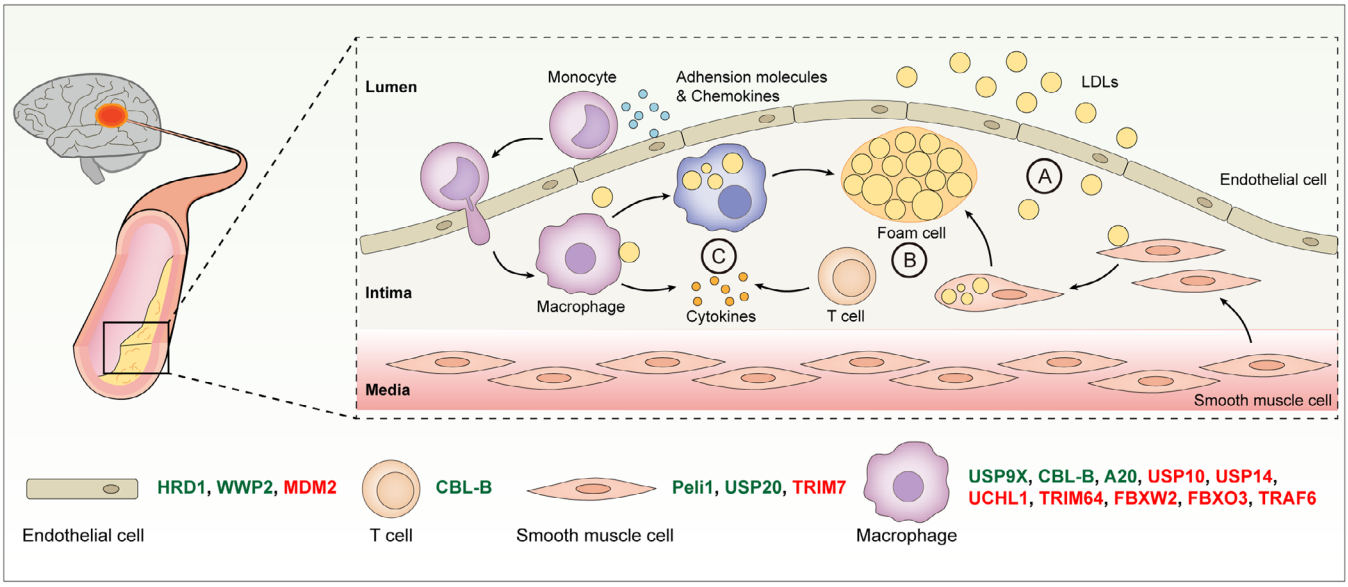

FIGURE 4 The role of UMEs in atherosclerosis. Atherosclerosis is a primary risk factor for stroke. During the initial stages of atherosclerosis, LDLs are transported across dysfunctional endothelial cells to the sub-endothelial space of arteries (A). LDLs in the artery intima are engulfed by macrophages through scavenger receptors. After ingesting overdose LDLs, macrophages laden with lipids become foam cells. Apart from macrophages, smooth muscle cells can also become foam cells after ingesting LDLs (B). Accumulation of foam cells further leads to the formation of atherosclerotic plaques. Besides, plaque formation is strongly promoted by pro-inflammatory cytokines produced by macrophages and T cells (C). UMEs can influence the pathogenesis and development of atherosclerosis by regulating various cell populations including endothelial cells, T cells, macrophages, and smooth muscle cells. UMEs inhibiting atherosclerosis are in green and UMEs promoting atherosclerosis are in red.

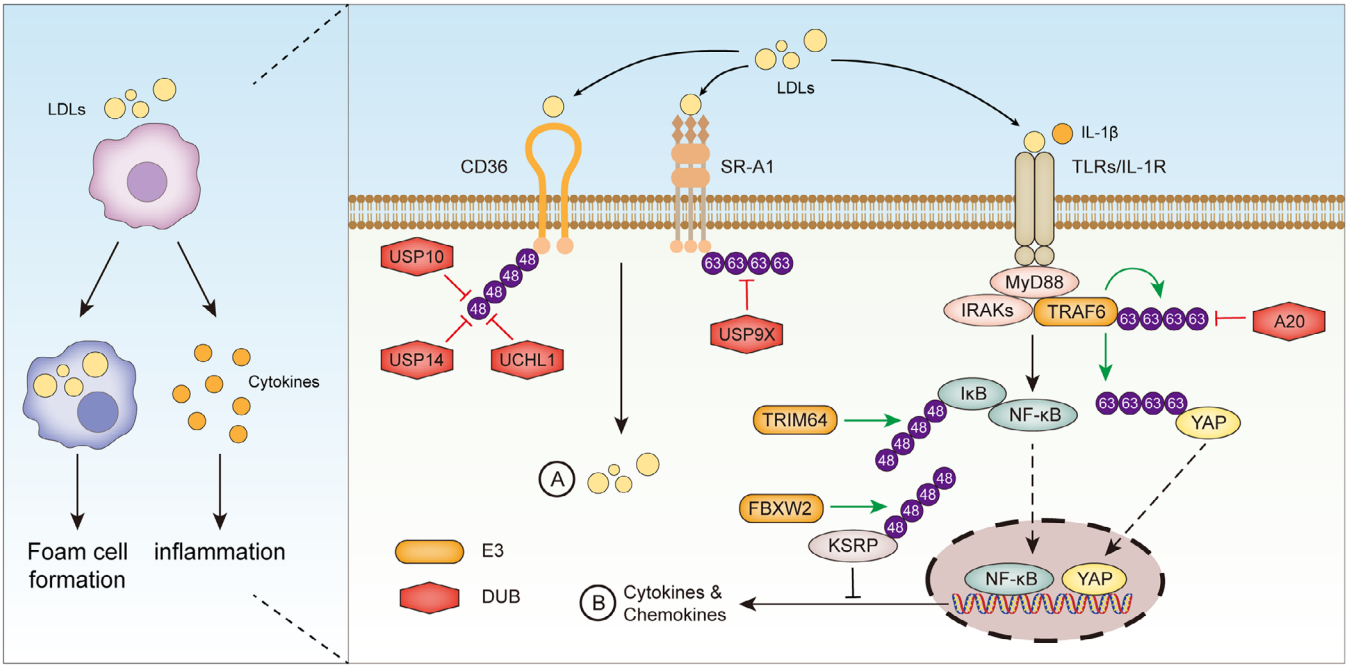

FIGURE 5 UMEs regulate macrophage functions in atherosclerosis. Macrophages are a key cell population promoting atherosclerosis. On the one hand, macrophages ingest LDLs to become foam cells (A). On the other hand, macrophages produce pro-inflammatory cytokines in response to LDLs (B). The two processes are closely controlled by UMEs.

(Figure 5A). Inhibition of USP10, USP14 or UCHL1 reduces CD36 protein abundance in macrophages and thereby diminishes OxLDL-induced foam cell formation.[136–138] Apart from macrophages, another important source of foam cells is VSMCs. The E3 ligase TRIM7 promotes the proliferation and migration of VSMCs in atherosclerosis, and the downregulation of TRIM7 alleviates atherosclerosis in ApoE$^{-/-}$ mice.[139] On the contrary, the E3 ligase Peli1 inhibits atherosclerosis progression by reducing inflammation and the transition of VSMCs to foam cells.[140]

1.4.3 | UMEs regulate inflammation in atherosclerosis

Atherosclerosis is characterized by continuous low-grade inflammation in the artery wall, and inflammation accelerates plaque expansion and destabilization[141,142] (Figure 4C). Macrophages are the predominant source of pro-inflammatory molecules in atherosclerosis and macrophage-mediated inflammatory responses are regulated by UMEs including A20, TRIM64, FBXW2, FBXO3, TRAF6[143–147] (Figure 5B). A20, a special UME with both DUB and E3 ligase activities, is an NF-κB inhibitor and critically regulates inflammatory responses in various diseases.[99,148,149] A20 was found to play a protective role in atherosclerosis by suppressing the expression of NF-κB target genes including cytokines and adhesion molecules.[143] As compared with control ApoE$^{-/-}$ mice, atherosclerotic lesions are increased in A20-haploinsufficient mice and decreased in A20-overexpressing mice.[143] In contrast, OxLDL-induced NF-κB-dependent inflammation in macrophages is promoted by the E3 ligase TRIM64, which enhances IκBα degradation by ubiquitinating IκBα at the K67 residue.[144] The E3 ligase FBXW2 is an F-box protein and acts as a substrate-binding component of the E3 ligase complex termed Skp1-Cullin-F-box protein (SCF) complex.[145] FBXW2 is upregulated in macrophages in atherosclerotic plaques. FBXW2 enhances the production of pro-inflammatory factors by mediating the ubiquitination and degradation of KSRP, an RNA-binding protein that negatively regulates the synthesis of a subset of cytokines and chemokines. Consistently, myeloid cell-specific ablation of FBXW2 mitigates atherosclerosis in mice, accompanied by reduced expression of pro-inflammatory factors in atherosclerotic lesions.[145] Another F-box protein of the SCF complex, FBXO3, can also promote atherosclerosis by enhancing inflammation.[146] FBXO3 is predominantly expressed in macrophages in human carotid atherosclerotic plaques, and FBXO3 depletion in macrophages diminishes OxLDL-induced inflammatory responses. Intriguingly, individuals carrying a hypo-functioning FBXO3 variant are less susceptible to atherosclerosis.[146] YAP is an essential signaling molecule of the Hippo pathway and it was recently shown to exacerbate atherosclerosis by promoting chemokine production in macrophages.[147] YAP expression is upregulated in macrophages in mouse and human atherosclerotic lesions, and myeloid cell-specific YAP overexpression aggravates atherosclerosis in mice. Upon stimulation with IL-1β, a key pro-inflammatory cytokine involved in atherosclerosis, YAP is K63 ubiquitinated by the E3 ligase TRAF6 at the K252 residue, leading to its protein stabilization and nuclear translocation. This study found that IL-1β enhanced YAP-mediated chemokine production in macrophages by activating TRAF6, highlighting a pivotal role of TRAF6 in the inflammation-driven progression of atherosclerosis.[147]

Akin to macrophages, T cells are a dominant immune cell type in atherosclerotic plaques and mediate the inflammatory responses underlying atherosclerosis[150,151] (Figure 4C). In both human and mouse atherosclerotic plaques, the E3 ligase CBL-B is mainly expressed in infiltrating macrophages and T cells.[152] CBL-B deficiency exacerbates vascular inflammation in mice by increasing the abundance and cytotoxicity of CD8$^+$ T cells as well as macrophage activation, resulting in aggravated atherosclerosis.[152] In addition to macrophages and T cells, VSMCs can also contribute to inflammation in atherosclerosis. The DUB USP20 has been shown to inhibit IL-1- and TNF-evoked inflammatory responses in VSMCs by deubiquitinating RIPK1. In vivo, specific overexpression of USP20 in VSMCs significantly reduces vascular inflammation and ameliorates atherosclerosis.[153]

Collectively, these reports demonstrate that UMEs regulate various key aspects in the pathogenesis and progression of atherosclerosis. Therefore, enhancing the beneficial functions and/or inhibiting the detrimental functions of UMEs may impede the progression of atherosclerosis, preventing the occurrence of more severe CVDs such as stroke.

1.5 | UMEs as therapeutic targets and tools

Considering that UMEs serve as versatile and critical regulators in CVDs, potent and specific UME inhibitors/agonists may become efficacious drugs for the prevention and treatment of CVDs. For example, the USP14 inhibitor IU1 has been shown to attenuate neurological deficits caused by ischemic stroke.[154,155] Since inhibition of USP14 diminishes foam cell formation, IU1 may also ameliorate atherosclerosis.[137] Of note, compared with E1, E2, and E3 ubiquitinating enzymes, DUBs are more favorable targets for the development of

small-molecule inhibitors.[156,157] In the past two decades, DUBs have emerged as novel drug targets for cancer and immune disorders.[158] In the foreseen future, UME inhibitors, particularly DUB inhibitors, may also enrich the therapeutic armamentarium for CVDs. Notably, in the NF-κB signaling, which critically regulates inflammation and cell death in multiple CVDs, stimulus-specific polyubiquitin scaffolds provide the docking sites for key upstream signaling molecules including IKKs.[69,70] In light of this, compared with the conventional "target-centric" inhibitors that inhibit single UMEs, "network-centric" inhibitors, which inhibit ubiquitin-mediated assembly of signaling complexes, may be more specific and effective.[159] In addition, given that UMEs tightly control the abundance, location, and activation of key proteins involved in CVDs, UMEs can also be applied to treat CVDs by precisely inhibiting detrimental proteins and enhancing beneficial proteins. Indeed, techniques based on UMEs, such as deubiquitinase-targeting chimera (DUBTAC) and proteolysis-targeting chimera (PROTAC), are gaining increasing attention as innovative therapeutic methods.[160–162] Therefore, UMEs may become novel drug targets and therapeutic tools, opening up new possibilities for the prevention and treatment of CVDs.

2 | CONCLUSION AND PERSPECTIVE

CVDs are a leading cause of disability and death in both developing and developed countries. In 2020, CVDs caused 7.08 million deaths worldwide, surging from 6.6 million deaths in 2019.[116,163] Recent studies have elucidated the pivotal roles of UMEs in CVDs, shedding light on the mechanism and therapy of these medical emergencies. Despite these advances, several critical aspects concerning UMEs in CVDs remain to be strengthened. First, more effects are needed to delineate the disease linkage of UMEs with CVDs. Inflammation is of particular importance in the progression of CVDs. Some UMEs, such as Pellino, are impactful regulators of inflammatory signaling, but their roles in CVDs remain largely unknown. Besides, no UME has been found to regulate cerebral small vessel diseases to date. It is intriguing and meaningful to identify new CVD-regulating UMEs. Second, the function of some UMEs in CVDs has yet to be clarified. Although RNF213 has been established as an essential protein in MMD, its exact function in MMD remains unclear. In the future, the in-depth investigation of new biochemical functions, interacting partners, and substrates of RNF213 may unravel the pathogenic mechanisms of MMD and inspire new therapies for MMD. Third, the clinical relevance of UMEs with human CVDs should be confirmed. Given that animal models cannot fully recapitulate human diseases and most studies have explored the function of UMEs in CVDs using animal models, these findings cannot be simply extrapolated to clinical situations. Comprehensive studies involving clinical research or humanized mice are more favorable for concluding the function of UMEs in CVDs. Fourth, the research and development of therapeutic approaches and drugs for CVDs based on UMEs should be accelerated. Despite recent advances, the study on UME inhibitors/agonists and PROTAC/DUBTAC is still in its infancy. Further studies in this burgeoning field may improve or even revolutionize the treatment for CVDs.

AUTHOR CONTRIBUTIONS

Conceptualization: Xu Wang. *Data collection and analysis*: Jingyong Huang, Zhenhu Zhu, and Xu Wang. *Writing-original draft*: Jingyong Huang, Zhenhu Zhu, and Xu Wang. *Writing-review and editing*: Jingyong Huang, Zhenhu Zhu, Dirk Schlüter, Kate Lykke Lambertsen, Weihong Song, and Xu Wang. *Funding acquisition*: Weihong Song and Xu Wang.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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