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TITLE: Biochemical Mechanisms of Hedgehog Signal Transduction Through Primary Cilia in Medulloblastoma

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The Hedgehog pathway is essential for development, but misactivation of the Hedgehog pathway can cause cancer1 . Medulloblastomas are rare pediatric brain tumors with an incidence of 0.2-0.6 cases per 100,000 population2 , and approximately one-third of medulloblastomas are caused by Hedgehog pathway misactivation. Hedgehog signals are transduced through primary cilia on the surface of most cells3. When the Hedgehog pathway is off, Patched1 (PTCH1) localizes to cilia and inhibits Smoothened (SMO). Upon pathway activation, Hedgehog ligands such as Sonic Hedgehog (SHH) bind to PTCH1, removing PTCH1 from cilia, and allowing SMO to accumulate in cilia to activate the GLI family of transcription factors. How PTCH1 leaves cilia, how SMO accumulates to cilia, and how PTCH1 inhibits SMO to regulate oncogenic Hedgehog signaling is unknown.

15. SUBJECT TERMS
None listed.

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INTRODUCTION: The Hedgehog pathway is essential for development, but misactivation of the Hedgehog pathway can cause cancer. Medulloblastomas are rare pediatric brain tumors with an incidence of 0.2-0.6 cases per 100,000 population, and approximately one-third of medulloblastomas are caused by Hedgehog pathway misactivation. Hedgehog signals are transduced through primary cilia on the surface of most cells. When the Hedgehog pathway is off, Patched1 (PTCH1) localizes to cilia and inhibits Smoothened (SMO). Upon pathway activation, Hedgehog ligands such as Sonic Hedgehog (SHH) bind to PTCH1, removing PTCH1 from cilia, and allowing SMO to accumulate in cilia to activate the GLI family of transcription factors. How PTCH1 leaves cilia, how SMO accumulates to cilia, and how PTCH1 inhibits SMO to regulate oncogenic Hedgehog signaling is unknown. Our objective is to define the molecular mechanisms underlying PTCH1 and SMO accumulation and activity in cilia, which represent major unresolved questions for understanding development and cancer. PTCH1 is homologous to membrane transport proteins that control lipid compartmentalization, and ciliary lipids that activate SMO and are enriched in Hedgehog-associated medulloblastomas. Thus, PTCH1 may control SMO by regulating ciliary lipids. Our central hypothesis is that cilia are important for Hedgehog signaling because they enable protein interactions that are required for PTCH1 and SMO accumulation and activity in cilia (Aim 1), and because they enable lipid compartmentalization that is required for PTCH1 to inhibit SMO (Aim 2).

KEYWORDS: Medulloblastoma, brain cancer, Hedgehog, cilia

ACCOMPLISHMENTS:

- **What were the major goals of the project?**

  Major Task 1: Perform APEX reactions in PTCH1^{APEX} and SMO^{APEX} cells at critical timepoints for PTCH1 and SMO trafficking and activity in cilia.

  Major Task 2: Validate and functionally interrogate candidate PTCH1 and SMO interactors

  Major Task 1: Define the impact of PTCH1 and PTCH1 lipid binding domains on ciliary Hedgehog signal transduction.

- **What was accomplished under these goals?** We performed APEX reactions for PTCH1^{APEX} and SMO^{APEX} using human Hedgehog-associated medulloblastoma cells (DAOY, UW228, and ONS76). We performed APEX reactions for PTCH1^{APEX} and SMO^{APEX} using human Hedgehog-associated medulloblastoma cells lacking cilia. Examples of our results from these experiments are shown in Figure 1d, Figure 1e, Figure 2a, and Figure 2b. By focusing on the candidates that were present in proximity to PTCH1 or SMO from medulloblastoma cells with cilia, we generated a candidate list of PTCH1 and SMO interactors from Subtasks 1 and 2 for validation in Major Task 2. To that end, we performed colocalization immunofluorescence and coimmunoprecipitation experiments to validate interactors, including Serpinh1, Nomo1, and Nceh1 (the most promising candidates). Results from cancer cells were successfully validated in NIH3T3 cells, a robust and broadly applicable cell line for studying Hedgehog signal transduction. Examples of our results from these experiments are shown in Figure 1f-l and Figure 2c-k.

The main findings of these experiments are that PTCH1 and SMO interact with Serpinh1, Nomo1, and Nceh1. We also performed QPCR to validate the functional impact of interactors from Major Task 1. Finally, we generated cells lacking PTCH1, performed lipidomic mass spectrometry using the experimental and control cells we generated, and performed rescue experiments using exogenous PTCH1 constructs expressed in these cells. At the conclusion of the funding period, the analyses from these experiments remained in progress, and therefore our results are not summarized in the figures but will be reporting in a forthcoming scientific peer-reviewed publication supported (in part) by this award. The main findings of these experiments, to date, are that PTCH1 control lipid compartmentalization in cells.

- **What opportunities for training and professional development has the project provided?** Nothing to report.
- **How were the results disseminated to communities of interest?** The findings of the research program have been incorporated into figures for a manuscript we intend to submit for peer review and eventual publication by March 2023.
- **What do you plan to do during the next reporting period to accomplish the goals?** Nothing to report. Funding period has ended.

IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?** As anticipated, this research program revealed novel protein interactors underlying PTCH1 and SMO localization to cilia during inhibition or activation of the Hedgehog pathway, respectively. These discoveries establish a resource for future mechanistic, functional, and pharmacologic interrogation of the Hedgehog pathway in health and disease. In sum, this proposal shed light on how PTCH1 and SMO accumulate and function in cilia, and how PTCH1 inhibits SMO. Understanding mechanisms underlying Hedgehog signal transduction is critical for developing new treatments for Hedgehog-associated cancers, such as medulloblastoma.
- **What was the impact on other disciplines?** Nothing to report.
- **What was the impact on technology transfer?** No technology was transferred
- **What was the impact on society beyond science and technology?** Nothing to report.

CHANGES/PROBLEMS
Nothing to report.

PRODUCTS
Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

  David Raleigh, PI, 0.6 Cal months

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report.

- **What other organizations were involved as partners?**

Nothing to report

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

APPENDICES: N/A

LIST of Equipment Purchased with Award Funds: N/A

List of Residual Inventory of unused supplies exceeding $5,000 in value: N/A

Transition Plan: None

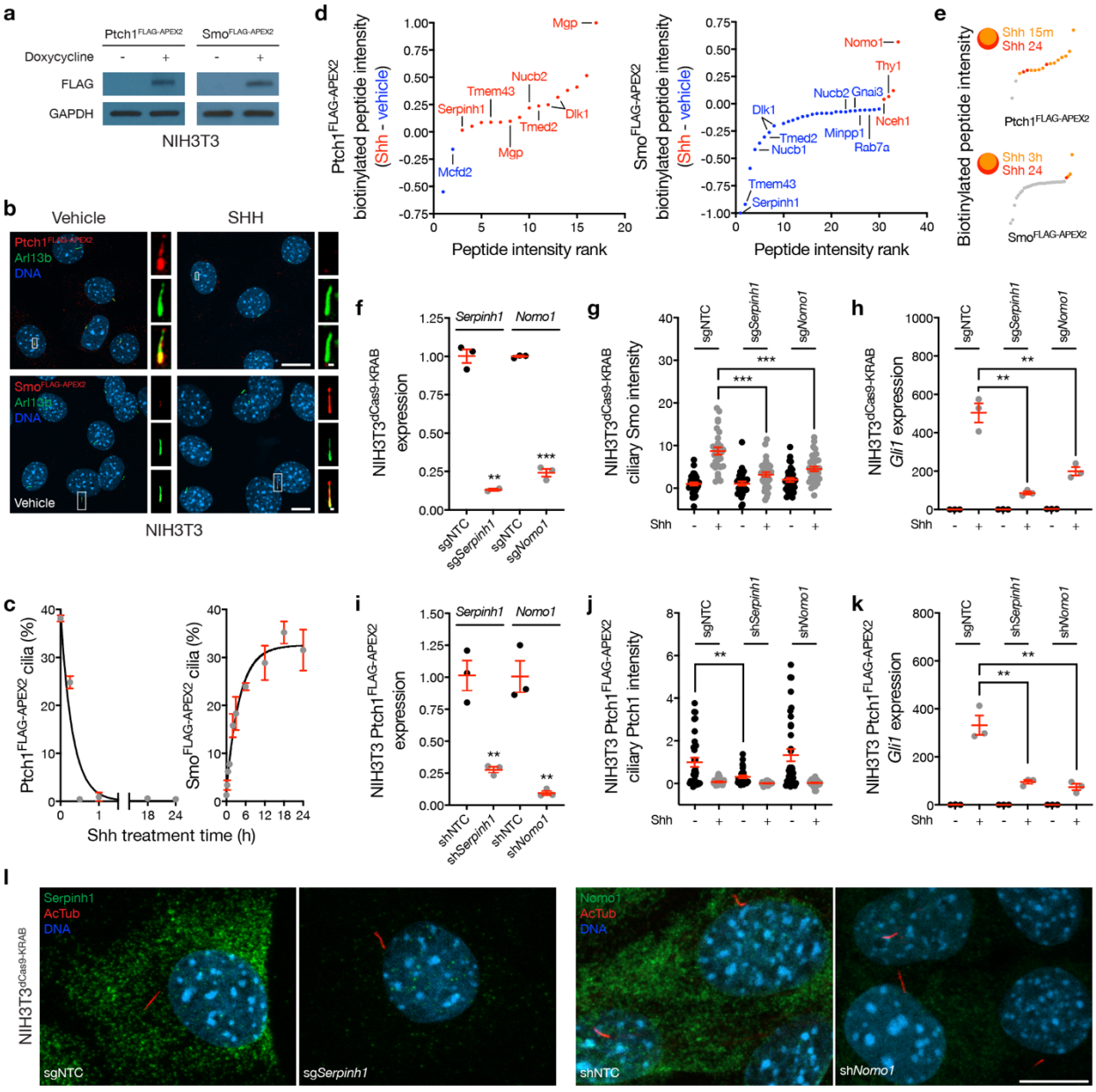

Figure 1. PTCH1 and SMO interact with Serpinh1 and Nomo1. (a-c) Generation of cells expressing PTCH1 or SMO constructs expressing FLAG-APEX2 tags for proximity labeling proteomic mass spectrometry. Immunoblots (a), immunofluorescence (b), and kinetics of ciliary trafficking of contructs to/from cilia with Hedgehog pathway stimulation from immunofluorescence (c) are shown. (d-e) Ranked candidates from PTCH1 or SMO proximity labeling proteomic mass spectrometry. (f-k) Validation of Serpinh1 and Nomo1 as important PTCH1 and SMO interactors for Hedgehog signal transduction using QPCR and immunofluorescence.

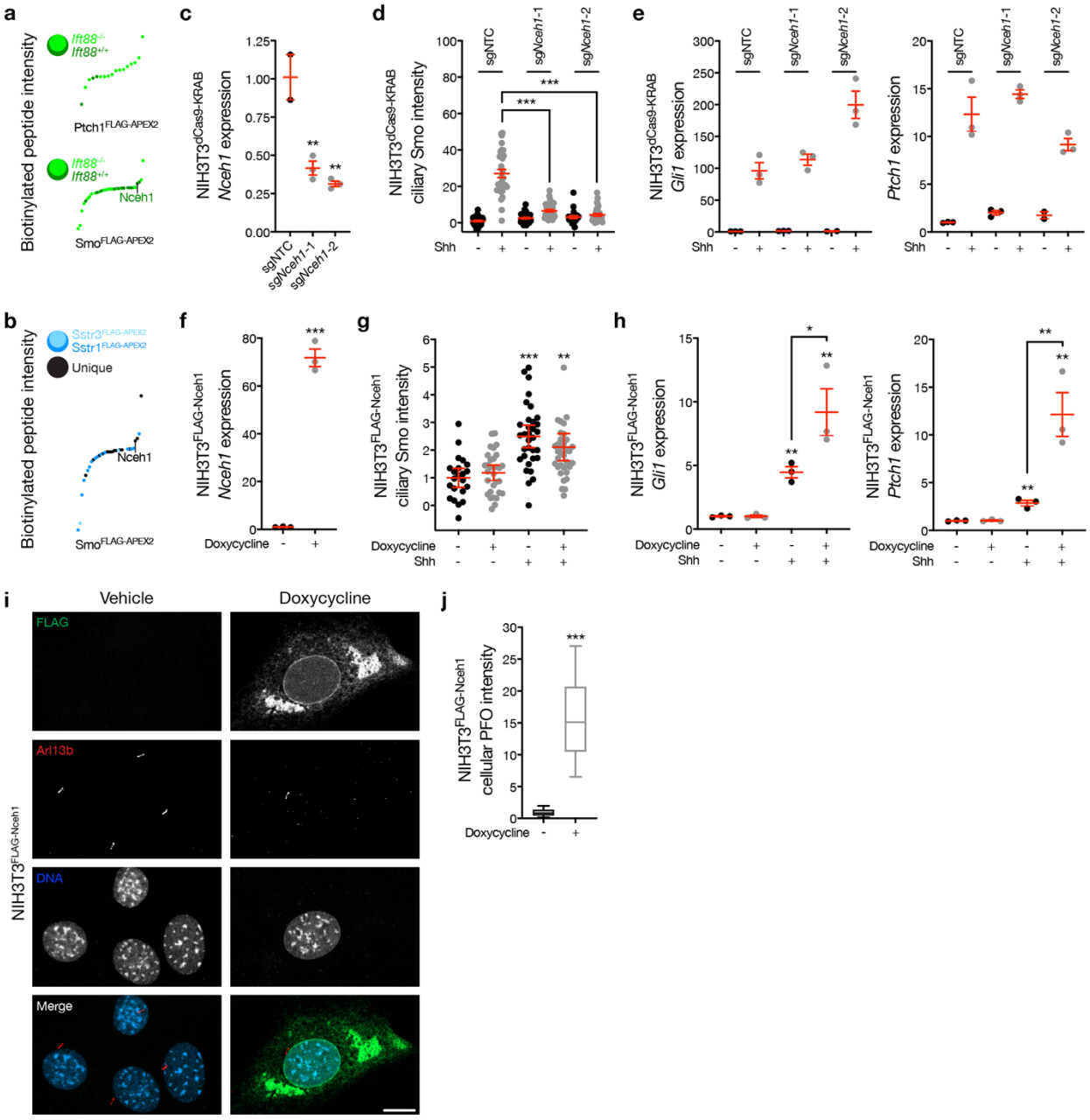

Figure 2. SMO interact with Nceh1. (a-b) Re-analysis of ranked SMO proximity labeling proteomic mass spectrometry using filtering of interactors in cells without cilia (Ift88-knockout) or of interactors that were identified in proximity to other GPCRs (SSTR) reveals a unique interaction with Nceh1. (c-k) Validation of Nceh1 as a SMO interactor that is important for Hedgehog signal transduction using QPCR and immunofluorescence.