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Secret alter ego of well-known protein fights leaky blood vessels

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With every heartbeat, a gallon and a half of blood pulses through the body's network of veins and arteries. The force of that blood flow helps keep the cells that line the blood vessels, called endothelial cells, healthy; when blood flow is disrupted, such as during surgical procedures or a stroke, the vessels start to leak, which can cause a host of inflammatory responses that lead to cell damage and disease. Scientists at the Wyss Institute at Harvard University set out to solve the mystery of how blood flow keeps the vessels intact and, to their surprise, discovered a completely new cell signaling pathway that is a promising target for drugs to treat a variety of debilitating conditions.

"We found that the well-known Notch protein is responsible for keeping blood vessels from becoming leaky, and does so through a secondary signaling pathway that operates in a completely different manner than its known transcription-based pathway," says Chris Chen, M.D., Ph.D., Associate Faculty member of the Wyss Institute and Professor of Biomedical Engineering at Boston University, who is the corresponding author of the paper. "Not only is this new pathway exciting from a discovery perspective, it could ameliorate some of the side effects of cancer and cardiovascular drugs to make them safer and more effective." The study is published today in *Nature*.

The endothelial cells that line blood vessels are linked tightly together through connections called adherens junctions to form a barrier that keeps the blood inside the vessel and regulates how easily other substances can pass in and out of it. To study this barrier and determine

why a lack of blood flow causes it to leak, the researchers built a blood-vessel-on-a-chip model consisting of a channel lined with a layer of human endothelial cells surrounded by extracellular matrix within a microfluidic device, which allowed them to easily simulate and control the flow of blood through a vessel and evaluate the cells' responses.

Endothelial cells that experienced blood flow displayed increased activity of the transmembrane protein Notch1, while cells exposed to static blood did not. When the researchers added a chemical that blocks Notch1 activation, they observed that the vessel started to leak, which they determined to be caused by the disruption of adherens junctions between neighboring endothelial cells and the reorganization of actin fibers within each cell, confirming that activation of Notch1 by blood flow is necessary for the formation and maintenance of blood vessels' endothelial barrier.

Curiously, blocking Notch1's known mechanism of action - the detachment of its intracellular domain from the rest of the protein - did not make the vessels leak, which implied that some other part of the protein was responding to blood flow. This suspicion was strengthened by in vivo experiments in which the scientists injected mice with a chemical that blocked Notch1 activation along with blue dye, and saw that the dye leaked out of the blood vessels of treated mice at a much faster rate than expected. "[The intracellular domain's function of] transcribing a gene into a protein that then performs some function within the cell generally takes about two hours, but we were seeing leakage within 30 minutes of blocking Notch1, further suggesting that whatever process controls the permeability of the barrier is operating via a completely different mechanism," says Bill Polacheck, Ph.D., Postdoctoral Fellow at the Wyss Institute and co-first author of the paper.

Once they established that the intracellular domain was not involved in

regulating the endothelial barrier, the scientists scanned other parts of Notch1 for activity. They used CRISPR/Cas-9 to delete various sections of the Notch1 gene, and found that deleting the section that codes for the intracellular domain had no effect on permeability, while deleting the section that codes for the tiny transmembrane domain (TMD) caused vessel leakage to increase under flow conditions. "This is the first time the biological function of the Notch TMD has ever been evaluated," says Matthew Kutys, Ph.D., a Visiting Fellow at the Wyss Institute and co-first author. "It was largely assumed to be inert and just kind of disappear after activation, and most textbooks and research papers don't even show it as a distinct portion of Notch receptors." Through further testing, they figured out that when Notch1 is activated and its intracellular domain is released, its TMD assembles a complex in the membrane with the proteins VE-cadherin, Rac1, LAR, and Trio, which collectively assemble and maintain the adherens junctions between cells and distribute actin fibers against the cell membrane to support those junctions.

"In retrospect, we rolled the dice with this project, because by choosing to investigate Notch we were entering one of the most crowded research areas in biology. But our engineering-based approach let us study it in a new way, without the influence or bias of past work, which I think is what made us open-minded enough to observe and characterize this new, unexpected pathway," says Polacheck. "Knowing that Notch1 regulates cell adhesion [through the new TMD-controlled pathway] in addition to cell differentiation [through its previously described transcription pathway] also offers a new framework for understanding the coordination of complex cellular processes, in that single molecules like Notch can play multiple roles," adds Kutys.

The revelation that Notch1 serves different functions, and knowing which parts of the protein govern each function, allows for the development of new drugs that are both more effective and less toxic.

"Notch is a target for some cancer therapies, but those drugs are known to cause edema [the collection of fluid in the body] and other problems. Now, we're actively working on separating Notch's two pathways so that we can create drugs that target the intracellular domain alone, sparing the TMD and thus preserving the integrity of the blood vessels," says Karen Hirschi, Ph.D., Professor of Medicine and Genetics at the Yale School of Medicine, who collaborated on the study. Knowing that Notch governs vessel permeability makes it a candidate for new drugs to treat cardiovascular diseases as well, and the team is also investigating the TMD as a potential therapeutic agent itself, as cell models that were exposed to leak-inducing inflammation displayed a dramatic reduction in leakage when they were engineered to express the TMD.

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