Published in final edited form as:

Science. 2018 October 05; 362(6410): 26–27. doi:10.1126/science.aav1025.

Smoothing out the Patches:

New Patched structures suggest roles for cholesterol transport in Hedgehog signaling

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Hedgehog (Hh) proteins control core signaling pathways that direct development of all metazoans – employing unique molecular mechanisms that remain elusive. More than two decades after their discovery, a series of recently-determined structures of key transducers of Hh signals, Patched (Ptch) and Smoothened (Smo), are now beginning to reveal the transmembrane signaling events triggered by Hh. On page XXX of this issue, Qi *et al.* (1) describe key cryo-electron microscopy (cryo-EM) studies that harmonize recent reports (1–3) on the structure of the Hh receptor Ptch – an important tumor suppressor (4). Together with recent crystal structures of the 7-transmembrane-spanning (7TM) protein Smo (5,6), they shed important new light on molecular events in Hh signaling and suggests new opportunities for targeting this pathway in cancer and other diseases (4).

Smo was first identified genetically as the transducer of Hh signals across the membrane, and belongs to the F class of G-protein coupled receptors (GPCRs) – like Frizzleds, the receptors for Wnts. It subsequently became clear, however, that the physical receptor for Hh is actually Ptch (7) – and that Hh regulates Smo indirectly through Ptch. In the absence of Hh, Ptch inhibits Smo signaling. This inhibition is relieved upon Hh binding to Ptch, in a catalytic manner that does not require direct Ptch/Smo interaction (8) – arguing that a mediator is likely to be involved. Since Smo binds cholesterol-like molecules (5–7) and Ptch resembles the Niemann-Pick type C1 (NPC1) cholesterol transporter (8), sterols have become the prime suspects.

The new cryo-EM structures of human Ptch1 (1–3) range in resolution from 3.5Å to 3.9Å, and confirm the structural similarity of Ptch1 to NPC1. They detail how the 12 transmembrane (TM) α -helices (arranged as two groups of six) and the two homologous extracellular domains (ECDs) form a two-fold pseudo symmetric structure. Importantly, cholesterol-like densities are seen in two locations in Ptch1. One is sandwiched between the two ECDs. The other occupies a membrane-facing cavity in the transmembrane region's 'sterol-sensing domain' (SSD) that is analogous to a cholesterol-sized pocket seen in the NPC1 cholesterol transporter (9). These densities appear to be connected by a contiguous 'tunnel' across Ptch1 through the membrane, of sufficient size to accommodate cholesterol (1). This tunnel could allow transport of sterol-like molecules from the SSD within the membrane (and/or other intramembrane 'gates') to the extracellular space. Indeed, previous studies have implicated Ptch directly in cholesterol export (10).

Importantly, the structures also reveal for the first time how the N-terminal signaling domain of the human Hh orthologue Sonic (ShhN) binds Ptch1. Initially adding to the field's

uncertainties, however, the first two structures (2,3) showed ShhN bound to Ptch1 in two quite different ways – using distinct and non-overlapping parts of the ligand's surface. Unexpectedly, this is resolved in the newest structure from Qi *et al.* (1), which shows a single ShhN molecule bound simultaneously to two of Ptch1.

In Hh/Ptch complexes formed using a 'native' form of ShhN (3), the palmitoylated N-terminus of ShhN dominates the interface with Ptch1. The N-terminal ~15 amino acids of the ligand project between the two Ptch1 ECDs. This places the N-terminal palmitoyl moiety in a cavity between the ECDs at the membrane lipid headgroup level and occludes the putative tunnel through the Ptch1 molecule – so should inhibit cholesterol transport. Non-palmitoylated ShhN binds to a very similar region on the Ptch1 surface (mostly involving ECD1), but uses the opposite side of the ligand (2), including a surface at which divalent cations are bound in all Hh family structures (11). Gong *et al.* suggest that this mode of ShhN binding will also limit sterol egress from the tunnel – by 'sealing' the ECD sterol binding site (2). This second mode of Ptch1 binding appears to be the weaker of the two (3), but its importance is demonstrated by the fact that an antibody (5E1) that recognizes precisely the same surface (2,3) is an effective inhibitor of Hh signaling. On the other hand, a palmitoylated N-terminal ShhN fragment of just 22 amino acids can partially activate Hh signaling with no contribution from the globular part of ShhN (12). Thus, there is no basis for dismissing either Ptch1 binding mode.

The most recent structural study (1) appears to resolve this conundrum – arguing that both interfaces are used. Interestingly, Ptch1 showed a tendency to oligomerize in preparations used for each study (1–3). When Qi *et al.* (1) added 1mM Ca²⁺ (not present in their previous study (3)), they observed a complex in which two Ptch1 molecules bind a single native ShhN – using both of the previously-observed interfaces. Mutations affecting only one or other of the two ShhN surfaces argue that both interfaces are important for signaling – implicating this 2:1 (Ptch:ShhN) complex as a key signaling entity.

The new views of Ptch1 gel nicely with recent advances in understanding Smo regulation and the role played by cholesterol as Smo activator (4–6,13). A crystal structure of active Smo suggests that it too has a tunnel, open to the membrane at one end and to the extracellular cysteine-rich domain (CRD) at the other (5) – including a region analogous to the agonist-binding site in other GPCRs. This tunnel is large enough to allow cholesterol to transit from the inner leaflet of the membrane to the agonist-binding site and/or to the Smo CRD, reorienting it and allosterically activating Smo in the process (5,6).

In the model that seems to be emerging, unligated Ptch locally depletes cholesterol from the inner leaflet of the membrane to inhibit Smo signaling activity. Indeed, cholesterol is required for Smo activity in a reconstituted system (13), and inhibition of Smo by Ptch1 in this system requires the presence of a transmembrane Na⁺ gradient (13) – consistent with the homology that Ptch1 (and NPC1) share with cation gradient-driven efflux pumps (8). When ShhN binds to Ptch1 the path for cholesterol efflux is thought to be occluded. The resulting inhibition of cholesterol transport by Ptch1 would lead to re-accumulation of cholesterol in the membrane, and restoration of Smo signaling to Gli transcription factors.

Naturally, numerous important questions remain – and this is only a working model. It still remains formally possible that Ptch1 transports an inhibitor that shuts off Smo signaling rather than starving Smo of a key activator by depleting cholesterol. Inhibiting Ptch-mediated transport would still promote Smo activity in this scenario. Moreover, the importance of having two Ptch1-binding sites on ShhN is difficult to understand. The resulting bivalence will certainly enhance ShhN avidity for cell surface Ptch1. It is not completely clear from all of the structures that both interaction modes would inhibit cholesterol efflux equally. Might Ptch1 dimerization also play a signaling role? If inhibition of Ptch1 transporter function is incomplete, the second binding mode could aid signaling by promoting Ptch1 internalization to remove it from the membrane (12). Intriguingly, numerous other Hh-interacting proteins that regulate this signaling axis bind to the divalent cation-containing surface of Hh proteins (11), and may regulate signaling by modulating formation of the 2:1 (Ptch1:ShhN) complex.

The new Ptch1 structures enrich our understanding of this important signaling system, but may also suggest therapeutic opportunities. Inhibition of deregulated Hh signaling with Smo antagonists like vismodegib is important in treating medulloblastoma and advanced basal cell carcinoma (4). Smo agonists have also been developed, which may have value for regenerative therapies. Mechanistic details of Ptch function may suggest new ways of modulating this pathway. Finally, it is also interesting to speculate that the current model for sterol regulation of Smo, supported by the new results with Ptch, might be mirrored for regulation of the closely related Wnt-binding Frizzleds (14) by free fatty acids that bind independently to their CRDs (15).

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Hedgehog regulation of Smoothened

In the absence of Hedgehog (Hh), Patched (Ptch) exports cholesterol from the membrane through a 'tunnel' identified in its structure – locally depleting cholesterol from the membrane. Since Smoothened (Smo) requires cholesterol for its signaling, Smo is inactive. Hh binds two Ptch molecules, occludes the tunnels in both apparently in different ways. Cholesterol levels in the membrane are restored, and activate Smo by traversing its own tunnel and reaching the extracellular domain – promoting Smo signaling to activate the Gli transcription factors.