RESEARCH HIGHLIGHT

Linear ubiquitin chains break blood vessel branches

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New work published in *Molecular Cell* by Fu et al. reveals that the protein kinase ALK1 is deregulated when the enzymes assembling and disassembling Met1-ubiquitination, LUBAC and OTULIN, are inhibited or missing. This latest non-degradative role for ubiquitin impacts on Smad1/5 signaling and deregulates angiogenesis downstream of bone morphogenetic proteins, adding to the growing list of Met1-polyubiquitin-regulated cellular pathways.

Protein ubiquitination is the most versatile posttranslational modification in eukaryotic cells.[1,2] Non-degradative Met1-linked or linear ubiquitin chains are assembled by the Linear Ubiquitin Chain Assembly Complex, LUBAC, comprised of the Met1-linkage specific E3 ligase HOIP and two adaptor proteins HOIL-1L and Sharpin, and dismantled by two highly active deubiquitinases (DUBs), OTULIN and CYLD, that are responsible for low levels of Met1-ubiquitination in unstimulated cells.[3,4] While CYLD cleaves both Lys63- and Met1-linkages, OTULIN exclusively targets Met1-chains.[3,4] Hence, whenever OTULIN and LUBAC are involved in a cellular pathway, it is safe to assume that Met1-ubiquitination constitutes a cellular signal. Mutations in LUBAC, OTULIN, or CYLD in humans lead to severe inflammatory diseases and cancers. Especially in inflammation, LUBAC and OTULIN provide a delicate balance of Met1-signals, that when disrupted, derails responses to cytokines such as TNF-α. Consistently, the monogenic disorder OTULIN-related autoinflammatory syndrome (ORAS) can be halted by anti-TNF-α treatment.[3,4]

Fu et al. reveal a new signaling pathway regulated by Met1-ubiquitin signaling. In a spectacular paper[5], they reproduce the previously noted angiogenesis phenotype in embryonically lethal *Otulin* mutant knock-in (KI) mice[6] and show that endothelial cell-specific *Otulin* deletion (*Otulin*$^{LoxP/LoxP}$ *Tie2-Cre*$^{+}$) causes an identical phenotype and embryonic lethality. Wnt signaling does not appear to be grossly deregulated, and NF-κB signaling is only mildly affected in endothelial cells. Instead, OTULIN loss leads to ubiquitin-dependent deregulation of activin receptor-like kinase 1 (ALK1), a Ser/Thr protein kinase that is an integral part of bone morphogenetic protein (BMP) signaling.

ALK1 works in conjunction with BMP receptor type 2 (BMPRII) and other co-receptors to form the transmembrane receptor for key angiogenesis regulators BMP9 and BMP10[7] (Fig. 1). Upon activation, ALK1 activity triggers phosphorylation of the transcription factors Smad1 and Smad5, leading to their association with Smad4, translocation to the nucleus, and initiation of transcription.[7,8] *Alk1*-deficient mice develop arteriovenous malformations, defects in the vascular system caused by abnormal connections between arteries and veins as a result of deregulated Smad signaling.[8] In addition, mutations of ALK1 in humans cause type 2 hereditary haemorrhagic telangiectasia (HHT2), an autosomal dominant vascular disease.[8]

Fu et al. show that ALK1 modification with Met1-linked polyubiquitin, e.g., upon *Otulin* deletion, leads to loss of phosphorylated Smad1/5.[5] Strikingly, LUBAC only targets ALK1 but not other ALK kinases, and ubiquitinated ALK1 does not appear to be degraded due to ubiquitination. Instead, increased ubiquitination of ALK1 in overexpression or in vitro experiments inhibited ALK1 kinase activity, while inhibition or deletion of HOIP reduced ALK1 ubiquitination, leading to increased Smad1/5 phosphorylation and transcription of Smad1/5 target genes. HOIP directly binds and ubiquitinates the ALK1 kinase domain at four lysine residues, likely interrupting ATP binding.

Also, OTULIN binds directly to the ALK1 kinase domain via its OTU domain, and cleaves Met1-ubiquitin chains on ALK1 in vitro and in cells. Knockdown or knockout of OTULIN increases ubiquitinated ALK1, which again results in a loss of Smad1/5 phosphorylation and target gene expression. Finally, the authors show that BMP9 treatment appears to dynamically regulate ubiquitination levels of ALK1 in conditions where OTULIN is depleted.

It is somewhat curious that Met1-linked ubiquitin chains are important to regulate ALK1 activity. Indeed, kinase inhibition would likely be achieved by single ubiquitin molecules on ALK1. Moreover, OTULIN appears to revert ALK1 to the unmodified form, which is surprising, since OTULIN is mechanistically dependent on cleaving Met1-linked chains[9] and should be inefficient in removing the 'first' substrate proximal ubiquitin on ALK1. These interesting questions warrant future molecular studies.

Is the new regulatory loop involving ALK1 ubiquitination physiologically important? All data point towards its relevance in health and disease. For example, BMP9 treatment of *Otulin*-knockout mice alleviates vascularization defects, and embryonic lethality is delayed past day E13.5. A second way to delay embryonic lethality is via expression of a constitutively active ALK1 mutant (knock-in of Alk1 Q200D), likewise rescuing angiogenesis (Fig. 1). These insights provide very strong evidence that the ALK1/Smad axis is indeed regulated by Met1-ubiquitination.

The authors also wondered whether HHT2-associated ALK1 mutations result in distinct Met1-ubiquitination of ALK1. In fact, several ALK1 mutants appeared to be excessively Met1-ubiquitinated in expression studies pointing towards stronger association with LUBAC or loss of OTULIN binding. Significantly, the HOIP inhibitor 11a[10] rescued Smad1/5 phosphorylation for affected mutants, normalizing responses to BMP9 signaling.

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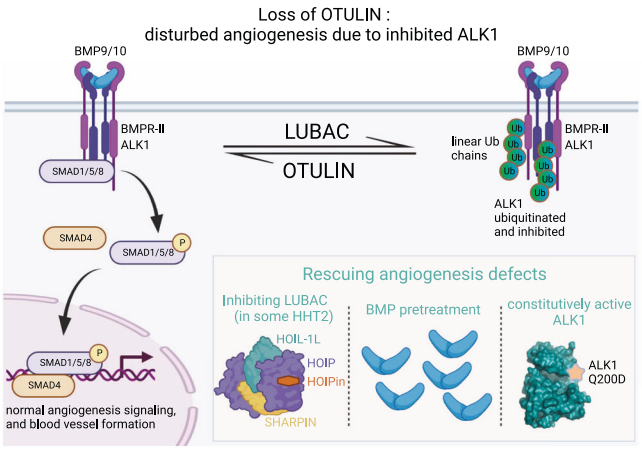

Fig. 1 Regulation of ALK1 signaling and angiogenesis by linear ubiquitin chains. BMP9/10 are recognized by a transmembrane receptor that includes the kinase ALK1 to initiate a signaling cascade to activate Smad transcription. LUBAC and OTULIN are highly specific ubiquitination/deubiquitination enzymes that add or remove a specific, non-degradative ubiquitin signal, Met1-linked polyubiquitin chains. Fu et al. report that excessive ALK1 ubiquitination upon OTULIN loss inhibits ALK1 kinase activity, stalling Smad transcription and deregulating angiogenesis. They further report three mechanisms to overcome the new regulatory loop with potential therapeutic application. Figure created with Biorender.com.

Translation of these studies into a clinical setting could reveal a potential new avenue for the utility of a HOIP inhibitor.

The work by Fu et al. provides ample evidence for adding a new, non-inflammatory pathway being regulated by Met1-linked ubiquitin chains. The discovery was enabled by deleting the DUB, OTULIN, which led to robust visualization of the ubiquitinated substrate. As with any study that breaks new ground, many questions remain. For instance, it is not clear how LUBAC and OTULIN sense activated BMP signaling. In inflammatory settings, HOIP exploits its ubiquitin-binding capability to arrive at and further modify ubiquitinated cytokine receptor complex components;[3,4] this mechanism does not seem to be at play here. It will be interesting to further scrutinize the proposed complexes that ALK1 is forming upon activation. Secondly, OTULIN loss in humans leads to ORAS, but to date in the few cases reported, an angiogenesis defect has not been prominent, and vascularization defects in ORAS patients should be investigated. Overall, the data yet again highlight the importance of Met1-polyubiquitin in regulating tightly controlled and pathologically important signaling pathways; it appears that Met1-ubiquitination is used as a precision tool in cell signaling.

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COMPETING INTERESTS

DK serves on the Scientific Advisory Board of BioTheryX.

ADDITIONAL INFORMATION

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