Smoothened transduces Hedgehog signals via activity-dependent sequestration of PKA catalytic subunits

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BACKGROUND INFORMATION

- The Hedgehog (Hh) pathway controls the development of nearly every vertebrate organ [1–4]. It is essential for organ development, homeostasis, and regeneration. Dysfunction of this cascade drives several cancers.
- Insufficient pathway activation during embryogenesis gives rise to birth defects [7], whereas ectopic pathway activity drives several malignancies, including basal cell carcinoma of the skin and pediatric medulloblastoma.
- To control expression of pathway target genes, the G protein–coupled receptor (GPCR) Smoothened (SMO) activates gliomaassociated (GLI) transcription factors. It is known that Hedgehog pathway regulates Smoothened, but the mechanism through which Smoothened regulates PKA is not yet well known.
- This paper provides a possible mechanism as to how Smoothened regulates PKA.

RESEARCH QUESTION

- **Through what mechanism does Smoothened regulate PKA, and thus how does Hedgehog (Hh) signal transduction at the cell membrane link to GLI activation in the nucleus?**
- We hypothesise that Smoothened is the key which activates GLI through an inhibition of PKA, and Smoothened has the ability to regulate PKA in a G-protein independent manner**.**

METHODOLOGY

- **HEK293 model system to study SMO regulation of PKA (Fig. 1):**
  - CREB assay monitors the activity of cAMP/PKA signaling pathway in cultured cells using CRE luciferase reporter.
  - Less PKA would cause decreased CREB pathway activation, leading to less activation of Luciferase and light detected.
  - CREB is a soluble transcription factor regulated by PKA phosphorylation. However, CREB is not known to be affected by any other mechanisms that regulate GLI activity Hence, any effects of SMO on CREB transcription would provide evidence that SMO can control PKA.

- **Applicability of HEK293 cell model to primary cilia:**
  - To find out whether the findings from HEK293 model applied to primary cilia. which is the endogenous subcellular location for Hh signal transduction, SMO and PKA-C localisation in inner medullary collecting duct cells was studied.

- **Subcellular localisation of PKA-C in HEK293 cells:**
  - As a positive control, SMO colocalisation with nanobody NbSmo2 was examined.
  - As a negative control, the non-SMO-binding Nb did not colocalise with SMO.

- **Bioluminescence resonance energy transfer (BRET) as an assay to study vertebrate Hh signal transduction (Fig. 2):**
  - SMO was fused to a luciferase energy donor and PKA-C or other interactors to a YFP acceptor.
  - On interaction, light produced by luciferase excites YFP. YFP/luciferase emission ratio was used as a normalised metric for measuring protein interaction with SMO.
  - BRET was used for a number of reasons. For example, BRET was employed to examine the impact of small molecule SMO ligands on SMO interactions with either PKA-C or NbSmo2 (which strongly prefers to bind active SMO over inactive SMO).

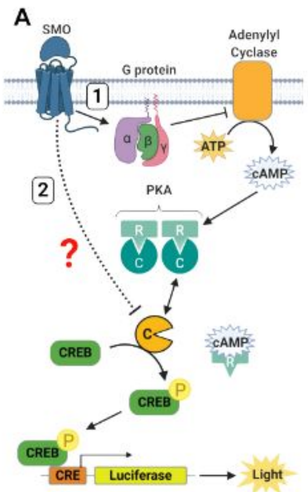

Fig 1. SMO inhibits PKA substrate phosphorylation in a G protein–independent manner.

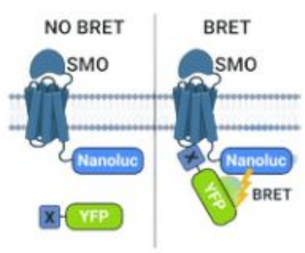

Fig 2. SMO pCT interaction with PKA-C.

RESULTS

1. **SMO inhibits PKA substrate phosphorylation in a G protein–independent manner.**
   a. PKA-C expression strongly activated the cyclic AMP response element (CRE) binding protein (CREB) reporter, indicating an excess of PKA-C over PKA-R.
   b. Cotransfection of SMO blocked PKA-C-mediated reporter activation, indicating that SMO can inhibit PKA-C in a G protein–independent manner

2. **SMO uses its essential proximal cytoplasmic tail (pCT) domain to recruit PKA-C to the membrane in HEK293 cells and in primary cilia. SMO pCT interacts with PKA-C, SMO interacts with free PKA-C subunits rather than PKA holoenzymes.**
   a. In the presence of SMO, PKA-C localised to the membrane, colocalising with SMO in the majority of HEK293 cells. Similar experiments yielded the same results in inner medullary collecting duct (IMCD3) cells.
   b. Vertebrate SMO recruits PKA-C to the membrane via direct protein-protein interactions, as evidenced by extremely strong bioluminescence resonance energy transfer (BRET) signals exceeding NbSmo2 positive control.
   c. YFP-tagged PKA-R exhibited only weak, nonsaturable BRET with SMO, and coexpression of untagged PKA-C did not increase this signal, thus indicating that PKA-C neither interacts with SMO via PKA-R, nor recruits PKA-R–containing holoenzymes to SMO.

3. **SMO/PKA-C interactions depend on SMO and GRK2/3 activity.**
   - The selective GRK2/3 inhibitor Takeda Compound 101 (Cmpd101) inhibited PKA-C colocalization and BRET with SMO, but did not affect BRET with NbSmo2.
   - SMO inhibition of PKA-C was drastically reduced in HEK293 cells harboring genetic knockouts of GRK genes, and this defect was reversed upon reintroduction of GRK2 into the knockout cells

4. **GRK2/3 phosphorylation of conserved SMO pCT residues mediates PKA-C binding.**
   - Phosphoprotein staining of purified SMO samples revealed SMO activity- and GRK2/3-dependent phosphorylation that required the pCT and sites were mapped using mass spectrometry.
   - Mutation of the 7 SMO activity- and GRK2/3-dependent phosphorylation sites to alanine substantially reduced SMO phosphoprotein staining and SMO BRET with PKA-C, demonstrating the importance GRK2/3 phosphorylation sites in the pCT domain for PKA-C interaction.

5. **Hh signal transduction is blocked when SMO cannot bind PKA-C in cell culture and in living organisms.**
   - Deletion of SMOΔ570–581 which lies within a region of pCT critical for interaction with PKA-C, abolishes activation of GLI in cultured Smo-/- fibroblasts without affecting SMO ciliary localization.
   - To assess whether SMO interactions with PKA-C also required this region, NbSmo2 was fused to the end of the CT, resulting in SMO-NbSmo2 failingto bind PKA-C, whereas SMO-Nbβ2AR80 still mediated strong transcriptional responses .
   - Meanwhile in zebrafish, expression of mRNA encoding wild-type SMO or SMO-Nbβ2AR80 restored Hh pathway–dependent muscle development to smo-/- embryos, whereas SMO-NbSmo2 did not.
   - Mutations in the 7 critical GRK2/3-dependent phosphorylation sites in SMO dramatically impaired SMO-dependent GLI transcription in cultured Smo-/- fibroblasts and also failed to restore normal muscle specification to smo-/- zebrafish embryos.

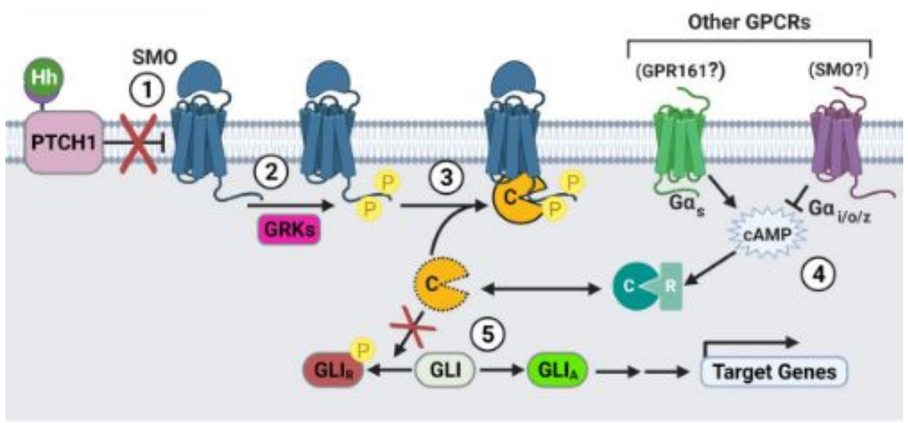

Fig 3. Hh signal transduction is blocked when SMO cannot bind PKA-C.

DISCUSSION

- It has been established before that Hh, through inhibition of PTCH1, activates SMO, thereby activating GLI and promoting cell growth. Though PKA is already known to be implicated in this Hh pathway, the exact molecular mechanism through which PKA is involved was unclear prior to this research.
- In this research, a novel interaction between SMO and PKA-C was identified, and it was also shown how this recruits PKA-C to membranes, and affects the activity of PKA-regulated transcription factors and GLI-dependent outputs in cells.
- The activities of proteins in this pathway were represented by CREB assays and visualised by fluorescent markers.

- **Proposed mechanism**:
  - Based on these findings, we propose the following mechanism. In the pathway “off” state, SMO is inactive and inefficiently binds PKA-C. PKA-C is thus available to phosphorylate and inactivate GLI. In the pathway “on” state, SMO activation triggers GRK phosphorylation of the pCT, increasing PKA-C binding and sequestering PKA-C to the membrane. SMO-bound PKA-C cannot access soluble GLI substrates. This leads to loss of inhibitory GLI phosphorylation.

- **Limitations:**
  - While this paper focused on SMO-mediated PKA-C membrane sequestration in activating GLI, a new research question can be about whether there are other biochemical mechanisms through which SMO affects PKA-C signalling, thereby altering GLI activity.
  - Additionally, although the specific interaction between SMO and PKA-C theorised in this reconstitution study has been shown to apply in vivo in zebrafish embryos, heterologous reconstitution may not necessarily capture all aspects of Hh signal transduction in vivo. Hence, another research question would be to study how other Hh pathway components may influence the effect of SMO on PKA-C signalling.

- **Clinical significance**:
  - Deeper research into these research questions can help us gain a better understanding of the pathway through which Hh may affect GLI, hence opening up new options for target-specific therapy in the Hh pathway to alter its activity, in cases which the pathway is pathologically overactive or underactive leading to disease. Of note, there are currently already SMO and GRK2 inhibitors in use clinically to target this pathway, though more novel drugs targeting this pathway may be invented with more research in this area