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Disturbed Flow-Induced Endothelial Proatherogenic Signaling *Via* Regulating Post-Translational Modifications and Epigenetic Events

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Abstract

Significance: Hemodynamic shear stress, the frictional force exerted onto the vascular endothelial cell (EC) surface, influences vascular EC functions. Atherosclerotic plaque formation in the endothelium is known to be site specific: disturbed blood flow (d-flow) formed at the lesser curvature of the aortic arch and branch points promotes plaque formation, and steady laminar flow (s-flow) at the greater curvature is atheroprotective. **Recent Advances:** Post-translational modifications (PTMs), including phosphorylation and SUMOylation, and epigenetic events, including DNA methylation and histone modifications, provide a new perspective on the pathogenesis of atherosclerosis, elucidating how gene expression is altered by d-flow. Activation of PKC ζ and p90RSK, SUMOylation of ERK5 and p53, and DNA hypermethylation are uniquely induced by d-flow, but not by s-flow. **Critical Issues:** Extensive cross talk has been observed among the phosphorylation, SUMOylation, acetylation, and methylation PTMs, as well as among epigenetic events along the cascade of d-flow-induced signaling, from the top (mechanosensory systems) to the bottom (epigenetic events). In addition, PKC ζ activation plays a role in regulating SUMOylation-related enzymes of PIAS4, p90RSK activation plays a role in regulating SUMOylation-related enzymes of Sentrin/SUMO-specific protease (SEN)2, and DNA methyltransferase SUMOylation may play a role in d-flow signaling. **Future Directions:** Although possible contributions of DNA events such as histone modification and the epigenetic and cytosolic events of PTMs in d-flow signaling have become clearer, determining the interplay of each PTM and epigenetic event will provide a new paradigm to elucidate the difference between d-flow and s-flow and lead to novel therapeutic interventions to inhibit plaque formation. *Antioxid. Redox Signal.* 25, 435–450.

Introduction

THE ENDOTHELIUM MODULATES a variety of biologic processes within the blood vessel wall, including active regulation of vascular tone and blood pressure through stimulation of nitric oxide, endothelin, and angiotensin II (84, 112, 125, 135); suppression of inappropriate activation of the coagulation system through production of antithrombotic factors (20, 121, 132); and regulation of cell proliferation and angiogenesis through secretion of various growth factors and vasoactive substances (31, 149, 150). Thus, endothelial cells (ECs) in general possess atheroprotective functions, indi-

cating that the endothelium plays a central role in the initiation and development of inflammatory atherosclerosis.

ECs covering the inner surface of vessels are continuously exposed to various forces as blood flows. Hemodynamic shear stress influences vascular pathologic conditions such as progression of atherosclerosis (18, 47, 49, 50), dilatation in the brachial artery (134), aneurysms (36), and arteriovenous malformations (68). Shear stress is imposed directly on the luminal surface of blood vessels, which is made up of a thin monolayer of ECs.

Various types of flows modulate endothelial structure and function through the activities of local mechanotransduction

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mechanisms, ultimately activating the shear stress response promoter elements and transcription factors that modulate endothelial gene expression (19, 57, 103, 133). For example, plaque formation is rare in areas exposed to steady laminar flow (s-flow). ECs exposed to s-flow (10–20 dyn/cm²) release factors that inhibit leukocyte inflammation, coagulation, and proliferation of smooth muscle cells while simultaneously promoting the survival of ECs (37, 114). In addition, some reports have indicated that s-flow increases secretion of NO, PGI₂, and tPA, which downregulate both thrombogenic and inflammatory cellular events (26, 27, 32, 74) and provide antiatherogenic effects.

In contrast, atherosclerotic plaques localize to areas of disturbed flow (d-flow) found at the lesser curvature of the aortic arch, as well as at vessel bifurcations and branch points. D-Flow is characterized as proatherogenic flow and it induces inflammation, apoptosis, proliferation, and reduction of vascular reactivity in the endothelium. The effects of d-flow are unique in that these four events are induced at the same time and location, which accelerates the turnover rate of ECs. This accelerated turnover rate of ECs may generate cells (daughter cells) that are already primed to respond to d-flow, eliciting a chronic atherogenic condition at the d-flow site (7, 54, 67, 136, 139). Thus, whereas s-flow protects against atherosclerosis, d-flow promotes atherosclerosis (39), and understanding how various signaling pathways in EC dysfunction are affected by d-flow and s-flow is crucial.

Epigenetics is the study of chromatin-based mechanisms that are important in the regulation of gene expression in a DNA sequence-independent manner (44, 104). Epigenetic events include DNA methylation (15, 23, 28, 76, 82, 113, 115) and histone modification (6, 8, 129). The use of epigenetic chromatin markers to identify shear stress response elements (SSREs) in the promoters of flow-responsive genes, as well as the idea that protein post-translational modifications (PTMs) play a critical role in the modulation of epigenetic states, has been suggested (28, 63, 143). However, how d-flow and s-flow regulate epigenetic events remains unclear. Our group and others have defined several signaling events involving kinases, SUMOylation, and DNA methylation, which are differentially regulated by d-flow and s-flow. Our group aims to determine how epigenetic events in the nucleus are differentially regulated by d-flow and s-flow. In this review, we focus on d-flow-induced signaling from the cell membrane cascade (mechanosensory systems) to the cascade in the nucleus (DNA modification events) and discuss the possible interplay among protein PTM events, including kinase-mediated phosphorylation, SUMOylation, DNA methylation, and histone modification.

To simplify the context, we divided the flow-induced signaling in the following four components from the top (mechanosensory systems at the cell surface) to the bottom (epigenetic events in the nucleus); Component 1: activation of PKC ζ and p90RSK (reactive oxygen species [ROS]-sensitive kinases) and inflammasome, Component 2: PKC ζ and p90RSK targeting of ERK5 and SUMOylation-related enzymes, Component 3: SUMOylation of ERK5 and p53 or PTMs of DNA methylation enzymes, and Component 4: modification of histones. We believe that the very different and unique physiological consequences induced by d-flow and s-flow are incited by the filtering of these four different

components (Fig. 1A). Therefore, to study the interplay among these four components would be crucial.

Mechanosensory Systems of d-Flow and s-Flow

The involvement of PECAM-1, VE-cadherin, VEGFR, and integrin receptor in mechanosensory systems of d-flow and s-flow, located in the cell membrane, has been reported. In particular, PECAM-1 has been established as a first-line mechanosensor (131). PECAM-1 is a type 1 transmembrane glycoprotein containing one transmembrane domain. It has six Ig-like homology domain in its extracellular region and two immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which recruit Src homology 2 (SH2) domain-containing proteins after phosphorylation of ITIM tyrosines (111). Interestingly, it has been reported that in the lesser curvature of the aortic arch (d-flow area), PECAM-1 can accelerate the formation of atherosclerotic lesions (126), whereas in the descending thoracic and abdominal aorta (s-flow area), PECAM-1 can reduce atherosclerotic lesions (41), suggesting that PECAM-1 may play a role as a mechanosensor for both d-flow and s-flow.

Osawa *et al.* have reported that direct application of mechanical force to PECAM-1 on the EC surface causes its tyrosine phosphorylation at these sites and recruits SH2 domain-containing proteins such as the SH2 domain-containing protein tyrosine phosphatase 2 (SHP-2) and subsequently activates ERK1/2 (109). Tzima *et al.* reported that VE-cadherin is associated with PECAM-1 at intercellular junctions, and once PECAM-1 is phosphorylated by flow, PECAM-1-associated VE-cadherin recruits VEGFR2, Src kinase phosphorylates VEGFR2, and phosphorylated VEGFR2 subsequently activates Akt and ERK1/2 kinases (131). However, the formation of the PECAM-1-VE-cadherin-VEGFR2 complex under the two different types of flows has not been well evaluated. Therefore, we expect that d-flow and s-flow take different binding partners with PECAM-1, and this may cause unique responses to each flow type. However, further investigation is needed to clarify this issue. There are many other proposed mechanosensors, including Piezo1 (Piezo-type mechanosensitive ion channel component 1) (83), p130 Crk-associated substrate (Cas) (120), and syndecan 4 (5). How these mechanosensors coordinately regulate flow signaling will also be the future challenge.

d-Flow Signaling After Mechanosensory Activity, Component 1: Activation of PKC ζ and p90RSK (ROS-Sensitive Kinases), and Inflammasome

The mechanosensory machinery localized on the surface of ECs, including caveolin/caveolae, integrins, and PECAM-1, can be activated by d-flow, which induces various proatherogenic responses in ECs (131). Especially, the contribution of d-flow-induced ROS production in regulating endothelial dysfunction and consequent atherosclerosis has been well established (34, 146). In this section, as the first component of d-flow signaling after sensing signals at the cell surface, we will discuss the role of ROS, as a second messenger, and how the mechanosensory machinery-mediated ROS production is involved in the initiation of proatherogenic responses by activating ROS-sensitive kinases. It has been well established that d-flow-induced ROS production,

FIG. 1. Primary structure of p90RSK and ERK5 and their regulation by shear stress. (A) Four major components and signaling events that are uniquely regulated by d-flow. (B) p90RSK are widely expressed Ser/Thr kinases characterized by two functional kinase domains, which are the N-terminal kinase domain belonging to the AGC group of kinase (*i.e.*, PKA and PKC) and the C-terminus kinase domain belonging to the calcium/calmodulin-dependent kinase group. Several phosphorylation sites both within and outside of the RSK kinase domain, including Ser221, Ser363, Ser380, tHR573, and S732, are important for kinase activation. (C) ERK5 is twice the size of other MAPKs and hence the largest kinase within its group. It possesses a catalytic N-terminal domain, including the MAPK-conserved threonine/glutamic acid/tyrosine (TEY) motif in the activation loop with 50% homology with ERK1/2, and a unique C-terminal tail, including transactivation domains. The activation of ERK5 occurs *via* interaction with and dual phosphorylation in its TEY motif by MEK. On the other hand, inflammatory stimuli or atheroprone flow (d-flow) leads to ERK5 deactivation *via* phosphorylation of Ser486 or Ser496, respectively. The N-terminus K6 and K22 sites with SUMO modification inhibit its own transactivation. (D) After ERK5 kinase activation induced by MEK5 binding or atheroprotective flow (s-flow) stimulation and TEY motif phosphorylation with deSUMOylation, ERK5 transcriptional activity at the C-terminus region is fully activated. In contrast, d-flow increases ERK5 SUMOylation and ERK5 Ser496 phosphorylation and inhibits ERK5 transcriptional activity. Reprinted and modified from Abe and Berk (1). eNOS, endothelial nitric oxide synthase; KLF, Kruppel-like factor; MAPKs, Mitogen-activated protein kinases; p90RSK, p90 ribosomal S6 kinase; PKA, protein kinase A; PKC ζ , protein kinase C- ζ ; PPAR, peroxisome proliferator-activated receptor; s-flow, steady laminar flow; SUMO, small ubiquitin-like modifier. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

loss of bioavailable NO production, and increased levels of ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and ONOO^- , are highly linked to the development of atherosclerosis (21, 40). ROS could increase PECAM-1 tyrosine phosphorylation and recruit SHP-2 (93). Among various ROS-producing enzymes, NADPH oxidase 4 has been identified as the major ROS-producing enzyme generated in blood vessels in response to d-flow (42).

We and other groups have found that the activity of two kinases, PKC ζ and p90RSK, is uniquely upregulated by their activity only under d-flow, and both kinases are sensitive to ROS, as explained below. PKC isozymes are serine/threonine kinases that phosphorylate multiple proteins, which in turn regulate intracellular signaling (22). The pseudosubstrate autoinhibitory sequence (amino acid [aa] 116–122) of PKC ζ binds the kinase domain (aa 268–587) and inhibits its

catalytic activity, and the release of the kinase domain from this autoinhibitory domain leads to PKC ζ activation (105, 124). PKC ζ was found to be highly activated in ECs in d-flow areas of porcine aortas (94). We also found that ONOO $^-$ played an important role in d-flow-mediated PKC ζ activation. ROS and NO can interact and generate ONOO $^-$, increasing nitrosative stress (55), and we and other groups have found that nitrosylation was increased in the d-flow area in mouse aortic arches (33, 51), where PKC ζ activation was increased. Although the direct effect of ONOO $^-$ on PKC ζ remains unclear, three upstream events may be involved in the activation of PKC ζ . First, ONOO $^-$ elicits protein tyrosine nitration, which can regulate a variety of kinases, including receptor tyrosine kinases (73, 87, 147). Second, ONOO $^-$ can activate Src by displacing Tyr527 from its binding site to the SH2 domain (118). Third, ONOO $^-$ can inhibit phosphatases *via* oxidation of cysteine-bound thiols (128).

p90RSK is a serine/threonine kinase containing two functional kinase domains (Fig. 1B) (35). The N-terminus kinase is a member of the AGC group of kinases (*i.e.*, PKA and PKC), and the C-terminus kinase is a member of the calcium/calmodulin-dependent kinase group. p90RSK is one of the downstream kinases of the Raf-MEK-ERK1/2 signaling pathway (10), and ERK1/2 activates the C-terminus kinase of p90RSK, leading to full activation of the N-terminus kinase and subsequent substrate phosphorylation. However, the involvement of an ERK1/2-independent pathway and a fyn kinase that regulates ROS-induced p90RSK activation has also been suggested (2). Recently, we reported that p90RSK is activated by d-flow, but not by s-flow (50). These findings suggest that d-flow-induced ROS production plays a crucial role in ROS-sensitive kinase, such as PKC ζ and p90RSK activation.

Xiao *et al.* reported the critical role of sterol regulatory element-binding protein 2 (SREBP2) in d-flow-mediated NADPH oxidase 2 (Nox2) expression and this could subsequently upregulate NLR family, pyrin domain containing 3 (NLRP3) inflammasome activation (144). D-flow induced the mature form of SREBP2 (SREBP2-N), which transcriptionally upregulated Nox2 and NLRP3 expression, thereby leading to interleukin-1 β expression and atherosclerotic plaque formation. Interestingly, SREBP can directly be phosphorylated by AMP-activated protein kinase (AMPK), which suppresses protein processing, nuclear translocation, and target gene expression. In addition, sirtuin 1 (SIRT1) can directly deacetylate SREBP-1c and increase its degradation (122). Of note, the specific activation of AMPK and induction of SIRT1 by s-flow have been reported (14). However, it is not clear whether s-flow-induced AMPK and SIRT1 induction inhibit SREBP2 activation and nuclear localization of SREBP2.

Further studies will be necessary to clarify, especially the following two issues: (i) how mechanosensory components such as PECAM-1/VE-cadherin/VEGFR are differentially regulated by d- and s-flow and (ii) how mechanosensory components can upregulate ROS production under d-flow.

d-Flow Signaling Component 2: PKC ζ and p90RSK Targeting of ERK5 and SUMOylation-Related Enzymes

Possibly, there are multiple downstream targets of PKC ζ and p90RSK. In this section, we will focus on two molecular targets of ERK5 and SUMOylation-related enzymes as the

downstream events of PKC ζ and p90RSK activation. The involvement of these two molecular events in d-flow-induced atherosclerosis formation is now getting evident.

ERK5

ERK5 is part of the mitogen-activated protein kinase family. ERK5 is unique in that it is not only a kinase but also a transcriptional coactivator with a unique C-terminus transactivation domain (Fig. 1C) (3, 70). s-Flow-induced ERK5 activation increases peroxisome proliferator-activated receptor γ transcriptional activity and Kruppel-like factor (KLF)2/4 expression, inhibits induction of inflammatory genes, and upregulates endothelial nitric oxide synthase (eNOS) expression (Fig. 1D) (3, 110). ERK5 regulates eNOS expression not only transcriptionally but also post-transcriptionally. In fact, we found that PKC ζ binds directly to ERK5 *via* the PKC ζ catalytic domain and phosphorylates ERK5 at S486 (106), which causes eNOS protein degradation, although the exact regulatory mechanism of eNOS degradation *via* the PKC ζ -ERK5 module remains unclear. We have also reported that p90RSK directly phosphorylates ERK5 S496 and inhibits ERK5 transcriptional activity (79).

Future studies will be necessary to clarify how d-flow-induced S486 and S496 phosphorylation coordinately regulates ERK5 function and ERK5 downstream events.

SUMOylation-related enzymes: PIAS4 and SENP2

Protein modifications with small ubiquitin-like modifier (SUMO) have been found to play a key role in d-flow-induced signaling and subsequent formation of atherosclerosis (46, 47, 50, 142). SUMO proteins covalently modify certain residues of specific target substrates and change the function of these substrates. Both conjugation and deconjugation enzymes mediate a dynamic and reversible process of SUMOylation (85, 145) (Fig. 2A). In particular, we found that PIAS4 (an SUMO E3 ligase) is regulated by PKC ζ , and Sentrin/SUMO-specific protease (SENP2; a deSUMOylation enzyme) is regulated by p90RSK. As explained above, release of the kinase domain from this autoinhibitory domain leads to PKC ζ activation (Fig. 2B) (105, 124). We found that PKC ζ activation induced by d-flow is associated with PIAS4 and increased PIAS4 SUMO E3 ligase function. The C-terminus kinase domain of PKC ζ (aa 401–587) was shown to be a PIAS4 binding site, and deletion of the N-terminus autoinhibitory domain (aa 1–200) was shown to increase PKC ζ -PIAS4 association (51). However, we could not detect PKC ζ -mediated PIAS4 phosphorylation using an *in vitro* kinase assay. Therefore, in addition to PKC ζ protein kinase activation, the subsequent release of the PKC ζ N-terminus autoinhibitory domain is necessary for the PKC ζ -PIAS4 association.

The PIAS family contains (i) an SP-RING domain containing SUMO E3 ligase activity; (ii) an SAP (scaffold attachment factor-A/B, apoptotic chromatin condensation inducer in the nucleus, and PIAS) domain, which leads to PIAS transcriptional repression activity; (iii) a Pro-Ile-Asn-Ile-Thr motif (PINIT) domain, which leads to nuclear retention; and (iv) an SUMO-interacting domain. PKC ζ associates with the catalytic site, the RING domain, of PIAS4, which forms the PIAS4/substrate complex by recruiting the cognate E2-conjugating enzyme to facilitate SUMO conjugation. Therefore, the association of

FIG. 2. The scheme of the SUMOylation pathway and regulation of PKC ζ to increase EC dysfunction by atheroprone flow. (A) Protein SUMOylation is achieved by a recycle system consisting of conjugation and deconjugation pathways (50, 51, 79, 106). SUMO proteins covalently modify certain residues of specific target substrates and change the function of these substrates. Both conjugation and deconjugation enzymes mediate a dynamic and reversible process of SUMOylation. First, the E1-activating enzymes, SAE1-SAE2 heterodimers, activate the mature form of SUMO (66). SUMO is then transferred to Ubc9, an E2 conjugase, forming a thioester bond between Ubc9 and SUMO (65). Last, SUMO E3 ligases, including a family of protein inhibitors such as activated STAT (PIAS1-4), regulate SUMO transfer to the target substrate containing the free ϵ -amino group of a lysine residue mediated by Ubc9 (64). DeSUMOylation enzymes are also involved in the process of SUMOylation. SENPs (SEN1-7) catalyze the deconjugation of SUMOylated substrates or edit the SUMO precursor into a matured form, which terminates with a pair of glycine residues (85, 145). Reprinted and modified from Woo *et al.* (140). (B) Atheroprone flow (d-flow) uniquely activates PKC ζ , which increases PKC ζ -PIAS4 binding at the SP-RING domain and PIAS4 SUMO E3 ligase activity, subsequently increasing p53 SUMOylation. PIAS, protein inhibitor of activated STAT; SAP, scaffold attachment factor-A/B, acinus, and PIAS domain; PINIT, Pro-Ile-Asn-Ile-Thr motif; SP-RING, Siz/PIAS-RING domain. Reprinted and modified from Abe and Berk (1). EC, endothelial cell; SENPs, Sentrin/SUMO-specific proteases. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

PKC ζ with PIAS4 may alter the structure and enzymatic activity of PIAS4. Depletion of PIAS4 from mouse embryonic fibroblasts inhibited p53-SUMOylation and retrovirus-associated DNA sequence-induced senescence (9). We will explain the role of p53-SUMOylation in d-flow signaling below.

In contrast to PIAS family enzymes, SENPs catalyze deconjugation of SUMOylated substrates. In addition, SENPs can edit the SUMO precursor into a matured form by terminating it with a pair of glycine residues (Fig. 2A) (145). SENPs contain a conserved C-terminus domain with the characteristic His-Asp-Cys catalytic triad, and the non-conserved N-terminus region of SENPs is thought to determine the subcellular localization and substrate specificity (71). Both the nuclear localization signal and the nuclear export signal exist in the SENP2 sequence.

In resting ECs, SENP1 forms a complex with an antioxidant protein, thioredoxin, in the cytoplasm. Tumor necrosis factor releases SENP1 from thioredoxin, and freed SENP1 translocates to the nucleus. This nuclear translocation process is specifically blocked by antioxidants such as N-acetylcysteine, suggesting that SENP1 nuclear translocation induced by TNF is ROS dependent (86). Therefore, it is possible that SENP1 has a significant role in mediating d-flow signaling, but the contribution of SENP1 in d-flow signaling remains under investigation. SENP2 is primarily localized in the nuclear envelope and is associated with the nuclear pore complex. SENP2 also has the nuclear localization signal and nuclear export signal within the N-terminus region, which enable SENP2 to shuttle between the nucleus and the cytoplasm. This shuttling is blocked by mutations in the nuclear

export signal (60), but the exact regulatory mechanism of SENP2 shuttling remains unclear.

We found that reduced SENP2 expression in *Senp2*^{+/-} mice accelerated EC inflammation and dysfunction by inducing SUMOylation of endothelial p53 and ERK5 (Fig. 3) (47). p53 and ERK5 SUMOylation elicits proatherogenic events, including apoptosis, inflammation, and reduction of eNOS, and details of the pathophysiologic role of p53 and ERK5 SUMOylation in the formation of atherosclerosis will be explained in a later section. We utilized a double knockout *Senp2*^{+/-}/*Ldlr*^{-/-} mouse model and examined the extent of plaque formation after feeding them with a high-cholesterol diet for 16 weeks. The lesion area in both the aortic arch and the descending aorta was significantly larger in *Senp2*^{+/-}/*Ldlr*^{-/-} mice than in *Senp2*^{+/-}/*Ldlr*^{-/-} mice. Interestingly, the extent of increase in the lesion size in the aortic arch of *Senp2*^{+/-}/*Ldlr*^{-/-} mice was much larger than that in the descending aorta. The acceleration of the lesion size induced by the depletion of SENP2 was much more significant in the aortic arch (d-flow area) than in the s-flow area, suggesting that SENP2 plays a key role in the formation of atherosclerosis under d-flow conditions. We found that SENP2 expression was not affected by different flow patterns (*i.e.*, d-flow and s-flow) (47). Therefore, we proposed that PTM of SENP2 contributes to the regulation of the deSUMOylation function in SENP2.

In fact, recently, we found that p90RSK could directly phosphorylate SENP2. Using liquid chromatography–tandem mass spectrometry, we identified T35, S38, and T368 as SENP2 sites phosphorylated by p90RSK (50). We mutated each phosphorylation site to alanine, expressed each mutant SENP2 separately in ECs, and determined the effect of each site on p90RSK-mediated p53 SUMOylation. Inhibition of p53 SUMOylation induced by wild-type (WT) SENP2 was completely lost in cells cotransfected with p90RSK. Among the three SENP2 phosphorylation site mutants, the SENP2-T368A mutant, but not the T35A and S38A mutants, was able to completely block this inhibitory effect. We also confirmed that T368 phosphorylation of endogenous SENP2 was induced by d-flow. This T368 phosphorylation was completely abolished by Ad-DN-p90RSK, indicating the crucial role of p90RSK in regulating d-flow-induced SENP2 T368 phosphorylation.

To determine the role of p90RSK in ECs *in vivo*, we generated EC-specific WT-*p90rsk* transgenic mice using mice expressing tamoxifen-inducible *Cre*-recombinase under the regulation of the *VE-Cad* promoter, herein referred to as WT-*p90rsk*-ETg mice (102). We found increased levels of SENP2-T368 phosphorylation and cleaved caspase 3 and also the expression of inflammatory adhesion molecules at both the protein and mRNA levels in mouse ECs isolated from WT-*p90rsk*-ETg mice compared with those from non-transgenic littermate control mice after 4-OHT injection. In contrast, eNOS expression was downregulated in the WT-*p90rsk*-ETg mice. This study revealed the key role of the p90RSK-SENP2 module in d-flow-induced EC signaling. Specifically, we found that p90RSK regulates SENP2 deSUMOylation function by phosphorylating SENP2-T368, and that modulating the function of p53 and ERK5 as we will discuss in the following section results in d-flow-induced EC inflammation, apoptosis, and consequent plaque formation (Fig. 3).

FIG. 3. Regulation of p90RSK-SENP2 to increase EC dysfunction by atheroprone flow. p90RSK regulates SENP2 deSUMOylation function by phosphorylation of SENP2 Thr368, modulating the function of p53 and ERK5. These result in d-flow-induced EC inflammation, apoptosis, and subsequent atherosclerotic plaque formation. The acceleration of the lesion size induced by the depletion of SENP2 crossed to *Ldlr*^{-/-} mice after 16 weeks on a high-cholesterol diet was much more significant in the aortic arch (d-flow area) than those in the s-flow area, suggesting the key role of SENP2 under d-flow. Aorta images were reprinted from Heo *et al.* (46). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

d-Flow Signaling Component 3: SUMOylation of ERK5 and p53, and PTMs of DNA Methylation Enzymes

In this section, we will discuss several SUMOylated molecules, including ERK5 and p53, and DNA methylation enzymes, which have been reported to have significant effects on d-flow-induced endothelial dysfunction and atherosclerosis formation.

ERK5 SUMOylation

As explained above, s-flow has a vasoprotective effect *via* increased ERK5-mediated KLF2 and eNOS expression (106, 142). Our studies showed that d-flow and ROS significantly increased SUMOylation at ERK5 Lys6 and Lys22 residues and that this SUMOylation inhibited ERK5/myocyte enhancer factor-2 (MEF2) transcriptional activity and subsequent KLF2 promoter activity and KLF2-mediated eNOS expression (142). Of note, ERK5 SUMOylation inhibited ERK5 transcriptional activity, but this was independent of ERK5 protein kinase activity. We also found that ERK5 K6/22R SUMOylation mutant abolished the ROS-induced reduction of eNOS and KLF2 expression in ECs, suggesting that ERK5 SUMOylation may downregulate the vasoprotective effects of s-flow (142). Furthermore, we found that ERK5 SUMOylation was increased by d-flow, but decreased by s-flow (47). These data strongly suggest the important role of ERK5 SUMOylation in regulating endothelial inflammation and vascular tone.

p53 SUMOylation

The transcription factor p53, as a sensor for DNA damage, is a key molecule in determining cellular fate. Of note, p53 in the nucleus not only increases the expression of proapoptotic genes but also protects against cell death by upregulating p21 expression (38). Lin *et al.* reported that s-flow increased p53 expression and JNK-mediated p53 phosphorylation, which caused EC growth arrest by increasing GADD45 and p21^{cip1} expression (88). These data suggest that the atheroprotective effect exerted by s-flow increases p21 and pRb signaling *via* p53, inducing growth arrest and possibly inhibiting apoptosis simultaneously, as previously described (4).

Most p53 antiapoptotic effects have been explained by the function of p53 in the nucleus, especially under resting conditions (38). In fact, p53 was localized in the nucleus and reduced the number of apoptotic ECs in the area exposed to s-flow (51), supporting this general idea. In contrast to this, ECs exposed to d-flow have increased levels of extranuclear p53 localization and become apoptotic. In addition, the nuclear export of p53 can promote cellular proliferation by losing its antigrowth effect in the nucleus. Therefore, the nuclear export of p53 *via* SUMOylation can explain how d-flow can promote both EC apoptosis and proliferation simultaneously.

We have reported that PKC ζ plays a role in regulating p53 SUMOylation and subsequent nuclear export (51). Previously, Carter *et al.* reported that p53 SUMOylation played a role in regulating p53 localization (12). When p53 is unmodified, the COOH-terminus nuclear export signal of p53 is masked by its C-terminus region and this causes persistent nuclear localization. Once p53 is monoubiquitinated, this exposes the nuclear export signal, prompting p53 to interact with PIAS4 and causing further modification by SUMOyla-

tion, which leads to p53 nuclear export. In addition, Santiago *et al.* reported that p53 SUMOylation at the nuclear pore complexes unlocks the CRM1 (chromosomal region maintenance 1) Huntingtin-EF3-PP2A subunit-TOR1 9 loop to facilitate the disassembly of the transporting complex and release of p53 to the cytoplasm (119). These results indicate that p53 nuclear export is regulated by SUMOylation (12).

It has been reported that cytoplasmic p53 directly interacts with members of the Bcl (B cell lymphoma/leukemia)-2 family proteins, Bcl-xL and Bcl-2, and attenuates the well-known antiapoptotic function of these proteins, supporting the idea that cytosolic p53 performs nontranscriptional proapoptotic activities (98, 99). We found that p53 nuclear export, p53-Bcl-2 binding, and apoptosis were induced by d-flow in a p53 SUMOylation-dependent manner (51). As described above, we discovered that d-flow upregulated PKC ζ binding to the E3 SUMO ligase PIAS4 and stimulated p53 SUMOylation and, subsequently, EC apoptosis (51). Taken together, these findings indicate the crucial role of PKC ζ activation and subsequent PKC ζ -PIAS4 binding in regulating d-flow-induced p53 SUMOylation and EC apoptosis (51).

d-Flow-induced upregulation of DNA methylation enzymes and DNA hypermethylation of d-flow-related gene promoters

Epigenetic events include DNA methylation (15, 23, 28, 76, 82, 113, 115), histone modification (6, 8, 129), and the actions of small and noncoding RNAs (53, 91, 97). DNA methylation subjects the 5-position of cysteine (the fifth base of DNA) to dynamic postsynthetic covalent modification (43). In mammals, more than 98% of DNA methylation occurs in cytosine-phosphate-guanine (CpG) dinucleotides. About 50% of gene promoters are associated with a cluster of unmethylated CpG dinucleotides from genomic DNA, allowing transcription. Methylation of cytosine within or near the promoter region results in gene transcriptional silencing either through interference with transcriptional factor binding or induction of a repressive chromatin structure (61, 138). During embryogenesis and cell differentiation, the DNA methylation status establishes properties of cell identity that are essential to defining the broad areas of development, and defective DNA methylation and hypermethylation (transcriptional silencing) of tumor suppressor genes in cancer stem cells are leading epigenetic characteristics of cell proliferation (24). The DNA methylation landscape of the genome is established by methylation and demethylation enzymes (Fig. 4).

The main enzymes regulating DNA methylation are DNA methyltransferases (DNMTs) encoded by different genes on distinct chromosomes: DNMT1, DNMT3A, and DNMT3B. *De novo* methylation during early embryonic development is catalyzed by DNMT3A and DNMT3B, which are crucial for the establishment of DNA methylation patterns, whereas DNMT1 ensures the maintenance of methylation through replication (108). Another methyltransferase, DNMT3L, which is related to DNMT3A and DNMT3B, lacks catalytic activity and is inactive, but is important for the stabilization of DNMT3A and associated assembly complexes during *de novo* methylation in germ cells (143). Methylation by DNMTs is counterbalanced by passive and active DNA demethylation, in which the ten-eleven translocation (TET)

FIG. 4. DNA methylation/demethylation enzymes. Methylation of the promoter regions of genes significantly suppresses transcription by direct inhibition of transcription factor binding and recruitment of methyl-CpG-binding proteins within their recognition sites of transcription factors. DNA methylation occurs at carbon 5 of cytosine (5-methylcytosine [5mC]) in CpG dinucleotides. DNMT1 maintains DNA methylation patterns during cell proliferation *via* methylation of a hemimethylated nascent DNA strand. DNMT3A and DNMT3B are required for genome-wide *de novo* methylation and play crucial roles in the establishment of DNA methylation patterns. Methylation by DNMTs is counterbalanced by DNA demethylation. TET oxidizes 5mC to 5-hydroxymethylcytosine (5hmC) and subsequently to 5-formyl cytosine (5fC) and 5-carboxy cytosine (5caC). The carboxyl group of 5caC is excised by TDG to restore cytosine. An active demethylation pathway through consecutive oxidation of 5-methylcytosine (5mC) mediated by TET proteins and subsequent BER in mammalian system DNA methylation dynamics. BER, base excision repair; CpG, cytosine-phosphate-guanine; DNMT1, DNA (cytosine-5-)-methyltransferase 1; TDG, thymine DNA glycolase; TET, ten-eleven translocation. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

methylcytosine dioxygenase gene pathway has been suggested to play a central role in oxidizing 5-methylcytosine to 5-hydroxymethylcytosine (108).

Aberrant DNA methylation patterns are associated with several human diseases, including various cancers (17, 100, 116, 117), immune system disorders (62), and neurodegeneration (16, 95). Recently a dynamic role for flow-mediated endothelial DNA methylation in vascular homeostasis and transition to pathologic states has been reported. Jiang *et al.* showed changes in DNA methylation in ECs isolated from swine aortas and human aortas exposed to d-flow and s-flow (63). They found that d-flow induced DNA methylation of CpG islands within the KLF4 promoter, which abolished KLF4 transcription. Both premature and mature (*i.e.*, comprising only exons) KLF4 mRNAs were examined, and the inhibitory effect of d-flow on premature KLF4 was completely recovered by RG-108 and 5-Aza (DNMT inhibitors), suggesting that DNMT activity plays a critical role in regulating d-flow-induced reduction of KLF4 transcription. Because the inhibitory effect of DNMT inhibitors on mature KLF4 was partial, another post-transcriptional inhibition of

KLF4 mRNA induced by d-flow has been suggested (28). In addition, DNMT activity and subsequent DNA hypermethylation of KLF4 promoter induced by d-flow have been found to contribute to the inhibition of eNOS, thrombomodulin, and monocyte chemoattractant protein 1 expression (28).

Dunn *et al.* and Zhou *et al.* have also reported that DNMTs contribute to d-flow signaling, but there are some discrepancies between these studies, especially in terms of the expression of isoforms of DNMTs (28, 148). Dunn *et al.* used partial ligation of the mouse carotid artery to generate d-flow and compared gene expression and DNA methylation in these arteries with that of nonligated carotid arteries (exposed to s-flow) (28). They found that both DNMT1 transcription and protein expression were increased by d-flow. Zhou *et al.* also found that DNMT1 mRNA expression and DNMT1 nuclear localization increased after d-flow (148). In contrast, Jiang *et al.* did not observe any significant changes in DNMT (DNMT1, 3A, and 3B) expression or cytosine demethylation enzyme mRNA (TET1-3, TDG1, GADD45B, MBD4, and SMUG1) expression after d-flow. Interestingly, however, a significant increase in DNMT3A protein levels without any change in mRNA levels has been reported (63).

Dunn *et al.* also reported that 5-Aza significantly inhibited d-flow-induced formation of atherosclerosis (28). In addition, they found that d-flow in partially ligated carotid arteries induced a significant increase in DNA methylation in 11 gene promoters, which was reversed by treatment with 5-Aza. Using a systemic biological analysis of MetaCore, the authors found that cAMP response element-binding protein (CREB1) can comprehensively regulate these 11 genes. Furthermore, the differentially methylated regions in 5 of the 11 genes contained a CRE site, and the authors confirmed that the promoters of HoxA5, Klf3, Cmlklr1, and Acvrl1 at the CRE CG site were hypermethylated by d-flow and this hypermethylation was inhibited by treatment with 5-Aza. In addition, a possible role for HoxA5 in vascular remodeling and angiogenesis *via* EC inflammation has been proposed (28). However, the pathophysiologic role of the remaining six genes in d-flow biology remains unclear.

DNMT SUMOylation

As explained above, a significant increase in DNMT3A protein levels without a corresponding change in mRNA levels has been reported. Jiang *et al.* and other groups have suggested that DNMT3A SUMOylation may contribute to this process because SUMOylation can stabilize DNMT3A substrate degradation (Fig. 5) (63, 89). In addition, it has been reported that SUMOylation of DNMT3A can disrupt the ability of DNMT3A to interact with histone deacetylases (HDACs) and repress transcription of a reporter gene (89). However, endogenous modification of these DNMTs and the enzymes involved in this process remains to be discovered. Although endogenous human DNMT1 SUMOylation-induced methylation activity in genomic DNA has been reported, the way in which this activity stimulates the methylation activity of DNMT1 is unclear (80). Because we found that d-flow downregulates SENP2 deSUMOylation enzyme function and increases ERK5 and p53 SUMOylation, it is very reasonable to speculate that d-flow induces DNMT SUMOylation, which may increase DNA hypermethylation. Further investigation is needed.

FIG. 5. Post-translational regulation of mammalian DNA methyltransferases. DNMT protein domain structure and SUMOylation. (A) DNMT1; DMAP1 domain, PCNA domain, NLS domain, DNA replication foci-targeting domain, CXXC- zinc finger region, bromo-adjacent homology domains (BAH1 and BAH2), and catalytic domain. More than 10 SUMOylation sites throughout DNMT1 sequence were suggested. (B) DNMT 3A and 3B; a proline-tryptophan-proline domain (PWWP), an ATRX-DNMT3-DNMT3L-type zinc finger domain (ADD), and catalytic domain. SUMOylation of the N-terminal regulatory region, including the PWWP domain, was reported. NLS, nuclear localization signal. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

DNMT phosphorylation and methylation

The critical role of DNMT phosphorylation in regulating DNMT activity has been reported. AKT and PKC can phosphorylate DNMT1, and AKT1-mediated Ser143 DNMT1 phosphorylation stabilizes DNMT1 and decreases the ability of DNMT1 to associate with PCNA and UHRF1, which are involved in sustaining DNA methylation patterns through the recruitment of DNMT1 (29). Recently, Deplus *et al.* investigated the role of casein kinase II (CK2) in regulating Dnmt3a activity (25). CK2 is a serine/threonine kinase, which is constitutively active and ubiquitously expressed in all mammalian tissue and cell types (30, 90, 123). CK2 activity is not directly affected by phosphorylation and is constitutively active. Therefore, biological activity of CK2 is mainly regulated by its level of expression and cellular localization (123). Uniquely, both adenosine triphosphate (ATP) and guanosine triphosphate (GTP) can be used by CK2 as phosphoryl donors (90, 96). More than 300 substrates can be phosphorylated by CK2, and it has been suggested that CK2 is responsible for up to one quarter of the eukaryotic phosphoproteome (96).

Therefore, CK2 can regulate a variety of biological processes, including inflammation, apoptosis, and proliferation (123). Deplus *et al.* showed that CK2 phosphorylates Ser386 and Ser389 located near the CK2 PWWP domain, which is the conserved Pro-Trp-Trp-Pro motif, and inhibits the ability of the PWWP domain to promote DNA methylation (25). As explained above, we found that d-flow significantly increased PKC ζ activation. Therefore, the possible role of PKC ζ in regulating DNMT1 phosphorylation function may need to be investigated. In addition, the crucial role of DNMT1 methylation induced by lysine methyltransferase Set7/9, which induces DNMT1 degradation *via* activating proteasomes, has been reported, and cross talk between this Lys142 monomethylation and Ser143 phosphorylation to regulate DNMT1

stability and degradation has also been suggested. These findings suggest that PTMs play an important role in DNMT activity, which would be the convergent point of d-flow signaling from s-flow signaling. In addition, these data suggest that the coordination of SUMOylated molecules induced by d-flow decides the EC response to d-flow.

d-Flow Signaling Component 4: Modification of Histones

Histones are one of the components of nucleosomes around which DNA is wound in chromatin. Eight core histone proteins of two dimers of H2A/H2B and two dimers of H3/H4 form nucleosomes. It is well known that PTMs of the histone N-terminus of histones can modulate histone-DNA interactions, which are the so-called histone code. These interactions change chromatin structure and show significant influence on the accessibility of transcriptional regulators to cis-DNA binding elements. We will briefly summarize the role of histone acetylation and phosphorylation and the possible role of methylation under d-flow.

Histone acetylation and phosphorylation

Histone acetylation is catalyzed by two types of molecules, histone acetyltransferases (HATs) and HDACs (58, 59, 75, 130). HATs catalyze the addition of acetyl groups to specific lysine residues of histone proteins, whereas HDACs do the opposite, catalyzing the removal of these groups. In most cases, histone acetylated lysine residues are associated with the activation of transcription (45, 58, 69). HATs are divided into three families, which are the GNAT, MYST, and CBP/p300 families. In contrast, gene silencing is provided by histone deacetylation (45, 69).

Illi *et al.* reported that aberrant flow, which increased the formation of the CREB/CREB-binding complex, induced H3

Ser10 phosphorylation and H3 lysine 14 acetylation (58). They also found that flow-induced c-fos expression was independent of H3 phosphorylation, but dependent on H3 lysine acetylation, and H3 lysine acetylation was regulated by PKA, p38, and mitogen- and stress-activated kinase-1. It is well established that d-flow activates CREB signaling, and this pathway may also apply to d-flow signaling (58). Although H3 Ser10 phosphorylation is not related to c-fos induction, the contribution of H3 Ser10 phosphorylation to Myc transcriptional activation and cell cycle progression has been reported (127, 151). Therefore, both H3 Ser10 phosphorylation and lysine 14 acetylation can be important under d-flow conditions.

Histone acetylation has been studied more in s-flow than in d-flow. Chen *et al.* demonstrated that s-flow increased NF- κ B subunits of p50 and p65 binding to the eNOS promoter. p300 HAT was activated by s-flow and acetylated both p65 and histones in proximity to the eNOS SSRE, opened the chromatin structure surrounding the SSRE, and induced eNOS mRNA expression (13). Huang and Chen have shown that Akt phosphorylation of p300 on Ser1834 is essential for Ser1834 histone acetyltransferase activity, which may also be involved in s-flow-induced eNOS expression (56). Not only HAT but also HDACs serve as one of the important mechanotransduction molecules and have significant impact on both d-flow- and s-flow-mediated signaling. Recent evidences obtained from both *in vitro* and *in vivo* studies have indicated that d-flow induced the expression and nuclear accumulation of both class I (HDAC-1/2/3) and class II (HDAC-5/7) HDACs in ECs (81). In addition, d-flow-induced association of HDAC-1/2/3 with NF-E2-related factor-2 (NRF2) and HDAC-3/5/7 with MEF2 and the expression of NADPH quinone oxidoreductase-1 as an antioxidant gene and KLF2 as an anti-inflammatory gene were downregulated (81).

Wang *et al.* investigated the possible involvement of HDAC5 in s-flow signaling (137). They demonstrated that s-flow induced HDAC5 Ser259/498 phosphorylation, reduced the inhibitory effect of HDAC5 on MEF2C by dissociating

HDAC5 from MEF2C, and promoted MEF2-dependent KLF2 gene transcription (137). In addition, HDAC4 and HDAC5 were shown to cooperate with p65/NF- κ B to inhibit MEF2 transcriptional activity and subsequent KLF2 expression after cytokine stimulation (77). Because we have reported that d-flow induced ERK5 SUMOylation and decreased ERK5 transcriptional activation, and because ERK5 is a well-known upstream regulator of NRF2 and MEF2-KLF2 as we explained above (47, 72, 79, 141), it is reasonable to postulate that this HDAC process may also be involved in both d-flow and s-flow signaling.

Histone methylation versus DNA methylation

Histone methylation is an important PTM that influences its widespread biological processes. Although histone methylation can occur on all basic residues, including arginines, lysines, and histidines, the most extensively studied types of histone methylation are H3 lysine 4 (H3K4), H3K9, H3K27, H3K36, H3K36, H3K79, and H3K20. Histone methyltransferases catalyze the addition of methyl groups donated from S-adenosylmethionine to histones. Three families of HATs of SET domain-containing proteins, as well as DOT1-like proteins (lysine methylation) and the protein arginine N-methyltransferase family (arginine methylation), have been reported to be involved in histone methylation. Two families of demethylases of amine oxidases and jumonji C domain-containing iron-dependent dioxygenases have been identified. The effects of histone methylation on the regulation of gene expression are context dependent. For example, H3K4me generally activates transcription, H3K27me3 represses chromatin, H3K4me1 is associated with enhancer function, H3K4me3 is linked to promoter activity, H3K79me2 is critical for proliferation, and H3K79me3 is related to the WNT signaling pathway (101). These issues were described and discussed in detail in recent reviews (11).

As discussed above, d-flow-induced DNA methylation has been studied extensively, but the role of histone methylation

FIG. 6. Overall scheme of disturbed flow-induced endothelial proatherogenic signaling. Signaling events involved in d-flow-specific signaling are (i) kinase activation, including PKC ζ and p90RSK, (ii) SUMOylation-related enzymes of PIAS1 and SENP2 to regulate ERK5 and p53 SUMOylation, and (iii) DNA methylation at KLF4 and HOX promoters. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

in d-flow signaling has not been well described. The enzymes that carry out DNA methylation and histone methylations are different. In addition, the biological relationship between these two modifications can work in both directions. First, it has been suggested that DNMTs can read histone modification, leading to the recruitment of DNMTs to nucleosomes carrying specific markers (24). For example, genome-wide analysis has shown that DNMT3A is excluded from active chromatin marked by H3K4me3, which leads to an inverse correlation between DNA methylation and histone H3K4 methylation (24). Next, there is also evidence that DNA methylation is important for maintaining patterns of histone modification, especially during cell division (78). DNMT1 and UHRF1 (the E3 ubiquitin ligase) uniquely recognize the methylated CpG residues of hemimethylated DNA, which are generated during DNA replication, and methylate the opposite strand (92). Through this mechanism, a DNA methylation profile can be sustained, and the DNA methylation profile of the parent cell can be reproduced in daughter cells (92). Therefore, the exact relationship between DNA methylation and histone methylation under d-flow conditions needs to be determined.

Conclusions

It has been well established that ECs sense and respond differently to s-flow and d-flow, and ECs exposed to d-flow show very unique proatherogenic phenotypes, including EC inflammation, proliferation, apoptosis, and reduction of vascular reactivity (Fig. 6). In this review, we have discussed mainly four major signaling components that appear to be differentially regulated by s-flow and d-flow patterns. Events that may be involved in d-flow-specific signaling include (i) kinase activation, including activation of PKC ζ and p90RSK, and inflammasome; (ii) SUMOylation-related enzyme activity, including that of PIAS1 and SENP2; (iii) DNA methylation; and (iv) histone modification, including phosphorylation, acetylation, and methylation (Fig. 6).

The study of each component has provided innovative insights into ways to prevent formation of atherosclerosis caused by EC dysfunction. In this review, we have also discussed the interplays among these components, especially we have attempted to more fully and specifically present the role of two different flow types in regulating PTM of target proteins by specific enzymes. Understanding this interplay under d-flow and s-flow conditions may be the key to understanding how the different types of flows can differentially regulate EC functions. In addition, ECs exposed to d-flow showed a uniquely accelerated turnover rate by enhancing both apoptosis and proliferation simultaneously. This increase in turnover rate may maintain d-flow-induced chronic proatherogenic conditions. We believe that the interplay among these four components signaling events under d-flow conditions makes this possible.

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Abbreviations Used

AMPK	= AMP-activated protein kinase
Bcl	= B-cell lymphoma/leukemia
CK2	= casein kinase II
CpG	= cytosine-phosphate-guanine
d-flow	= disturbed blood flow
DNMTs	= DNA methyltransferases
EC	= endothelial cell
eNOS	= endothelial nitric oxide synthase
HATs	= histone acetyltransferase
HDACs	= histone deacetylase
ITIMs	= immunoreceptor tyrosine-based inhibitory motifs
KLF-2	= Kruppel-like factor 2
MEF2	= myocyte enhancer factor-2
NLRP3	= NLR family, pyrin domain containing 3
Nox4	= NADPH oxidase 4
NRF2	= NF-E2-related factor-2
PIAS	= protein inhibitors such as activated STAT
PPAR	= peroxisome proliferator-activated receptor
PTMs	= post-translational modifications
RING	= really interesting new gene
ROS	= reactive oxygen species
SENP	= Sentrin/SUMO-specific protease
s-flow	= steady laminar flow
SH2	= Src homology 2
SHP-2	= SH2 domain-containing protein tyrosine phosphatase 2
SIRT1	= sirtuin 1
SREBP2	= sterol regulatory element-binding protein 2
SSRE	= shear stress response elements
SUMO	= small ubiquitin-like modifier
TET	= ten-eleven translocation
TNF	= tumor necrosis factor
WT	= wild-type