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SPOTLIGHT

Rab35 controls formation of luminal projections required for bile canalicular morphogenesis

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Hepatocytes display a unique biaxial polarity with shared apical luminal connections between adjacent hepatocytes that merge into a network of bile canaliculi. Belicova et al. (2021. *J. Cell Biol.* https://doi.org/10.1083/jcb.202103003) discovered that hepatocyte apical membranes generate Rab35-dependent extensions that traverse the lumen and are essential for bile canalicular formation and maintenance.

Many crucial functions of the hepatocyte, the predominant liver cell, are dependent on its unique "biaxial" polarity (1)—none more so than the generation and secretion of bile. Hepatocytes receive nutrients from venous blood via sinusoids and secrete bile into bile canaliculi (BC) that merge into larger ducts lined with cholangiocyte epithelial cells with simple "vectorial" polarity (1). Any disturbance in this complicated minute irrigation system can lead to severe and often fatal disease (2). Better understanding of the molecular mechanism of liver parenchymal morphogenesis will uncover therapeutic targets essential for developing effective treatments.

Hepatocyte membrane molecular exchange is extremely efficient due to the highly expanded canalicular surface and transporter proteins specifically localized to canalicular or basolateral membranes. Moreover, each hepatocyte generates multiple apices by creating connections with several adjacent hepatocytes. BC-like structures can be mimicked when cells are cultured in 3D; hepatocyte apical connections can become confluent and form continuous BC channels (3). Several pathways have been proposed to play key roles in BC morphogenesis such as mitosis, cytokinesis and endosomal trafficking (4, 5, 6), nevertheless the mechanistic studies are lacking. Belicova and colleagues set out to investigate the basic mechanisms for BC formation (7).

The authors generated a cell model of lumen morphogenesis by terminally differentiating primary mouse hepatoblasts isolated from embryonic livers into hepatocytes. The cultured differentiated cells were able to generate BC structures. Using time-lapse microscopy, it was possible to observe how single hepatocytes formed the initial individual lumina by interacting with neighboring cells. From a small point contact, the lumina elongated spanning the whole surface of the cell–cell interaction. Astonishingly, the authors detected in real-time how two lumina fused and thus created an elongated channel (Fig. 1 A). Furthermore, one lumen could branch forming a three-cell contact. At the same time, to their great surprise, they saw stripes being formed in a transverse direction to the lumen elongation. The stripes contained a high concentration of F-actin and were localized as projections into the BC lumen rather than formations outside of it.

The authors then used EM and 3D reconstruction of serial sections to establish the nature of these newly discovered intracanalicular projections. The reconstructions showed that each cell participating in a luminal contact generated projections that met in the middle of the lumen and their connection was sealed by tight junctions (TJs) thus forming a structure that completely traversed the BC lumen. They hypothesized that such structure would provide additional tensile strength to the luminal wall just like bulkheads strengthen a boat shell against pressure. Belicova et al. found that the processes they named "bulkheads" had plate-/ridge-like 3D shapes and never completely isolated the BC compartments, thus allowing continuous bile flow along the canaliculus. To test whether bulkheads were present in vivo, they studied EM sections of embryonic day 15.5 (E15.5) and adult mouse livers as well as cultured primary mouse hepatocytes. The 3D reconstruction again showed the presence of bulkheads that originated from the opposing hepatocyte luminal membranes and their contact points sealed by the TJs.

To understand whether bulkheads are essential for BC morphogenesis and the molecular mechanisms governing their formation, the authors performed an RNAi screen in their BC cell model. They selected 25 genes of potential interest due to their known involvement in hepatocyte polarity pathways such as apical junction formation, cytoskeleton regulation, and polarized trafficking. One of the identified phenotypes was seen to be of particular interest. The knockdown of small GTPase Rab35 did not affect cell polarity marker localization but changed lumen morphology (Fig. 1 B). Silencing of Rab35 produced elongated tubes

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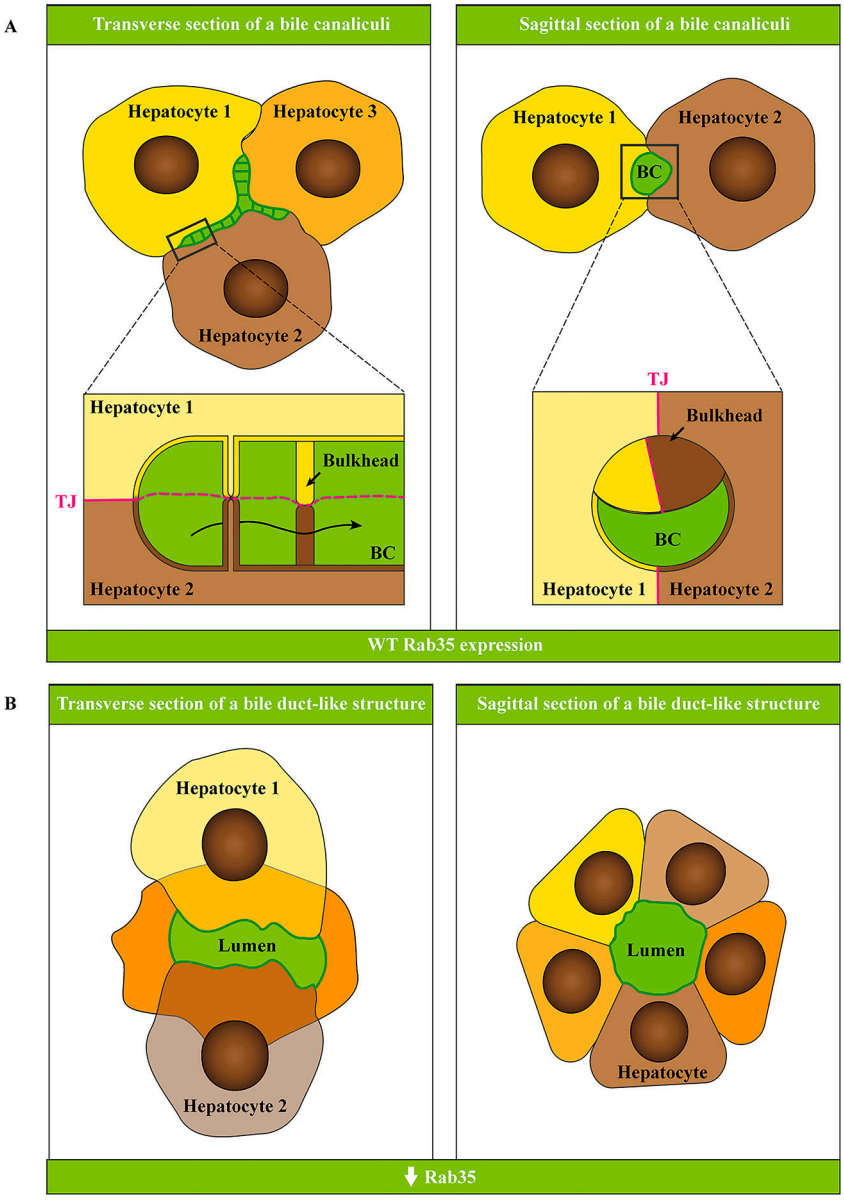

Figure 1. **Rab35 impacts the BC formation and stability. (A)** WT levels of Rab35 allow the formation of bulkheads in the BC lumen facilitating the hepatocyte biaxial polarity and elongation of BC between neighboring hepatocytes. The formed BC have a radii <6 µm and are not divided into discrete compartments by the bulkheads. **(B)** Reduction of Rab35 expression by RNA interference leads to bulkheads decreasing in size. This creates luminal bile duct–like structures with radii >6 µm surrounded by 4–5 conical shape hepatocytes. TJ, tight junctions.

with increased radius and large cysts that were still connected to the remaining BCs and their appearance resembled formations of cholangiocytes, although they did not express cholangiocyte marker Sox9. Reintroduction of Rab35 reduced the lumen to the original size.

Live-cell time-lapse microscopy was used to investigate the function of Rab35 in BC morphogenesis. Rab35 is typically an endosomal protein, and in the liver cell model it was enriched at the apical surface, lateral plasma membrane, and cytoplasmic vesicles. Moreover, it was also present at the transverse connections within forming lumina. Upon Rab35 depletion, hepatocytes reshaped their apical surface, forming multicellular cysts lined by cells with vectorial polarity. This spherical expansion of the lumen was associated with the absence of actin-rich bulkheads.

To test whether the cell model findings stay true in vivo, they targeted E13.5 mouse embryo livers, injecting lipid nanoparticle-coated siRNA against Rab35. 3D tissue reconstruction showed the changes in tissue architecture in siRNA-injected livers in striking detail. As predicted, large tubule formation was seen in contrast to the normal BCs seen in the mock-injected liver parenchyma. The 3D analysis demonstrated that the enlargement of the tubular lumina was not due to pure dilatation but the tissue reorganisation from typical biaxial polarity into a bile duct–like structure with vectorial polarity where multiple (4–5 in number) cells lined the single lumen (Fig. 1 B). Furthermore, the cells changed shape from a typical hepatocyte octagon to a conical profile while they still expressed the usual hepatocyte markers and not those typical of cholangiocytes.

Bile canalicular morphogenesis requires anisotropy of surface tension and/or rigidity of the wall to progress from the initial point contact of apical surfaces of the two adjacent hepatocytes toward elongation, branching, and fusion with other canalicular lumina (8). The authors discovered that presence of transverse connections (bulkheads) was associated with maintenance of narrow lumen, which elongates under inner pressure. Thus, similar to their use in boatbuilding, BC bulkheads provide mechanical stability to the elongating BC lumen. It is possible that bulkheads could provide directionality to the bile flow in liver parenchyma. Positional location of the bulkheads may be determined by the sensing mechanisms coupled to the tension and curvature through actin mesh. Primary cilia are assigned a function in sensing directional flow and controlling tissue morphogenesis. Unlike cholangiocytes and many other epithelial cells, hepatocytes lack cilia and the molecular components of the tension sensing mechanism in hepatocytes remain to be determined.

Belicova and colleagues showed that Rab35 is required for bulkhead formation and hepatocyte lumen shape. Rab35 was previously found to regulate endosomal recycling and thus it may control intracellular trafficking of proteins required for bulkhead formation, such as the components of TJs. It may also support the fusion of the two projections along the T-shaped TJs. Furthermore, the authors report that their unpublished observations suggest that clusters of Rab35-positive vesicles are seen at the base of the bulkhead, and it may coordinate trafficking and organize actin cytoskeleton or regulate actin remodelling as previously described in neurons (9).

Elucidation of the mechanism of hepatocyte canalicular morphogenesis is one of the important research questions highly relevant to understanding the basic processes in both healthy and diseased liver. Liver cirrhosis was one of the top 10 causes of death in low- and middle-income countries in 2019 (10). Cirrhotic changes in the liver architecture include destruction of BC, and the new information provided by Belicova and colleagues may eventually lead to discoveries of effective treatments for devastating liver diseases.

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