Characterization of sPDZD2-GPR161 interaction in the negative regulation of Hedgehog signaling

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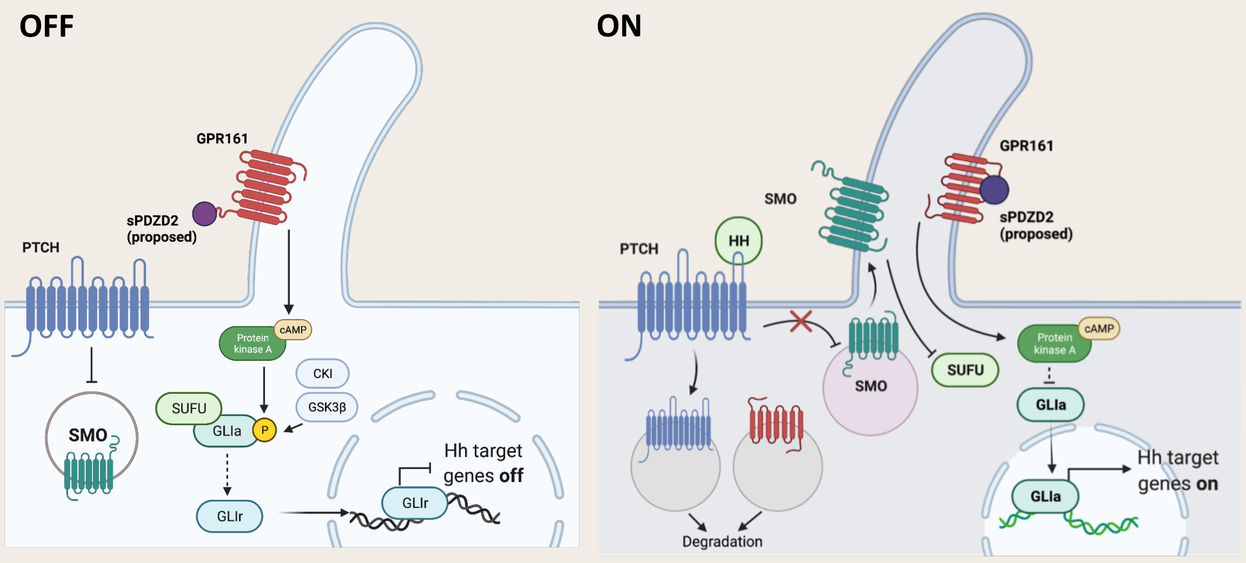
Abstract

The Hedgehog signaling pathway is an evolutionarily conserved pathway essential in early embryonic development. It was previously demonstrated in the chicken neural tube that the secreted protein, sPDZD2, can negatively regulate the Hedgehog signaling pathway; however, how it achieves its negative regulation is unclear. GPR161, a cell surface receptor, has also been identified as a negative regulator and attenuator of Hedgehog signaling by activating protein kinase A (PKA) through upregulating cyclic AMP (cAMP). The activated PKA then phosphorylates the GLI3 transcription factor, promoting its processing into the truncated, repressor form. Because of their similar regulatory roles, mechanisms of regulation, and the close proximity of expression patterns in mouse embryos, it is hypothesized that sPDZD2 exerts its negative regulation on Hedgehog signaling through binding to GPR161. In this project, the interaction between sPDZD2 and GPR161 is characterized. Co-immunoprecipitation assay identified and demonstrated the protein-protein interaction, and co-immunoprecipitation between v5-sPDZD2 and deletion mutants of myc-GPR161 further demonstrated the interaction interface between v5-sPDZD2 and myc-GPR161. The C3H10T1/2 mouse embryonic fibroblast cell line is utilized to knockout *Gpr161* by CRISPR/Cas9-based genome editing, such that downstream regulators of the pathway can be further characterized. The negative regulation exerted by the sPDZD2-GPR161 interaction is expected to be important in early embryonic development, and would be able to provide insights into drug target identification in cancer treatment.

Introduction

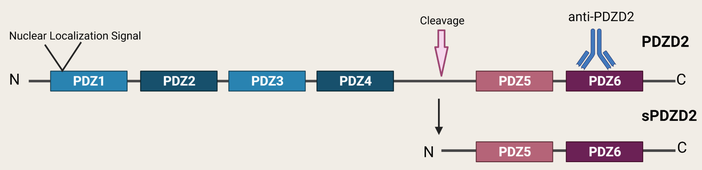
The Hedgehog Signaling Pathway



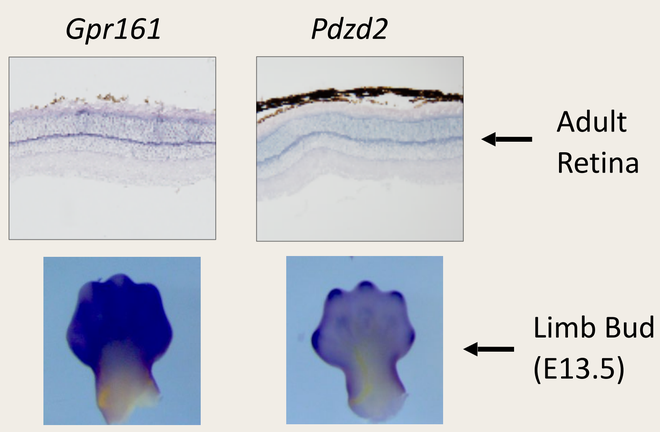
Hedgehog signaling is activated when the Hedgehog (HH) ligands bind to the cell surface receptor, Patched (PTCH), releasing PTCH's inhibition on Smoothened (SMO). In the absence of HH binding and activation, the GLI transcription factors are phosphorylated and targeted for partial degradation into the repressor forms. In the presence of HH binding, SMO inhibits GLI phosphorylation, promoting the full-length GLI to induce target gene expressions as activators.

sPDZD2, GPR161, and Hedgehog Signaling

PDZD2 is a protein expressed in multiple tissues. It was previously characterized that PDZD2 undergoes proteolytic cleavage to produce the secreted form, **sPDZD2**, which acts as a signaling molecule. It was reported that sPDZD2 can induce expression of insulintropic genes in β cells by activating PKA.



GPR161 is a cell surface receptor that has been identified to be a negative regulator of the Hedgehog signaling pathway by activating PKA through upregulating cAMP. However, GPR161 remains an orphan receptor without an identified ligand.

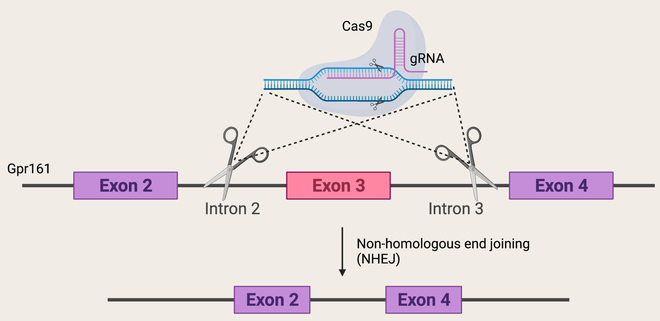


The link between GPR161 and sPDZD2 is drawn by the two disease loci identified in Brachydactylyl type A 1 (BDA1), the two proteins' similar regulatory roles of Hedgehog signaling and regulatory mechanisms, as well as their proximity of expression patterns.

Methodology

Functional Studies

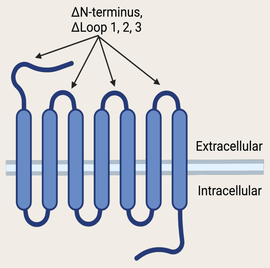
Employ CRISPR/Cas9-based genome editing to knockout *Gpr161* in C3H10T1/2 cells by deleting the third exon of the gene



Interaction Studies

Co-immunoprecipitation with full-length myc-GPR161 and v5-sPDZD2 overexpressed in HEK293 cells

Co-immunoprecipitation with deletion mutants of GPR161 to define its interaction interface with sPDZD2



Results

Preliminary understanding of GPR161 and its regulation of Hh signaling

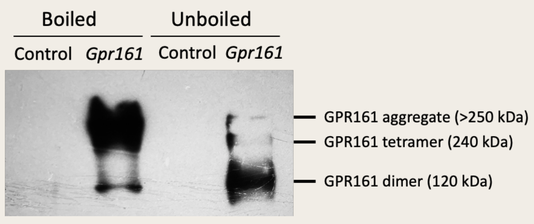


Figure 1 (left). Immunoblotting shows the behavior of the transmembrane protein, GPR161, under different denaturing conditions *in vitro*.

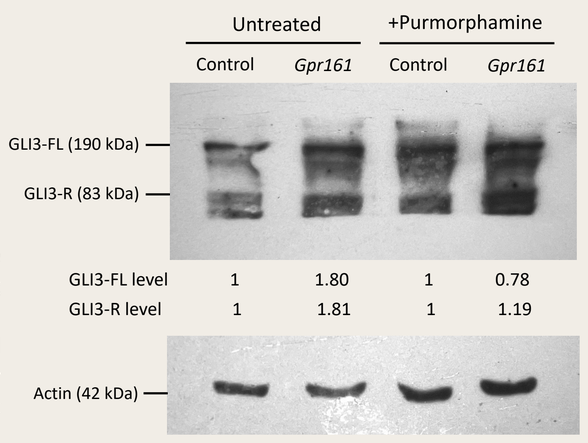


Figure 2 (right). Immunoblots showing levels of GLI3-FL and GLI3-R in C3H10T1/2 cells under different treatments (with or without Hh activation by purmorphamine, and with or without *Gpr161* overexpression).

Interaction and interaction interface identified by co-immunoprecipitation

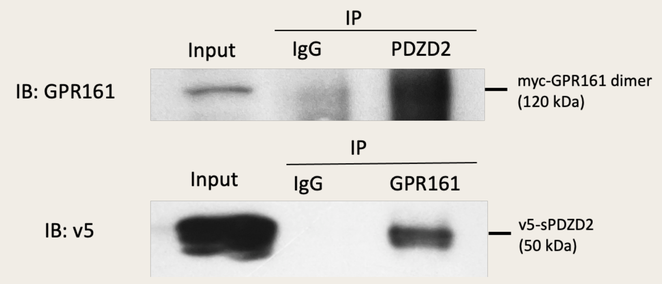


Figure 3 (left). Reciprocal co-immunoprecipitation demonstrated protein-protein interactions between myc-GPR161 and v5-sPDZD2.

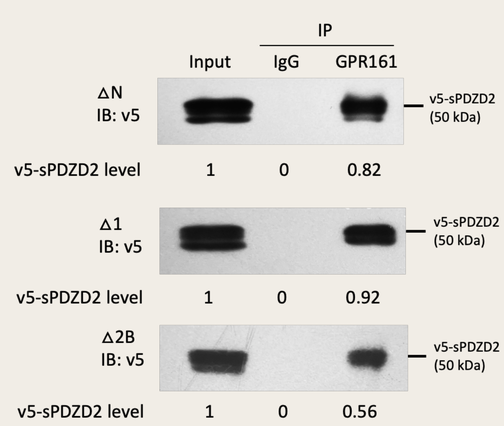


Figure 4 (right). Co-immunoprecipitation between full-length v5-sPDZD2 and the deletion mutants of myc-GPR161 at its extracellular domains suggested two proteins interact partially through the second extracellular domain.

Knockout of *Gpr161* from C3H10T1/2 cells by CRISPR/Cas9

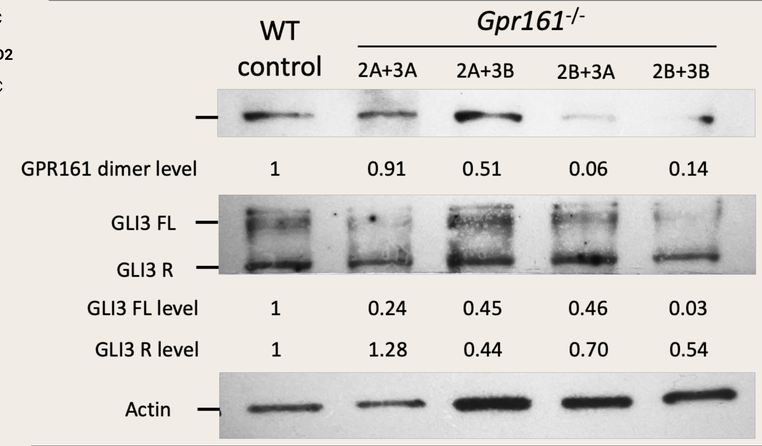


Figure 5. Immunoblotting showing the protein levels of GPR161 dimer, GLI3-FL, and GLI3-R in total cell lysates from untreated wildtype (WT) C3H10T1/2 cells and four *Gpr161*$^{-/-}$ C3H10T1/2 cells treated with four different pairs of gRNAs targeting intron regions flanking exon 3 of *Gpr161*.

Conclusion, Discussion, & Future Directions

- GPR161's negative regulation of basal and attenuating effect on activated Hh signaling
- Co-IP assays demonstrated protein-protein interactions, and indicated that the second extracellular loop is involved in the interaction
- CRISPR/Cas9-mediated deletion of Gpr161's third exon by targeting flanking intron regions was effective but not efficient
- Downstream characterizations of sPDZD2's regulation of Hh signaling to be done in the absence of GPR161.
- Functional relevance of the interaction to be done in the chicken neural tube

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