
Reviewer A:

Scientific merit:
The scientific merit of the **AOP Report** is

high.

3R relevance:
The **AOP Report** addresses

- reduction of animal experiments.
- refinement of animal experiments (reduction of pain, suffering, distress and harm; improvement of animal welfare).
- replacement of animal experiments.

Overall impression:
The AOP Report

- discusses critically and objectively.
- considers the relevant literature.
- provides a vision for future developments.
- is targeted at an expert audience.

Language and structure:
Language and structure of the AOP Report

are excellent.

Figures:
Figures included in the AOP Report

are well designed and explained.

Comments for editor and authors

Please answer the following charge questions on the **AOP** in detail:

1. Scientific quality:
a) Does the AOP incorporate all appropriate scientific literature and evidence?
b) Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

2. Weight of evidence (WoE):
a) Is the WoE judgement/scoring well described and justified based on the evidence presented? If not, please explain.
b) Please consider WoE for each Key Event Relationship (KER) and the for the AOP as a whole.

1a. Yes, this AOP uses more recent studies. Particularly, updating that not just concentration but duration of SHH signal is affecting the AO in question is important to addressing the AO in question.
1b. Yes, it is up to date.
2a. Yes, more attention needs to be paid to the temporal dynamics of SHH signaling.
2b. They are accetpable

Reviewer B:

Scientific merit:
The scientific merit of the **AOP Report** is

high.

3R relevance:
The **AOP Report** addresses

- reduction of animal experiments.
- replacement of animal experiments.

Overall impression:
The AOP Report

- provides a good overview of the state of the art.
- discusses critically and objectively.
- considers the relevant literature.
- provides a vision for future developments.
- is targeted at an expert audience.

Language and structure:
Language and structure of the AOP Report

are mostly good (specific comments below).

Figures:
Figures included in the AOP Report

are well designed and explained.

Comments for editor and authors

Please answer the following charge questions on the **AOP** in detail:

1. Scientific quality:
a) Does the AOP incorporate all appropriate scientific literature and evidence?
b) Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

2. Weight of evidence (WoE):
a) Is the WoE judgement/scoring well described and justified based on the evidence presented? If not, please explain.
b) Please consider WoE for each Key Event Relationship (KER) and the for the AOP as a whole.

The AOP does seem to incorporate relevant scientific literature and evidence, although the subject matter is outside of my field of expertise. The scientific content seems to reflect the current state of the science.

The authors rationale for their weight of evidence calls is well explained and seems reasonable.

Please see attached file for my detailed review and suggestions for improvement.

We appreciate the detailed and constructive review of this work. We have worked to address the comments and have updated both the manuscript and the AOP-Wiki. We have focused on correcting wording of the KEs and KERs to ensure that they use consistent terminology and that all KEs and KERs are modular. We feel that these corrections have strengthened the AOP and the manuscript we have put together for it. We have included additional responses for the questions below.

Review of Altex AOP Report 460

The authors have developed an AOP linking antagonism of the smoothened receptor to orofacial cleft (OFC). Given the prominence of OFC as a birth defect, the AOP is of high relevance, and as the authors suggest, could help establish the relevance of NAMs for identifying pharmaceuticals or contaminants that could cause OFCs as a result of developmental exposures. The authors development conveys a fairly detailed understanding of the mechanistic processes which are organized into a series of key events and relationships to form the AOP. While much is known about the molecular and cellular processes of SHH signaling, the authors may want to consider whether quite as much detail is needed in terms of separate events for GLI translocation versus its subsequent effect on gene expression. However, lumping versus splitting is at the discretion of the authors and should be based on consideration of what type of indicator endpoints are likely to be measured. The graphical representation of the AOP includes a Key Event for “decrease palatal shelf outgrowth” but in the AOP-Wiki this is titled “decrease outgrowth” only. Adding the specificity for palatal shelf outgrowth is important relative to the way the KERs are structured and the way they are used in the AOP. In my opinion “decrease outgrowth” is not specific enough. Given their focus on the AOP and role of SHH signaling, many of the relationship descriptions either break modularity by making reference to other parts of the AOP that aren’t specifically involved in that relationship. That is inconsistent with the AOP framework and the goal of being able to share KEs and KERs among multiple AOPs and building AOP networks. Similarly, the terminology used doesn’t always match the level of resolution at which the events and relationships are described. While biologically, speaking terms like SHH signaling and GLI mediated expression may be more or less synonymous, for readers that are non-experts, using terms different than those in the Event and Relationship

titles may lead to considerable confusion. I would encourage the authors to really focus on aligning their text with the subject of each independent Event and Relationship page. With some additional revision, I believe this can be a strong and impactful contribution to the AOP-Wiki and the peer-reviewed literature. Specific comments are provided below, starting with the manuscript (comments identified by line number), then the AOP-wiki pages (comments identified by page ID and sub-heading).

1. Line 19. Can "GLI" be defined further, for example as glioma-associated oncogene? Perhaps Gli is the most widely used term.

   Corrected

2. Line 24. "NAMs" (new approach methodologies) should be defined on first use.
   Corrected
3. Line 25. Data are plural. The sentence should read "…data used to support this AOP were generated…."
   Corrected
4. Line 30. Consider something like "This AOP report assembles evidence that links antagonism of the Smoothened (SMO) receptor to orofacial clefts (OFCs)."
   Corrected
5. Line 33. While "facial prominences" is a correct technical term here, I'm not sure it is the optimal term to use in the plain language summary. Perhaps something like "early embryonic facial features"
   Corrected
6. Line 39. Consider using key words that aren't already in the title to increase findability during search.
   Keywords
   Sonic Hedgehog, cleft lip/palate, smoothened receptor, Craniofacial morphology , adverse outcome pathway

7. Line 54. Suggest either "…genetics and environment in the etiology…" or "…genetics and environmental factors in the etiology…."
   Corrected
8. Line 82. Suggest "clear approach" instead of "clear mechanism"
   Corrected
9. Line 83. "AOP wiki" should be "AOP-Wiki" throughout.
   Corrected
10. Line 83. (AOPs, 2024) – unclear what this citation is referring to.
    Corrected
11. Line 109. The abbreviation EMi is not used frequently in the text. Consider just spelling it out for clarity.
    Corrected
12. Line 133. Should "HH" be "SHH" here? If not, it is likely to create confusion to use both HH and SHH as abbreviations in the paper.

Corrected to SHH

13. Line 152. The sentence "The schematic of AOP 460 appears…." can be deleted. Fig. 1 can be cited parenthetically right after "This AOP…" on line 151.
    Corrected
14. Line 153. The project number the authors cite will be meaningless to most readers. Either provide more details about what kind of project you're referring to (an AOP development project under the OECD Advisory Group on Emerging Science for Chemical Assessment (ESCA)), or remove reference to a specific project and just refer to a larger network of SHH-related AOPs.
    Corrected
15. Line 157. Should use "KEs" instead of "Kes"
    Corrected
16. Line 161. A significant portion of this sentence is redundant with that on lines 154-155.
    Corrected paragraphs combined
17. Lines 161-169. This paragraph could be combined with the previous to provide the same information more concisely without redundance.
    Corrected paragraphs combined
18. Line 172. I don't think it is necessary to list the "Development status" in the AOP ID Box. By definition, all AOPs that have not undergone a peer review according to OECD guidance on the scientific review of AOPs are regarded as "under development". After the present review is completed, the status will change.
    Deleted
19. Line 180. Provide a link or citation for PubMed.
    Link added
20. Lines 180-181. Consider something like "details of the search terms employed and dates of each search are provided in Supplementary Table 1." Alternatively, just delete the sentence "searches are organized by…" and replace it with the last sentence of this paragraph (Lines 184-185).
    Corrected
21. Line 190. The sentence "Table 1 below…." Could be replaced with a simple parenthetical citation of Table 1 at the end of the preceding sentence.
    Corrected
22. Lines 194-198. Perhaps more of a curiosity question, but are any of these SMO antagonists relevant as environmental contaminants, or would use of pharmaceuticals be the primary route of human exposure?
    Good question. Of the chemicals mentioned, PBO is probably the most likely to have human exposure. The 2021 Rivera-Gonzalez paper we cite summarizes the route of exposure and sources of contamination. It is also important to note that Cyclopamine was identified through natural exposure when cyclopia in sheep was found to be linked to cyclopamine in the plan Veratrum californicum.
23. Line 201. Define GD as "gestational day" on first use.
    Corrected
24. Line 203. Consider separately addressing both the empirical domain of the evidence (i.e., limited to mice), and the plausible domain (likely applicable to an array of mammals, perhaps

even other vertebrates). See "Development tip 6" of the AOP Developer's Handbook - **Development tip 6 – *Domain of applicability***: When defining domain of applicability, it is useful to think about it in two ways **Empirical domain of applicability**: Species, sexes, life stages, for which there is already demonstrable evidence that the measurement can be made (KEs), the relationship applies (KERs) or the AOP in its entirety is relevant (AOPs). **Biologically plausible domain of applicability**: The broad range of species, sexes, life stages for which the measurement (KE), relationship (KER), or AOP is likely to apply based on scientific reasoning (i.e., molecular conservation of targets/pathways; phylogenetic relatedness; similarity in life history; analogy). Authors are encouraged to present both, and to clearly distinguish between the two based on the "evidence calls" made in the structured table and/or the explanatory text provided in the free text field.

Good idea. We have added text to include the biologically plausible domain.

25. Line 227. SANT – undefined abbreviation

    Corrected
26. Line 251. "…few studies have measured by outgrowth…." – awkward phrasing – perhaps some words missing?

    Phrasing corrected
27. Line 281. Suggest deleting "for" from "…through binding studies for including…."

    Corrected
28. Line 283-285. "while the level of support for most KERs is low…." – should that be the level of empirical support is low. Would you consider plausibility high, but empirical support being the type of evidence that is lacking? Plausibility is generally considered the strongest line of evidence. An AOP with strong plausibility and strong non-adjacent empirical support, but weaker empirical support in the adjacent KERS can often still be considered a well supported AOP.

    Corrected
29. Line 289. The sentence "A summary of the dose-concordance…." could be replaced with parenthetical citation of Supplementary Table 2 at the end of the preceding sentence.

    Corrected
30. Line 290. "Many studies were found to use a single exposure" – by "single exposure" do you mean a single concentration/dose of the test chemical?

    Corrected
31. Lines 298-314. Temporal concordance primarily pertains to the empirical evidence supporting various KERs. The argument that is presented here is largely a plausibility-based argument. If there are a lack of studies where the temporal concordance could be assessed empirically, it is fine to state that. Many studies only measure outcomes at a single time point, so these data are often absent. Lines 301-307 are also highly redundant with similar statements already made in prior text.

    Corrected- we have added text describing a lack of temporal concordance data and have removed the redundant text.
32. Lines 315-318. Not sure whether this adds much value.

    Agreed section removed

33. Lines 319-338. Rather than a bulleted list of all the KERs for which there are data gaps, consider a more narrative discussion of which endpoints are rarely if ever measured, and the types of studies, currently lacking, that would be desired.
34. Line 345. Add year information for Corbit et al.
    Corrected
35. Line 358. (53) should use author/year format consistent with the rest of references cited in the paper
    Corrected
36. Lines 375-377. The following sentence could be made much more concise "Most of the data found through the literature was obtained from doses at a single dose and was not conducted with dose-response or time-course in mind.
    Corrected
37. Lines 387-389. The following sentence could be more clear and concise "There is a need for development of NAMs to increase understanding of the…." – as currently written, I'm not sure it really adds much to the paragraph, and it is difficult to both understand what point the authors are trying to make and how it links to the sentences that follow. Perhaps it could just be deleted.
    Corrected- sentence split and additional context added
38. Lines 382-434. This section as a whole seems to lack focus. The final sentence (lines 427-428) was the most direct, and to the point sentence among the whole section. Line 383 "This AOP can help serve as a guide linking *in vitro* chemical testing data to traditional *in vivo* adverse outcomes" – that is basically the entire point of the AOP framework. The authors seem to touch on the AOPs as a way to establish the relevance of NAMs for detecting/characterizing chemicals with potential to cause OFC. However, they also speak multiple times to using the AOPs to guide the development of NAMs and their organization into an IATA or defined approach. Those are all reasonable applications, but as currently structured, I find it hard to follow and the authors seem to dance around their point without directly stating it. Suggest revising this section to make it more clear and concise as a whole. Right now it seems like the authors were struggling to define their intended application(s).
    We agree that the initial submission lacked focus. We have updated the section to increase the focus and make the points clear to the reader.
39. Table 1. Support for essentiality of the KEs section. The second two bullets don't seem to pertain to essentiality per se. In general, evidence is evidence and I don't thinks its productive to get too hung up on exactly the type of evidence, so I leave it to the authors to decide where these points fit. The key is to really critically assess how well the overall body of evidence supports a causal relationship between the sequence of KEs.
    We agree that these points may not be the perfect fit for this table. After review we decided to leave them in as we felt they needed to be included and did not feel there was a more appropriate place.
40. Table 1. Empirical evidence for relationship 2894. **Dose-response:** Multiple studies demonstrate a dose dependent incidence of clefting – dose response concordance is not just a measure of whether the downstream event occurs in a dose-dependent manner. Rather, it considers the relative doses at which the upstream and downstream events occur. At what concentration is smoothened antagonized versus the concentrations that cause OFC. Concentrations that elicit the downstream effect should be equal to or higher than those that cause the effect defined by the upstream event (MIE in this case).
    Good point. We have added additional text to make this distinction clear.

41. Supplementary Table 2. Useful. Highlights the approach to AOP development taken by the authors and helps to reinforce the authors points about the lack of studies designed for evaluation of temporal concordance, dose-response concordance, etc. (which is not uncommon).
42. AOP Page 460, Abstract: "This decrease in gene expression which causes a…." – could delete "which"

    AOP-Wiki updated
43. AOP Page 460, Context: "The etiology of OFCs is complex with approximately 50% of CPO and 70% of CL/P considered non-syndromic (2011)." – add author information to the reference citation.

    AOP-Wiki updated
44. AOP Page 460, Context: As per the Developer's Handbook, Context is intended to define "Why" this AOP was developed. The information provided here is useful biological context/background, but it does not describe the authors' motivations and goals. From the Handbook "This subsection describes key elements of *why* the AOP was developed and for whom (e.g., funding sources; stakeholders; etc.)." - https://aopwiki.org/handbooks/5#section-1-%E2%80%93-aop-description-27

    This AOP was developed as part of a larger network of AOPs linking disruption of SHH signaling with OFCs (OECD Advisory Group on Emerging Science in Chemicals Assessment (ESCA) workplan project 1.101.). This was the first AOP of the network to be developed and was selected due most stressors of the SHH pathway being believed to work at the level of SMO. Development was led by the Johnson lab at Michigan State University and coached by Dr. Judy Choi. This AOP serves as the primary literature for graduate student Jacob Reynolds' dissertation project. This work was supported by the National Institutes of Health R00-ES028744 and the National Institute of Environmental Health Sciences P42ES004911.

45. AOP Page 460, Strategy: As per the Handbook, the Strategy section is intended to describe " *how* the AOP was developed. Specifically, what was the strategy, focus and workflow for identification and assembly of relevant evidence to meet the objective/envisaged application?" – think of this as sort of the Methods section of an AOP development project. The early part of this section "*This AOP was developed as part of a larger network of AOPs linking disruption of SHH signaling with OFCs (EAGMST workplan project 1.101.). Orofacial clefts (OFCs) are one of the most common human birth defects and occur in approximately 1-2/1,000 live births (Lidral, Moreno et al. 2008). Early orofacial development involves epithelial ectoderm derived SHH ligand driving tissue outgrowth through an induced gradient of SHH dependent transcription in the underlying mesenchyme, which is thought to drive mesenchymal proliferation (Lan and Jiang 2009, Kurosaka 2015). The SHH pathway is sensitive to chemical disruption at multiple molecular targets along the signaling cascade, with exposure during critical windows in development leading to OFCs (Lipinski and Bushman 2010, Heyne, Melberg et al. 2015). The molecular targets of this disruption include SHH ligand modification with cholesterol and palmitoylate, ligand secretion, mesenchymal reception, and signal transduction (Jeong and McMahon 2002, Lauth, Bergström et al. 2007, Petrova, Rios-Esteves et al. 2013).*" Would probably align better with Context. Starting at "This AOP focuses on the disruption to SHH signaling resulting in antagonism of the SMO receptor. To select the key events for the AOP, we used…." The text is relevant to strategy.

Wiki updated- To select the key events for the AOP, we used existing knowledge of the pathway along with reviews of the SHH pathway to assemble a path that was physiologically plausible. Care was taken to select events that would be of direct regulatory relevance (i.e. a method to quantify exists). To identify sources and data for each Key Event Relationship (KER), Pubmed was used. Initially results were screened for relevance off title/abstract and any of suspected relevance were reviewed in full to determine their applicability for the KER. Each KER includes a table of relevant search information (date, search terms, citations, etc). It is the hope of the authors that this AOP is used as a tool for risk assessment for drug and chemical exposures during embryonic development when disruption to SHH through antagonism of SMO occurs.

46. AOP Page, 460. As per the handbook, "Prototypical stressors Prototypical stressors are stressors for which responses at multiple KEs in addition to the MIE have been well documented. Experiments with the prototypical stressor(s) may have provided much of the empirical support for the AOP and/or quantitative understanding of the KERs. Thus, prototypical stressors identified may serve as useful "positive controls" for evaluating responses of other stressors that may act on this pathway and/or provide insights into the types of structures or properties that may be relevant to the stressor domain that is relevant to this AOP. The relative potency of various other stressors, compared to the prototypical stressor(s) may also be informative relative to quantitative understanding of the KERs and associated applications of the AOP." Please critically evaluate whether all the stressors listed meet the definition. Based on some of the uncertainty around cyclopamine, for example, perhaps that should not be listed. The goal is not to provide a comprehensive list, but rather a small number of stressors that are emblematic of the AOP.
    We have reviewed the provided list and have removed cyclopamine and PBO from the prototypic stressors. While we are confident that these compounds antagonize SMO, we agree that they may not have the level of evidence to classify as prototypic stressors for this AOP.
47. AOP Page 460. Domain of applicability – chemical. PBO should be spelled out on first use to make it clear you're referring to piperonyl butoxide.
    Wiki updated
48. AOP Page 460. Domain of applicability – taxonomic. Would it more appropriate to indicate that the empirical domain of the applicability is mouse. Consider remarking on the plausible domain of applicability as well. Seems like it would apply pretty broadly to mammals. Not necessarily to non-mammalian vertebrates though.
    Wiki updated- Taxonomic: At present, the empirical taxonomic applicability domain of this AOP is mouse (mus musculus). Most of the toxicological data that this AOP is based on has used mice as their model organism. Mice are a good analog of human craniofacial development and undergo similar signaling by SHH. The plausible domain of applicability for this AOP is mammals due to the largely conserved mechanisms of orofacial development and embryonic pathway signaling.
49. AOP Page 460. Relationship 2726. "OFCs caused by disruption to SHH are believed to be due to a reduction in epithelial induced mesenchymal?" – sentence should end with a period, not a question mark.
    Wiki updated
50. AOP Page 460 – Biological plausibility section – "Multiple antagonists of the SMO receptor have been identified through [binding studies for including] cyclopamine, vismodegib, PBO, and the SANT compounds" - missing word(s) and/or punctuation in area indicated by brackets.
    Wiki updated- Multiple antagonists of the SMO receptor have been identified through binding studies. Identified SMO antagonists include cyclopamine, vismodegib, PBO, and the SANT

compounds (Lipinski, Dengler et al. 2007, Lipinski, Song et al. 2010, Wang, Lu et al. 2012, Everson, Sun et al. 2019, Rivera-González, Beames et al. 2021).

51. AOP Page 460 – Concordance of dose-response relationships – Concordance is evaluated under empirical evidence. In general, the section would benefit from some reorganization and streamlining. Currently doesn't flow well.
    Agreed, Wiki updated- There are a limited number of studies in which multiple key events were assessed in the same study following exposure to known SMO antagonists. These studies form the basis of the dose-response concordance of this AOP. A summary of the dose-concordance can be found in Supplementary Table 2. Many of the studies identified while researching this AOP were performed using a single dose of antagonist making the study not suited for dose response concordance. This AOP would benefit greatly from increased studies designed to explore the dose-response concordance of the proposed relationships. The concentration-dependence of the key event responses regarding concentration of known in vitro and/or in vivo for some of the KEs in this AOP is summarized below.
    - Concentration dependent clefting with cyclopamine exposure (Omnell, Sim et al. 1990)
    - Dose dependent binding to SMO (Chen, Taipale et al. 2002)
    - Concentration dependent decrease in SMO-ciliary accumulation in vitro for vismodegib exposure (Wang, Arvanites et al. 2012)
52. AOP Page 460. Temporal concordance. Much of this section pertains more to plausibility, chemical modulators. Its really only the final statement that has much to do with temporal concordance. Consider removing most of the extraneous text and keep this focused. Otherwise, the user of the AOP has to constantly sift through redundant background and over-exposition to find the key information they are looking for in each section.
    Wiki updated- extraneous text removed last sentence retained
53. AOP Page 460 – assessment of the quantitative understanding of the AOP: "Most of the data found through the literature search was obtained from doses at a single dose and was not conducted with dose-response or time-course in mind." - Perhaps instead something like "…was obtained from studies that employed a single dose…."
    Most of the data found through the literature search was obtained from studies that employed a single dose and were not conducted with dose-response or time-course in mind.
54. AOP Page 460 – considerations for potential applications of the AOP: This section on the AOP page is much better focused than that in the accompanying AOP report.
55. Event 2027 – life stages: "Aberrant activation of HH signalling is known to cause cancer (Dahmane, Lee et al. 1997, Kimura, Stephen et al. 2005)." The relevance of this defining the life stage applicability of this key event is not clear. Suggest deleting.
    Wiki updated- we felt that including both the embryonic and cancer angle supports the defined life stages. "Life stages- The Hedgehog pathway is a major pathway in embryonic development. While the pathway is largely inactive following development, aberrant activation of SHH signaling is known to cause cancer (Dahmane, Lee et al. 1997, Kimura, Stephen et al. 2005). For these reasons all stages of life are of relevance."
56. Event 2027 – taxonomic. If SMO is conserved in both vertebrates and invertebrates, why are the controlled vocabulary terms for taxonomic applicability limited to vertebrates?
    Wiki updated- this was a oversight since I was thinking only in the context of AOP 460
57. Event 2027 – How it is measured or detected. QPCR cannot be used to determine antagonism of SMO, therefore, should not be included here.
    Wiki updated- removed

58. Event 2044 – Life stage – can a decrease in SMO relocation and activation only be determined in embryos? If not, the KE itself should be applicable to more life stages, even if the rest of the AOP is not.
    Wiki updated- All life stages added
59. Event 2028 – Life stage – does translocation of GLI1/2 only occur during embryonic development? If it occurs later in life as well, the life stage applicability of this KE should be expanded, even if the rest of the AOP does not apply to later life stages. Also, the text contradicts the controlled vocabulary selection – which indicates all life stages are relevant.
    Wiki updated- All life stages added

60. Event 2040 – Sex – "….and differences in gene expression has not been demonstrated…." - "has" should be "have"
    Wiki updated
61. Event 2040. Life stages – only refers to HH pathway. In order for this to make sense to readers, the role of GLI1/2 as part of the HH pathway should be stated as part of this line of argument.
    Wiki updated- Life stages- The Hedgehog pathway with the main transcription factors of GLI1/2 is a major pathway in embryonic development. Aberrant activation of HH signaling is known to cause cancer (Dahmane, Lee et al. 1997, Kimura, Stephen et al. 2005). For these reasons all stages of life are of relevance

62. Event 2040. Key Event Description – "…on SMO is relieved. SMO this then able to…." – "this" should be "is"
    Wiki updated
63. Event 1262. I realize the current authors may not have been responsible for this Key Event description, but the following information violates the modularity of KE descriptions that is recommended in the AOP Developer's Handbook as well as other guidance on AOP development "Several stimuli such as hypoxia, nucleotides deprivation, chemotherapeutical drugs, DNA damage, and mitotic spindle damage induce p53 activation, leading to p21 activation and cell cycle arrest [Pucci et al., 2000]. The SAHA or TSA treatment on neonatal human dermal fibroblasts (NHDFs) for 24 or 72 hrs inhibited proliferation of the NHDF cells [Glaser et al., 2003]. Considering that the acetylation of histone H4 was increased by the treatment of SAHA for 4 hrs, histone deacetylase inhibition may be involved in the inhibition of the cell proliferation [Glaser et al., 2003]. The impaired proliferation was observed in HDAC1-/- ES cells, which was rescued with the reintroduction of HDAC1 [Zupkovitz et al., 2010]. The present AOP focuses on the p21 pathway leading to apoptosis, however, alternative pathways such as NF-kappaB signaling pathways may be involved in the apoptosis of spermatocytes [Wang et al., 2017]."
    We agree that this text of this KE violates the modulatory of the KE. We did not create or edit this event and do not feel it is our responsibility to edit this information. This event is shared by so many authors that getting consent from all authors that use this AOP would likely prove problematic and time-consuming. We brought this concern up with our Coach (Judy Choi) and she was going to bring this concern up to the OECD advisory group and the other AOP coaches.
64. Event 2043. Ok
65. Event 1821. Ok
66. Event 2041. Event component – since the process terms relate to failure to meet at midline and abnormal shelf fusion, shouldn't the action term in the event components be increased?

This event focuses on the outgrowth of the palatal shelves, not specifically fusion. We updated the event components to now include the process "palatal shelves fail to meet at midline" with objects of primary and secondary palate and action increased. Upon further review of the KE we decided to remove the components regarding fusion as the event focuses on tissue outgrowth, not on the process of fusion. We feel that while the event components are increased, the KE focuses on the outgrowth of the tissue which is this case is decreased.

67. Event 2042. Title – there should be an action term like "increased"
    Wiki updated- Increase, Orofacial clefting
68. Relationship 2734. Relationship description – the following sentence does not seem relevant to this specific relationship "This relocation then leads to signaling to effectors resulting in the activation of the GLI transcription factors and the subsequent induction of HH target gene expression (Alexandre, Jacinto et al. 1996, Von Ohlen and Hooper 1997)."
    Wiki updated- sentence removed
69. Relationship 2734 – in vitro – presumably um should be µM (micromolar, not micrometers)
    Wiki updated- u to µ
70. Relationship 2734 – in vivo – the first evidence bullet would be better suited for relationship 2894. "The presence of critical periods for disruption of SHH was investigated using C57BL/6J mice. Vismodegib was suspended at 3mg/ml in 0.5% methyl cellulose and 0.2% tween. Pregnant dams were administered 40mg/kg vismodegib at GD7.0, 7.25, 7.5, 7.75, 8.0, 8.25,8.5, 8.625, 8.75, 8.875, 9.0, 9.25, 9.5, 9.75, and 10.0. Cyclopamine was dosed at 120mg/kg/d via subcutaneous infusion between GD8.25-9.375. Pregnant dams were euthanized at GD17 and fetal specimens were collected and fixed for imaging. The control group consisted of fetuses exposed to 0.5% methyl cellulose and 0.2% tween at GD7.75, 8.875, or 9.5. Acute exposure to vismodegib resulted in a peak incidence of lateral cleft lip and palate at GD8.875 (13%). Exposure at GD9.0 and 10.0 resulted in clefts of the secondary palate only (34%). A higher penetrance (81%) was found for cyclopamine exposure (Heyne, Melberg et al. 2015)."
    We agree that this study is not directly relevant to KER 2734. We have removed it from KER 2734 and have made sure it is included in KER 2894.
71. Relationship 2734 – in vivo – the following line of evidence does not seem to directly pertain to SMO antagonism leading to SMO relocation. "To explore how a conditional loss of primary cilia on neural crest cells Kif3af/f Wnt1-Cre mice were used to explore the molecular basis of aglossia. Aglossia was found to be due to a lack of mesoderm derived muscle precursor migration. RNA-seq was used on E11.5 embryos on the mandibular prominces of wildtype and knock mice. The key SHH readout, GLI1 was downregulated two-fold in mutants (Millington, Elliott et al. 2017)."
    We agree that these data are not directly relevant to the KER in question. It has been removed and the Wiki updated.
72. Relationship 2735. "and that aberrant SHH signaling during embryonic development can cause birth defects including orofacial clefts (OFCs)" – this statement is specific to the AOP and does not pertain to this specific relationship. Suggest deleting to maintain modularity of the Relationship. Same comment for "Multiple ciliopathies are associated with clefting in humans including Meckel-Gruber syndrome (OMIM 249000) and Ellis-van Creveld syndrome (OMIM 225500)(Brugmann, Cordero et al. 2010)."
    Wiki updated
73. Relationship 2735 – in vivo – the fact that GLI1 was downregulated in mutants that develop aglossia does not seem pertinent to a relationship between decreased relocation of SMO and

activation of GLI1/2. None of the bullets under the in vivo subheading seem to speak directly to the two Events that make up this relationship.

We agree that the data reported here is not specific to the relationship in question. We have removed it and updated the wiki.

74. Relationship 2735 – uncertainties and inconsistencies. It would be helpful if the authors could add a sentence or two regarding why knowing the exact mechanism of SMO ciliary trafficking is critical to understanding whether or not SMO relocation will activate GLI1/2.

Good idea. We have added the following text to the Wiki. "Improving understanding of SMO ciliary trafficking will increase the fields' understanding of SHH signaling. Understanding the mechanisms at play will help identify when SMO relocation will affect the SHH signaling cascade and subsequent GLI1/2 translocation."

75. Relationship 2735 – quantitative understanding – it is not clear to me why the role of SUFU in SHH signal transduction is relevant to this particular relationship.

We have updated the section. " The data presented in support of this KER includes in vitro studies. The in vitro work offers data that SMO relocates to the tip of the primary cilium and that this plays a role in the translocation of the GLI transcription factors to the nucleus. The quantitative understanding of this linkage is low as studies including dose-response and time-course were not found."

76. Relationship 2721 – "The SHH pathway is well understood to be fundamental to proper embryonic development and that aberrant SHH signaling during embryonic development can cause birth defects including orofacial clefts (OFCs). For this reason, this KER is applicable to the embryonic stage with a high level of confidence." – the domain of applicability for the relationship should be evaluated solely on the taxa, species, and life stages for which the GLI translocation leading to decreased GLI target gene expression is relevant. The fact that the rest of the AOP leads to OFC which is only relevant during development does not affect the domain of applicability for this particular relationship, which can be shared with other AOPs (same comment applies to Relationships 2731,2732, 2724, 2882 as well).

Thank you for catching this. We have reviewed the mentioned events and have removed the problematic text to ensure modularity of the KERs.

2721, 2731,2732, 2724, 2882 - Wiki updated text removed

77. Relationship 2721 – in vivo. The following line of evidence does not appear to have any direct relationship to GLI (at least none is clearly stated). "To study whether SHH signalling regulates the developmental fate of the ecto-mesenchyme via regulation of gene activity in the facial primoridia, Wnt1-Cre;Smon/c, (removal of SHH signalling) and Wnt1-Cre;R26SmoM2 (activation of SHH signalling). Positive regulation from SHH activity was found for Foxc2, Foxd1, Foxd2, Foxf1, and Foxf2. The Fox genes were found to be dissimilar in expression pattern with spatial activation even with uniform activation of the SHH pathway. Foxc2 and Foxd1 were found to be expressed ubiquitously in the MNA except at the midline, while Foxf1 is expressed at the lateral ends. Foxd2 and Foxf2 are both expressed along the mediolateral axis with Foxd2 having an increasing gradient from medial to lateral and Foxf2 having an opposing gradient (Jeong, Mao et al. 2004)."

We agree that as originally written the connection to GLI1/2 is not apparent. We have added additional text including:" These data support that disrupting GLI1/2 translocation via disruption of the SHH signaling pathway disrupts transcription of Foxc2, Foxd1, Foxd2, Foxf1, and Foxf2." The Wiki has been updated.

78. Relationship 2731 – Here and elsewhere it may be confusing for readers if the authors use shh target gene expression and GLI target gene expression more or less interchangeably. The

relationship speaks to GLI – therefore the text should make it clear that what you're referring to is GLI-mediated expression. This is a significant issue that makes the relationship description and evidence hard to follow. The relationship between the broad term SHH signaling and the role of GLI may be obvious to experts in this biology, but it is critical that users of the AOP, who will not necessarily be experts on this topic, can follow the logic and lines of evidence.

We have reviewed the event and updated the text to be expand on the relationship between SHH and GLI and specify GLI mediated transcription.

79. Relationship 2731 – The following has nothing to do with this specific relationship. Breaks the modularity of the relationship description: "A network of reciprocal growth factor signaling between the epithelium and mesenchyme is required for proper growth and patterning of the early palatal shelves." Could start with "Activation of the SHH pathway…." – but even that isn't entirely modular, as the relationship is focused on GLI mediated gene expression, which seems more specific.

    We have removed this text from the Wiki.

80. Relationship 2731 – An explicit statement or list of "the following genes are thought to be regulated by GLI as a component of SHH signaling", or something to that effect, may be useful: e.g., BMP2, BMP4, Fgf10

    Wiki updated- Activation of the Sonic Hedgehog (SHH) pathway results in a downstream signaling cascade resulting in the relocation of GLI to the nucleus and subsequent gene transcription (Carballo, Honorato et al. 2018). This gene expression drives secondary messenger signaling for the pathway. The following genes are believed to be regulated by GLI as a component of SHH signaling: FGF10, BMP2, BMP4.

81. Relationship 2732 – "A network of reciprocal growth factor signaling between the epithelium and mesenchyme is required for proper growth and patterning of the early palatal shelves." – this sentence is outside of the scope of the current relationship. Same applies to "Activation of the SHH pathway results in a downstream signaling cascade resulting in the relocation of GLI to the nucleus and subsequent gene transcription (Carballo, Honorato et al. 2018)"

    Wiki updated- text removed

82. Relationship 2732 – Unclear how the current lines of evidence relate to cell proliferation "To determine if SHH can induce Fgf10, SHH overexpressing cells were implanted in the anterior region of the wing bud of chick embryos. By 27 hours, the expression of Fgf10 had significantly increased and expanded from the anterior mesenchyme to the bifurcating wing bud (Ohuchi, Nakagawa et al. 1997). To investigate whether MSX-1 is in the same pathway as Fgf10, MSX-1 expression was examined in Fgf10-/- mice and Fgf10 expression was examined in Msx-1-/- mice. No change in…."; "Fgf8 activity was found to sustain ccnd 2 expression in the neural groove and that the attenuation of fgf signalling is necessary for the up regulation of ccnd 1. This was conducted using chick embryos and replacing a small piece of the rostral presomitic mesoderm with an Fgf8 soaked bead. To test the necessity of the Fgf pathway, SU5402 treatment was used (Lobjois, Benazeraf et al. 2004). Cyclopamine treatment of stage 9-10 chick embryos in the neural tube and neural grove resulted in a strong down regulation of ccnd 1 transcripts as well as SHH target genes (e.g. Gli1). Toxicity was assessed using sox2 and effects due to non-specific toxicity were not found. Ccnd 2 expression was not affected by cyclopamine treatment. This suggests that the iniation of ccnd 1 in the neural groove is SHH dependent while ccnd 2 is not (Lobjois, Benazeraf et al. 2004).

    We have revised the list of evidence with a focus on relating to the specific relationship. The problematic lines noted have been removed.

83. Relationship 2724 – Title "decrease outgrowth" – outgrowth of what? Event title and associated relationship title should convey more clearly what kind of "outgrowth" the event

refers to. Remember, the event and relationship pages are modular and should be able to stand independent of the specific AOP they are linked to. Probably should be "decrease outgrowth of facial prominences" or "outgrowth of palatal shelf"

We have updated this to decrease, facial prominence outgrowth. While KE 2041 was titled correctly, we had the short name simply as decrease, outgrowth. This has been corrected and updated.

84. Relationship 2724 – KER description – without better defining what kind of "outgrowth" is being referred to, the paragraph on the development of the face seems out of context – or at least unclear why this is here.

    We have updated the title to include specificity to the facial prominences.

85. Relationship 2726 – More specificity to "decrease outgrowth" is needed for OFC to make sense. Also, because the short title of the Event is used for the KER title, I would recommend spelling out orofacial cleft even in the short title of the OFC event.

    Wiki updated- Decrease, facial prominence outgrowth leads to orofacial cleft

86. Relationship 2792 – This statement would make more sense here if outgrowth was specifically defined as outgrowth of facial prominences or palatal shelf "The SHH pathway is well understood to be fundamental to proper embryonic development and that aberrant SHH signaling during embryonic development can cause birth defects including orofacial clefts (OFCs). "

    Wiki updated- facial prominences specified

87. Relationship 2792 – The last sentence of the KER description is the only one that seems to make any reference to the role of apoptosis in the outgrowth process. The relationship to apoptosis should be better explained.

    Wiki updated- we agree that the original description was lacking. We have added additional text and references to better explain the role that apoptosis is believed to play in cleft formation.

88. Relationship 2792 – this specific relationship has nothing to do with SHH signaling (should be able to exist independent of its relationship to SHH signaling). Therefore, under biological plausibility "The SHH pathway is known to be associated with cell survival and that disruption of SHH signaling can lead to increased apoptosis." Doesn't really have anything to do with the current relationship.

    Wiki updated- text pertaining to SHH removed.

89. Relationship 2792 – the following lines of evidence have no direct relevance this this relationship, as they make no mention of apoptosis "SHH expressed in thickened palatal epithelium prior to palatal shelf outgrowth (E13.0-14.5) (Rice, Connor et al. 2006) SHH is expressed in oral epithelium and shown as a key signal for palatal shelf outgrowth in explant culture (Lan and Jiang 2009)."

    Wiki updated- we have removed the evidence that does not directly relate the events in question.

90. Relationship 2792 – the relationship is between apoptosis and outgrowth, so why is the "uncertainties and inconsistencies" section focused on SHH and cell survival. Again, this may seem obvious to the authors, but will be confusing for readers who are non-experts.

    Wiki updated- this was a problem with modularity. We have corrected the wiki to remove mention of SHH and focus only on apoptosis and orofacial development.

91. Relationship 2882 – The current relationship description has nothing to do with the two key events being linked here. The description should be completely revised.

    Wiki updated- we agree that the initial description was lacking. We have revised and updated it.

92. Relationship 2882 – the first sentence of "biological plausibility" is not relevant to this relationship. Delete or revise.
    Wiki updated- deleted
93. Relationship 2882 – in vivo – good, these lines of evidence are directly relevant to the two events being linked
94. Relationship 2894. The in vitro data basically establish the types of stressors that one would predict to lead to OFCs if this relationship holds up. Might want to add a sentence explaining that relationship to the in vivo evidence since OFC itself cannot be evaluated in vitro.
    Good idea. We have updated the Wiki to include text to explain this connection.
95. Relationship 2894. The relevance of this line of evidence to relationship 2894 is unclear "To explore how a conditional loss of primary cilia on neural crest cells Kif3af/f Wnt1-Cre mice were used to explore the molecular basis of aglossia. Aglossia was found to be due to a lack of mesoderm derived muscle precursor migration. RNA-seq was used on E11.5 embryos on the mandibular prominences of wildtype and knock mice. The key SHH readout, GLI1 was downregulated two-fold in mutants (Millington, Elliott et al. 2017)."
    Wiki updated- this entry has been removed.
96. Relationship 2894. Uncertainties. Whether or not cyclopamine blocks SMO relocation to primary cilia has no relevance to the pair of KEs linked by this relationship. Suggest removing from this KER. Similarly under time scale, it is unclear why the authors are focused on "relocation" which is not relevant to this particular KER.
    Wiki updated- we have removed the text relating to SMO relocation and have updated both the uncertainties and time scale sections.

Article type: AOP Report

Title: AOP 460: Antagonism of Smoothened receptor leading to orofacial clefting

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Summary

Sonic Hedgehog (SHH) is a major intercellular signaling pathway involved in the orchestration of embryogenesis, including orofacial morphogenesis. The SHH pathway is sensitive to disruption, including both genetic predisposition as well as chemical induced disruption at multiple molecular targets including antagonism of the SHH signal transducer Smoothened (SMO). Here we report the Adverse Outcome Pathway (AOP) that describes the linkage between antagonism of the SMO receptor, a key intermediate in the Hedgehog signaling and orofacial clefts (OFCs) (AOP 460 in the Collaborative Adverse Outcome Pathway Wiki). Multiple antagonists of SMO have been identified including natural compounds, synthetic pharmaceuticals, and a common pesticide synergist. Activation of the SHH pathway causes a signaling cascade that culminates with the transcription of genes driven by glioma-associated oncogene (GLI) transcription factors. When SMO is antagonized during normal development, the cascade is disrupted causing myriad phenotypes at different critical windows of exposure ranging from major structural defects and spontaneous abortion early in gestation to reduced outgrowth of the facial prominences and the formation of an OFC later in development. There is high evidence that antagonism of SMO causes OFCs that include a dose response relationship with incidence of clefting. Several emerging new approach methodologies (NAMs) offer the ability to monitor intermediate key events and test for temporal and dose response relationships *in vit*ro. While most data used to support this AOP were generated using mouse (*Mus musculus*) models during embryonic development, SHH and the development of the face is largely conserved between mouse and human making this AOP able to be extrapolated to risk assessment for human exposures.

Plain language summary

This AOP report assembles evidence that links antagonism of the Smoothened (SMO) receptor to orofacial clefts (OFCs). The Sonic Hedgehog (SHH) pathway, crucial for orofacial development, can be disrupted by various SMO antagonists. Inhibiting SMO during critical developmental windows disrupts the SHH pathway, leading to reduced growth of early embryonic orofacial features and the formation of OFCs. This AOP was found to have high biological plausibility, but with multiple data gaps in dose response or time course data. This AOP is intended to serve as a tool for risk assessment for drug and environmental exposures during embryonic development. It is hoped that the information presented

can help form a basis for the development of new approaches to testing to reduce the animal testing burden.

Keywords

Sonic Hedgehog, cleft lip/palate, smoothened receptor, craniofacial morphology, adverse outcome pathway

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1. Introduction and background

Orofacial clefts (OFCs) are one of the most common structural human birth defects and occur in approximately 1 in 700 live births (Mossey et al., 2009; Dixon et al., 2011a). Elucidating the complex etiologies that underly OFCs is paramount to working towards the goal of reducing OFCs. Many genetic sequencing studies on children with OFCs and their family have been completed, yet approximately 80% of OFCs are found to be of an unknown origin (Yaqoob et al., 2013; Feldkamp et al., 2017), reviewed in (Ye and Ahmed, 2022). Twin studies suggest complex genetics and environmental factors in the etiology where monozygotic twins have a 50% proband concordance and dizygotic twins have a concordance rate of 8% for cleft lip with or without cleft palate (CL/P) indicating a role of both genetics and environmental in the etiology of CL/P (Lin et al., 1999; Grosen et al., 2011).

Contributions to OFCs from environmental causes, including chemical exposures to human populations during critical windows of development is highly probable but remains underexplored. Using data from the 2014 US EPA ToxRefDB V1.0 dataset, we identified that 37 of 672, or 5.5% of all chemicals tested for prenatal developmental toxicity in animal models showed an increased incidence of cleft lip and/or palate (2014). In humans, maternal smoking has been shown to have a moderate role in etiology increasing the odds ratio for OFC incidence to 1.42 (reviewed in (Fell et al., 2022)). Variants in GSTT1 (glutathione S-transferase theta) or NOS3 (nitric oxide synthase 3) have been found to increase the risk of clefting when combined with maternal smoking indicating that gene-environment interactions also play a role in OFC etiology (reviewed in (Dixon et al., 2011b)).

There are tens of thousands of chemicals in commerce that have not undergone developmental and reproductive toxicity (DART) testing and new strategies are required to evaluate and prioritize further testing of these compounds. Both the critical window of orofacial development sensitive to exposure and multifactorial causes of disruption challenge elucidation of the underlying disruptions leading to OFCs, but some established mechanisms are known. Orofacial development requires precise signaling through multiple pathways including Sonic Hedgehog (SHH), transforming growth factor beta (TGF-ß), bone morphogenic protein (BMP), epidermal growth factor (EGF), Wingless (WNT), and other pathways (Bush and Jiang, 2012; Jiang et al., 2006; Lan et al., 2015). Environmental disruption of these primary morphogenetic signaling pathways in the embryo can lead to OFCs. The SHH pathway is known to have critical windows when signaling is sensitive to exposure (Heyne et al., 2015). For example, multiple studies in animal models exposed to higher concentrations of the Hedgehog (HH) pathway receptor Smoothened (SMO) antagonists, namely Vismodegib and Cyclopamine have high incidence of OFCs (Heyne et al., 2015; Lipinski et al., 2010a). In order to facilitate regulatory decision making on the testing and registration of new chemical entities, robust assays capable of detecting disruption at multiple places along these embryonic signaling pathways are needed to replace traditional animal testing. Thorough mapping of the pathways and identifying the endpoints of interest is the first step in creating these assays.

The Adverse Outcome Pathway (AOP) is an analytical framework that describes the biological pathway between exposure to a stressor (e.g. environmental toxicant) and an adverse outcome (AO) (OECD, 2024b). This framework provides a clear approach to continue mapping these pathways and organizes the information needed for assay development in a central repository (AOP-Wiki) (SAAOP, 2024). This AOP focuses on disruption of the SHH pathway through antagonism of the SMO receptor leading to OFCs. This AOP is intended to serve as a tool for risk assessment for drug and chemical exposures during

embryonic development, when disruption to SHH through antagonism of SMO occurs. It is hoped that increasing the fields' understanding of what exposures are risks for causing OFCs can lead to targeted prevention strategies and improved patient outcomes.

Development of the orofacial processes The development of the face occurs early in embryogenesis and involves precise coordination of multiple tissues (reviewed in (Som and Naidich, 2013)). Briefly, the oropharyngeal membrane appears early in the 4th week of human gestation and gives rise to the frontonasal process and the 1st pharyngeal arch. The frontonasal process is derived from the neural crest derived mesenchyme along with neuroectoderm and surface ectoderm. It in turn gives rise to two medial nasal processes and two lateral nasal processes that later fuse and form the intermaxillary process. The pharyngeal arch is derived from mesoderm and the neural crest. It gives rise to two mandibular processes and two maxillary processes (Som and Naidich, 2013). These processes are comprised of mesenchymal cells from neural crest migration and the craniopharyngeal ectoderm and are coated in an epithelium (Ferguson, 1988). The upper lip forms during early embryogenesis and fuses during weeks 5-7 (~E10-11.5 in mouse) when the maxillary processes grow towards the midline and fuses with the medial nasal (intermaxillary) processes that form the philtrum and columella (Kim et al., 2004; Warbrick, 1960; Lan and Jiang, 2022). The palate develops between week 6-12 (~E11.5-14.0 in mouse) from an intermaxillary process derived from the nasal process and a pair of lateral palatine processes (Lan and Jiang, 2022). The primary palate is formed from the posterior extension of the medial nasal (intermaxillary) processes. The lateral palatine processes arise as medial mesenchymal processes from both maxillary processes. These processes initially grow inferiorly until the tongue is pulled downwards by the elongation of the maxilla and mandible. Once above the tongue, the lateral processes grow medially until they make contact and fuse at the midline (Som and Naidich, 2014).

SHH in Orofacial Development . SHH is an important modulator of epithelial-mesenchyme interaction during embryonic development and disruption in animal models has been linked with OFCs (Heyne et al., 2015; Lipinski et al., 2010a). The epithelial derived SHH ligand drives orofacial development via a morphogenic gradient of high proximal and low distal binding in the underlying mesenchyme, relative to the epithelium (Kurosaka, 2015; Lan and Jiang, 2009). This morphogenic gradient induces cellular proliferation in the proximal mesenchyme and drives outgrowth of the facial prominences (Lan and Jiang, 2009). SHH signaling has also been implicated in microvasculature formation and stability through perivascular SHH signaling to the endothelium (Sun et al., 2020). This combination of tissue outgrowth, paired with the formation of the microvasculature, makes SHH signaling critical for proper formation of the lips and palate.

Reception and transduction of SHH ligand has been extensively studied, yet some details are still not fully understood. The interaction between the SHH cell surface receptors Patched (PTCH) and SMO continues to be elucidated. The SMO receptor is a Class F, G protein coupled receptor involved in signal transduction of the Sonic Hedgehog (SHH) pathway. It includes distinct functional groups including ligand binding pockets, cysteine rich domain (CRD), transmembrane helix (TM), extracellular loop (ECL), intracellular loop (ICL), and a carboxyl-terminal tail (C-term tail) (Arensdorf et al., 2016). SMO signaling is dependent upon its relocation to a subcellular location. In the absence of SHH ligand, PTCH suppresses the activation of SMO. When SHH ligand binds to PTCH, suppression on SMO is released and SMO can relocate, accumulate, and signal to intracellular effectors (Denef et al., 2000; Rohatgi and Scott, 2007). It has been shown that SMO localization to the tip of the primary cilia (PC) is essential for the SHH signaling cascade in vertebrates and typically occurs within 20 minutes of agonist stimulation (Rohatgi et al., 2009; Rohatgi et al., 2007; Corbit et al., 2005; Arensdorf et al., 2016; Huangfu and

Anderson, 2005). This signaling cascade leads to signaling to effectors resulting in the activation of the GLI transcription factors and the subsequent induction of GLI target gene expression (Von Ohlen and Hooper, 1997; Alexandre et al., 1996).

The SHH pathway is sensitive to chemical disruption and can be disrupted at multiple molecular initiating events (MIE) along the signaling cascade . The targets of this disruption include ligand production and post-translational modification, ligand secretion, downstream sensing, and transduction (Jeong and McMahon, 2002; Lauth et al., 2007; Petrova et al., 2013b). Disruption of SHH during critical windows of development causing OFCs is believed to work in an epithelial-mesenchyme interaction dependent, but epithelial-mesenchyme transition (Emt) independent manner due to a reduction in epithelial induced proliferation and the subsequent decrease in tissue outgrowth causing the failure of the facial processes to meet and fuse (Heyne et al., 2015; Lipinski et al., 2010a). Chemical modulators of the HH pathway have been identified including the natural alkaloid cyclopamine, both natural and synthetic pharmaceuticals, and a pesticide synergist (Piperonyl butoxide (PBO)) (Lipinski et al., 2007; Lipinski et al., 2010a; Wang et al., 2012a; Everson et al., 2019; Rivera-González et al., 2021). It should be noted that Indian and Dessert Hedgehog (IHH and DHH respectively) are other Hedgehog pathways that are active during development but are not believed to contribute significantly to orofacial development. These pathways signal in a similar manner through PTCH/SMO suggesting that exposures identified in the context of SHH should also be considered in the context of IHH and DHH. Due to the focus of this AOP on disruption to proper orofacial development, SHH will be the focus of discussion.

1. Brief description of AOP

This AOP links antagonism of the SMO receptor, MIE to the adverse outcome, orofacial clefting (**Fig 1**). This AOP is intended to fit within a larger AOP network for SHH disruption leading to OFCs being developed through the OECD Advisory Group on Emerging Science in Chemicals Assessment workplan. The SHH pathway is sensitive to chemical disruption at multiple MIEs along the signaling cascade, albeit only those that occur during critical windows in development can lead to OFCs (Lipinski et al., 2010a; Heyne et al., 2015). Other MIEs include postranslational SHH ligand modification with cholesterol and palmitoylate, ligand secretion, mesenchymal reception, and signal transduction (Jeong and McMahon, 2002; Lauth et al., 2007; Petrova et al., 2013a). This AOP covers one of these events; antagonism of the SMO receptor. Chemical antagonism of SMO is followed by a series of key events (KEs) ultimately leading to the adverse outcome (AO) orofacial clefting. This pathway occurs in the mesenchyme initially at the macromolecular level. The KEs were selected following a thorough review of the SHH pathway and by taking into consideration events that would be of regulatory interest and that would be possible to have a measurement/assay for. To select the key events for the AOP, we used existing knowledge of the pathway along with reviews of the SHH pathway to assemble a path that was physiologically plausible. Care was taken to select events that would be of direct regulatory relevance (i.e. a method to quantify exists).

Box1: AOP ID Box

- AOP title: Antagonism of Smoothened receptor leads to orofacial clefting
- AOP Authors: Jacob I. Reynolds, Brian P. Johnson
- AOP Contributors: Jacob I. Reynolds
- AOP number: 460
- OECD workplan number: Project 1.101: Disruption of the sonic hedgehog pathway during development leads to orofacial clefting
- List of Key Events
    - MIE 2027 Antagonism, Smoothened receptor
    - KE 2044 Decrease, Smoothened relocation, and activation
    - KE 2028 Decrease, GLI1/2 translocation to nucleus
    - KE 2040 Decrease, GLI1/2 target gene expression
    - KE 1262 Apoptosis
    - KE 2043 Decrease, Second messenger production
    - KE 1821 Decrease, Cell proliferation
    - KE 2041 Decrease, palatal shelf outgrowth
    - AO 2042 Orofacial clefting

2. Overview of AOP development approach

This AOP was developed using a "bottom up" (MIE-AO) approach. This AOP was developed by the Johnson Lab at Michigan State University as part of the OECD AOP Development Program Project n° 1.101 (OECD, 2024a). At the time of creation, the AOP-Wiki did not include any events for SHH signaling. With the exception of KE 1821 Decrease, Proliferation, and KE 1262 Apoptosis, all of the MIE, KE, KERs, and AO in this AOP were created and developed by the authors (OECD, 2021).

Pubmed was used as the primary database for evidence collection. Search results were initially screened through review of the title and abstract for potential for data relating a decrease in outgrowth and OFC. Each selected publication and its' data were then examined to determine if support or lack thereof existed for the KER in questions. Papers that did not show any data relating to the KER were discarded. The search terms, date of search, and references identified are organized in **Supplementary table 1**.

3. Summary of scientific evidence assessment

Overall Assessment

This AOP was assessed for its domains of applicability, the essentiality of the events, the empirical evidence presented, temporal concordance, dose-response concordance, consistency, and biological plausibility (**Table 1).**

Domain(s) of Applicability

Chemical: This AOP applies to antagonists of the SMO receptor. Chemical modulators of the SHH pathway have been identified, including the natural alkaloid cyclopamine, both natural and synthetic

pharmaceuticals (e.g. Vismodegib), and a widely used pesticide synergist (PBO) with established human exposures (Lipinski et al., 2007; Lipinski et al., 2010a; Wang et al., 2012a; Everson et al., 2019; Rivera-González et al., 2021).

Sex: This AOP is unspecific to sex.

Life Stages: The relevant life stage for this AOP is embryonic development. More specifically, the development of the craniofacial region which occurs between gestational day (GD) 10.0 and GD 14.0 in the mouse and week 4-12 in human.

Taxonomic: At present, the assumed taxonomic applicability domain of this AOP is mouse (*Mus musculus*). Most of the toxicological data that this AOP is based on has used mice as their model. Mice are a good analog of human craniofacial development and undergo similar signaling by SHH (Jiang et al., 2006). The biological plausible domain of applicability extends to mammals as the mechanisms of orofacial development and early embryonic signaling including SHH are largely conserved.

Essentiality of the Key Events

To date, few studies have addressed the essentiality of the proposed sequence of key events. Evidence linking SHH disruption through a decrease in proliferation exists. The hypothesized sequence of events has a high temporal concordance for canonical SHH signaling pathway and orofacial development.

- Studies have shown that SHH signaling is required for normal facial development and plays a critical role in the growth of the facial processes that form the upper palate and lip (Bush and Jiang, 2012; Kurosaka, 2015; Kurosaka et al., 2014).
- The epithelial derived SHH drives orofacial development through an induced gradient in the underlying mesenchyme (Kurosaka, 2015; Lan and Jiang, 2009; Kurosaka et al., 2014). This gradient of SHH induces cellular proliferation and outgrowth of the mesenchyme (Lan and Jiang, 2009).
- OFCs caused by disruption to SHH are believed to be due to a reduction in epithelial induced proliferation of the mesenchyme and the subsequent decrease in tissue outgrowth and the failure of the facial processes to meet and fuse (Heyne et al., 2015; Lipinski et al., 2010a).

Evidence Assessment

- KER ID-Title-[Adjacency], [Evidence], [Quantitative Understanding]
- Relationship 2734: Antagonism Smoothened (Event 2027) leads to Decrease, SMO relocation (Event 2044)-[Adjacent], [Moderate], [Low]-There is a high biological plausibility of this relationship and SMO localization to the primary cilia is essential for proper SHH signaling in vertebrates (Rohatgi et al., 2009; Rohatgi et al., 2007; Corbit et al., 2005). There is good evidence that the Smoothend Antagonist (SANT) compounds block the localization of SMO to the tip of the primary cilia. Contradictory *In vivo* data was found regarding whether cyclopamine blocks SMO relocation to the primary cilia. Further work is required to determine if SMO antagonism via cyclopamine results in decrease in SMO relocation.
- Relationship 2735: Decrease, SMO relocation (Event 2044) leads to Decrease, GLI1/2 translocation (Event 2028)-[Adjacent], [Moderate], [Low]- Moderate evidence is presented to support that a loss of SMO relocation to the primary cilia leads to a significant decrease in GLI1. GLI1 requires activation prior to nuclear translocation.

- Relationship 2721: Decrease, GLI1/2 translocation (Event 2028) leads to Decrease, GLI1/2 target gene expression (Event 2040)-[Adjacent], [Low], [Low]- There is high biological plausibility of this relationship but to date few studies were found to explore the relationship.
- Relationship 2731: Decrease GLI1/2 target gene expression (Event 2040) leads to Decrease, SHH second messenger production (Event 2043)-[Adjacent], [Low], [Low]-Coordinated signaling is paramount for proper embryonic development and the GLI signaling cascade drives feedback/forward loops with FGF and BMP signaling pathways. Support was found for SHH having a feedforward loop with FGF10 and BMP4, however further investigation into the interaction of these pathways and their crosstalk is required.
- Relationship 2732: Decrease SHH second messenger production (Event 2043) leads to Decrease, cell proliferation. (Event 1821)-[Adjacent], [Low], [Low]- SHH is a known mitogen and drives proliferation through its' secondary messengers. SHH was found to induce proliferation and FGF10 *In vivo*.
- Relationship 2724: Decrease, Cell proliferation (Event 1821) leads to Decrease, outgrowth (Event 2041)-[Adjacent], [Low], [Low]-SHH is a known mitogen that helps to drive the proper development of the face which includes the outgrowth of the facial prominences. To date, few studies have measured outgrowth of the facial prominences and proliferation. Hypoplasia of pharyngeal arch 1 was found in SHH-/- embryos supporting that outgrowth is driven by proliferation and is reduced when proliferation is decreased.
- Relationship 2726: Decrease, outgrowth (Event 2041) leads to OFC (Event 2042)-[Adjacent], [Moderate], [Low]- OFCs caused by disruption to SHH are believed to be due to a reduction in epithelial induced mesenchymal proliferation and the subsequent decrease in tissue outgrowth and the failure of the facial processes to meet and fuse (Lipinski et al., 2010b; Heyne et al., 2015). Mice with disrupted SHH signaling are found to have palatal shelves that are spaced apart supporting that the cleft results from an epithelial-mesenchyme dependent, but epithelial-mesenchyme transition (Emt) independent manner.
- Relationship 2792: Apoptosis (Event 1262) leads to Decrease, outgrowth (Event 2041)-[Adjacent], [Low], [Low]- SHH signaling is known to be associated with cell survival and there is a high biological plausibility that increasing apoptosis would cause a decrease in outgrowth. Supporting evidence is offered with increases in apoptosis in the mandibular arch seen in SHH signaling disrupted mice that exhibit decreased outgrowth.
- Relationship 2882: Decrease, GLI1/2 target gene expression (Event 2040) leads to Apoptosis (Event 1262) -[Adjacent], [Low], [Low]- To date few studies have examined the relationship of GLI1/2 target gene expression. There is a high biological plausibility that SHH plays a role in cell survival and death through GLI1/2 target gene expression. Decreased GLI1/2 target gene expression is seen in RA exposed dams alongside increased apoptosis on the cranial neural crest cells (CNCC).
- Relationship 2894: Antagonism Smoothened (Event 2027) leads to OFC (Event 2042)-[Non-adjacent], [High], [Moderate]- multiple studies have demonstrated *In vivo* that administration of SMO antagonists during critical windows of exposure leads to birth defects including OFC in a dose-dependent fashion.

Biological Plausibility

Biological plausibility refers to the structural and/or functional relationship that exists between the key events based on our understanding of normal biology. SHH signaling is largely conserved in mammals and is required for normal facial development and plays a critical role in the growth of the facial processes that form the upper palate and lip (Bush and Jiang, 2012; Kurosaka, 2015). Multiple antagonists of the SMO receptor have been identified through binding studies including cyclopamine, vismodegib, PBO, and the SANT compounds (Lipinski et al., 2007; Lipinski et al., 2010a; Wang et al., 2012a; Everson et al., 2019; Rivera-González et al., 2021). While the level of empirical support for most of the KERs is low, there is high empirical support for the non-adjacent relationship linking antagonism of SMO and OFC as well as high plausibility of the AOP.

Concordance of dose-response relationships

There are a limited number of studies in which multiple key events were assessed in the same study following exposure to known SMO antagonists. These studies form the basis of the dose-response concordance of this AOP (**Supplementary Table 2)**. Many studies were found to use a single concentration.

The concentration-dependence of the key event responses regarding concentration of known *in vitro* and/or *in vivo* for some of the KEs in this AOP.

- Concentration dependent clefting with cyclopamine exposure (Omnell et al., 1990)
- Dose dependent binding to SMO (Chen et al., 2002)
- Concentration dependent decrease in SMO-ciliary accumulation in vitro for vismodegib exposure (Wang et al., 2012b)

Temporal concordance

Temporal concordance refers to the degree to which the data supports the hypothesized sequence of Molecular Initiating Event (MIE) leading to the Adverse Outcome (AO) through a series of Key Events (KEs). This work has identified a lack of studies that address the temporal concordance of this AOP. While a lack of data for temporal concordance exists, there remains a high plausibility of the proposed relationship. Canonical SHH signaling through PTCH-SMO-GLI is well understood and our AOP remains consistent with the pathway. SHH signaling is required for normal facial development and plays a critical role in the growth of the facial processes that form the upper palate and lip (Bush and Jiang, 2012; Kurosaka, 2015). The epithelial derived SHH drives orofacial development through an induced gradient in the underlying mesenchyme (Kurosaka, 2015; Lan and Jiang, 2009). This gradient of SHH induces cellular proliferation and outgrowth of the mesenchyme (Lan and Jiang, 2009). The hypothesized sequence of events is supported by the existing data and follow the field's current understanding of the canonical SHH signaling pathway.

Consistency

The AO is not specific to this AOP. Many of the events in this AOP will overlap with AOPs linking disruption of SHH to OFC and some are expected to overlap with AOPs linking other developmental signaling pathways to OFCs.

Uncertainties, inconsistencies, and data gaps

This AOP would be strengthened by studies examining the dose-response and time-course relationships for these KERs. The main data gaps for this AOP exist in the lack of studies that have examined the relationship in the context of dose response or time course. Additional studies using mouse models along with the development of NAMs would help to strengthen this AOP.

Data gaps:

- Dose response and time course studies relating a Decrease, SMO relocation leads to Decrease, GLI1/2 translocation.
- Dose response and time course studies relating a decrease GLI translocation leads to decrease GLI target gene expression.
- Dose response and time course studies relating a Decrease, GLI1/2 target gene expression leads to Decrease, SHH second messenger production.
- Dose response and time course studies relating a Decrease, SHH second messenger production leads to Decrease, Cell proliferation.
- Dose response and time course studies relating a Decrease, Cell proliferation leads to Decrease, outgrowth.
- Dose response and time course studies relating a Decrease, outgrowth leads to OFC.
- Dose response and time course studies relating Apoptosis leads to Decrease, Outgrowth
- Dose response and time course studies relating a Decrease, GLI1/2 target gene expression leads to Apoptosis.

Inconsistencies:

- While it is well understood that cyclopamine is an antagonist of SMO, contradictory *In vitro* data was found regarding whether cyclopamine blocks SMO relocation to the primary cilia. Rohatgi et al used NIH 3T3s cell and found that cyclopamine did not inhibit the accumulation of SMO in the cilia even when dosed at 5-10um (>10 fold above Kd). The three antagonists (SANT-1, SANT-2, and cyclopamine) tested by Rohgati et al 2009 inhibited SHH pathway transduction and target gene expression (Rohatgi et al., 2009). In a 2005 study, Corbit et al used a renal epithelial MDCK (Madin-Darby canine kidney) line engineered to express Myc-tagged SMO. Following culture for 1hr in SHH conditioned media, SMO presence in the primary cilium is upregulated while cells cultured in the presence of cyclopamine see a downregulation of SMO in the primary cilia (Corbit et al., 2005). Further work is required to determine if SMO antagonism via cyclopamine results in decrease in SMO relocation.

Uncertainties:

- While we know that entry to the cilia is tightly controlled, the exact mechanism of SMO ciliary trafficking is not fully understood. The primary cilia (PC) is separated from the plasma membrane by the ciliary pockets and the transition zone which function together to regulate the movement of lipids and proteins in and out of the organelle (Rohatgi and Snell, 2010; Goetz et al., 2009). The SHH receptor PTCH contains a ciliary localization sequence (CLS) in its' carboxy tail. Localization of PTCH to the PC is essential for inhibition of SMO as deletion of the CLS in PTCH prevents PTCH localization as well as inhibition of SMO (Kim et al., 2015). SMO also

contains a CLS, but only accumulates in the PC upon ligand binding (Corbit et al., 2005). The entry of SMO into the PC is thought to occur either laterally through the ciliary pockets or internally via recycling endosomes (Milenkovic et al., 2009). Once inside the PC, SMO can diffuse freely, however it will usually accumulate in specific locations depending upon its' activation state. Inactive SMO will accumulate more at the base of the PC while active SMO will accumulate in the tip of the PC (Milenkovic et al., 2015).

- The relationships and feedback/feedforward loops that exist between SHH and its' secondary messengers primarily FGF10 and BMP4 are not well understood. More investigation into these relationships is warranted.
- The exact mechanism through which SHH promotes cell survival is not well understood (Cobourne et al., 2001). Further studies are needed to illuminate the mechanism that links SHH signaling with cell survival.
- The relationship between GLI1/2 target gene expression and increased apoptosis has a high biological plausibility although there is currently a lack of studies that address this relationship.

Assessment of quantitative understanding of the AOP:

The quantitative understanding for this AOP except for the non-adjacent relationship between Antagonism Smoothened leads to OFC is low. Most of the data found through the literature was obtained from studies that investigated a single compound that performed their investigation using a single concentration. Few studies were found to conduct either dose response or time course studies. For the non-adjacent relationship connecting Antagonism Smoothend to OFC there are several studies with dose response data showing a dose-dependent incidence of clefting were found. This AOP would benefit from the generation of additional data that addresses these relationships in a dose response and time course methodology to allow for an increased quantitative understanding of the linkage.

4. Potential applications

This AOP provides an opportunity from a regulatory standpoint, to facilitate the reduction or replacement animal testing for developmental toxicity testing (e.g., OECD test guideline 414). There are currently no OECD-validated *in vitro* assays to identify chemicals likely to cause a cleft prior to manifestation. Such developmental toxicants are primarily identified when structural abnormalities (e. g., OFC) had already occurred and detected in a developmental toxicity assay such as OECD Test No. 414: Prenatal Developmental Toxicity Study (OECD, 2018). AOP 460 provides an understanding of a mechanism leading to OFCs, such that if there were validated *in vitro* methods that could detect the earlier key events before the *in vivo* manifestation of OFCs, this would be highly relevant for the regulatory community.

Existing or new data from *in silico* and *in vitro* high-throughput screening assays (HTS) can also be applied to this AOP to guide early identification of chemicals for further investigation using more representative models of orofacial development using a tiered testing approach. For example, data has been generated by the National Center for Advancing Translational Sciences (NCATS) Toxicology in the 21st Century program (Tox21) for HH agonists and antagonists at the transcriptional level using a GLI3 luminescent reporter 3T3 line (Huang et al., 2018; Huang et al., 2016). A HTS assay has also been developed to detect potential inhibitors or activators of the auto processing that the SHH ligand undergoes upstream of SMO (Ciulla et al., 2022). Microphysiological models (MPMs) offer increased physiological relevance over traditional 2D cell culture providing the ability to capture disruption at

multiple MIEs. For example, research groups are already engineering models to facilitate the study of both normal and abnormal orofacial development including palatal fusion (Belair et al., 2018; Wolf et al., 2018; Belair et al., 2017; Wolf et al., 2023; Reynolds et al., 2022; Johnson et al., 2021). Leveraging these data and carefully designed *in vitro* models, there is the potential to aid developmental toxicity testing and reduce animal use.

This AOP can also serve as a reference for method developers to identify and incorporate the relevant and early biological endpoints in developmental and reproductive toxicology (DART), for which new assays (NAMs) could be developed. Our lab engineered one of the MPMs of orofacial development discussed above to study SHH signaling (Reynolds et al., 2022; Johnson et al., 2021). A major motivation for our completing this AOP is to apply a bottom-up approach to identify data gaps and guide experimental direction related to OFCs (Reynolds et al., 2024). Through development of this AOP and the larger AOP network we have identified data gaps of direct regulatory interest. As detailed above, most of the identified gaps involve a lack of studies addressing dose response or time course in an experiment designed to test the KER. The plan is to use the MPM of orofacial development our lab has developed and generate data to help fill these gaps. Understanding the data gaps and any inconsistencies or uncertainties is crucial both for risk assessment as well as for experiment planning. Pairing this increased understanding of the pathway and regulatory needs can guide application of engineered models and HTS assays.

Finally, the ability to detect disruption of the SHH pathway has broader consequences and impact than just OFCs. The SHH pathway is known to play a role in many aspects of embryonic development including patterning of the limbs, digit development, and development of the clinical phenotype, holoprosencephaly (Roessler et al., 1996; Scherz et al., 2007; Tickle and Towers, 2017; Sasai et al., 2019). The creation of complex in vitro NAMs that are sensitive to disruption of SHH has important implications in developmental toxicity testing including identifying chemicals that would be predicted to cause birth defects in these other areas. Mapping and in vitro modeling of the key intermediate events that are shared in these processes may broaden the applicability of these models furthering hazard identification. In summary, this AOP and the subsequent work can lead to improved methods or development of NAMs for DART testing and a shift away from traditional animal use.

5. Acknowledgement

We thank the OECD Advisory Group on Emerging Science in Chemicals Assessment (ESCA) for their inclusion of this project in their workplan (ESCA workplan project 1.101). We thank our coach Dr. Judy Choi for her mentorship in developing this AOP. We appreciate the help of Robert Lipinski and Tyler Beames for their feedback on the manuscript.

This work was supported by the National Institutes of Health R00-ES028744 and the National Institute of Environmental Health Sciences P42ES004911.

Conflict of interest: Brian Johnson owns equity in Onexio Biosystems, LLC

6. AOP Editors and Reviewers

7. Disclaimer

8. Data availability statement

Data, associated metadata, and calculation tools are available from the corresponding author (bjohnson@msu.edu). All review reports can be accessed at https://aopwiki.org/aops/460. The final snapshot pdf of this AOP can be accessed at https://aopwiki.org/aops/460/snapshots. The snapshot pdf that was used during the review process can be found at https://aopwiki.org/aops/460/snapshots.

Figure 1: Schematic of AOP 460. Adjacent and nonadjacent relationships are depicted as solid and dashed lines respectively.

Table 1: Assessment of the relative level of confidence in the overall AOP based on rank ordered weight of evidence elements.

Supplementary table 1: Organization of search terms and results for all KERs.

Supplementary table 2: Dose concordance for AOP. Studies were recorded for dose and any indication of any of the KEs in the AOP were noted.

9. References

Alexandre, C., Jacinto, A. and Ingham, P. W. (1996). Transcriptional activation of hedgehog target genes in drosophila is mediated directly by the cubitus interruptus protein, a member of the gli family of zinc finger DNA-binding proteins. *Genes Dev 10*, 2003-2013. doi:10.1101/gad.10.16.2003

Arensdorf, A. M., Marada, S. and Ogden, S. K. (2016). Smoothened regulation: A tale of two signals. *Trends Pharmacol Sci 37*, 62-72. doi:10.1016/j.tips.2015.09.001

Belair, D. G., Wolf, C. J., Wood, C. et al. (2017). Engineering human cell spheroids to model embryonic tissue fusion in vitro. *PLoS One 12*, e0184155. doi:10.1371/journal.pone.0184155

Belair, D. G., Wolf, C. J., Moorefield, S. D. et al. (2018). A three-dimensional organoid culture model to assess the influence of chemicals on morphogenetic fusion. *Toxicol Sci 166*, 394-408. doi:10.1093/toxsci/kfy207

Bush, J. O. and Jiang, R. (2012). Palatogenesis: Morphogenetic and molecular mechanisms of secondary palate development. *Development 139*, 231-243. doi:10.1242/dev.067082

Chen, J. K., Taipale, J., Cooper, M. K. et al. (2002). Inhibition of hedgehog signaling by direct binding of cyclopamine to smoothened. *Genes Dev 16*, 2743-2748. doi:10.1101/gad.1025302

Ciulla, D. A., Dranchak, P., Pezzullo, J. L. et al. (2022). A cell-based bioluminescence reporter assay of human sonic hedgehog protein autoprocessing to identify inhibitors and activators. *J Biol Chem 298*, 102705. doi:10.1016/j.jbc.2022.102705

Cobourne, M. T., Hardcastle, Z. and Sharpe, P. T. (2001). Sonic hedgehog regulates epithelial proliferation and cell survival in the developing tooth germ. *J Dent Res 80*, 1974-1979. doi:10.1177/00220345010800110501

Corbit, K. C., Aanstad, P., Singla, V. et al. (2005). Vertebrate smoothened functions at the primary cilium. *Nature 437*, 1018-1021. doi:10.1038/nature04117

Denef, N., Neubüser, D., Perez, L. et al. (2000). Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. *Cell 102*, 521-531. doi:10.1016/s0092-8674(00)00056-8

Dixon, M. J., Marazita, M. L., Beaty, T. H. et al. (2011a). Cleft lip and palate: Understanding genetic and environmental influences. *Nature Reviews Genetics 12*, 167-178. doi:10.1038/nrg2933

Dixon, M. J., Marazita, M. L., Beaty, T. H. et al. (2011b). Cleft lip and palate: Understanding genetic and environmental influences. *Nat Rev Genet 12*, 167-178. doi:10.1038/nrg2933

Everson, J. L., Sun, M. R., Fink, D. M. et al. (2019). Developmental toxicity assessment of piperonyl butoxide exposure targeting sonic hedgehog signaling and forebrain and face morphogenesis in

485 the mouse: An in vitro and in vivo study. *Environ Health Perspect 127*, 107006.
486 doi:10.1289/EHP5260
487 Feldkamp, M. L., Carey, J. C., Byrne, J. L. B. et al. (2017). Etiology and clinical presentation of birth
488 defects: Population based study. *BMJ 357*, j2249. doi:10.1136/bmj.j2249
489 Fell, M., Dack, K., Chummun, S. et al. (2022). Maternal cigarette smoking and cleft lip and palate: A
490 systematic review and meta-analysis. *Cleft Palate Craniofac J 59*, 1185-1200.
491 doi:10.1177/10556656211040015
492 Ferguson, M. W. (1988). Palate development. *Development 103 Suppl*, 41-60.
493 Goetz, S. C., Ocbina, P. J. and Anderson, K. V. (2009). The primary cilium as a hedgehog signal
494 transduction machine. *Methods Cell Biol 94*, 199-222. doi:10.1016/s0091-679x(08)94010-3
495 Grosen, D., Bille, C., Petersen, I. et al. (2011). Risk of oral clefts in twins. *Epidemiology 22*, 313-319.
496 doi:10.1097/EDE.0b013e3182125f9c
497 Heyne, G. W., Melberg, C. G., Doroodchi, P. et al. (2015). Definition of critical periods for hedgehog
498 pathway antagonist-induced holoprosencephaly, cleft lip, and cleft palate. *PLoS One 10*,
499 e0120517. doi:10.1371/journal.pone.0120517
500 Huang, R., Xia, M., Sakamuru, S. et al. (2016). Modelling the tox21 10 k chemical profiles for in vivo
501 toxicity prediction and mechanism characterization. *Nat Commun 7*, 10425.
502 doi:10.1038/ncomms10425
503 Huang, R., Xia, M., Sakamuru, S. et al. (2018). Expanding biological space coverage enhances the
504 prediction of drug adverse effects in human using in vitro activity profiles. *Sci Rep 8*, 3783.
505 doi:10.1038/s41598-018-22046-w
506 Huangfu, D. and Anderson, K. V. (2005). Cilia and hedgehog responsiveness in the mouse. *Proc Natl Acad*
507 *Sci U S A 102*, 11325-11330. doi:10.1073/pnas.0505328102
508 Jeong, J. and McMahon, A. P. (2002). Cholesterol modification of hedgehog family proteins. *The Journal*
509 *of Clinical Investigation 110*, 591-596. doi:10.1172/JCI16506
510 Jiang, R., Bush, J. O. and Lidral, A. C. (2006). Development of the upper lip: Morphogenetic and
511 molecular mechanisms. *Dev Dyn 235*, 1152-1166. doi:10.1002/dvdy.20646
512 Johnson, B. P., Vitek, R. A., Morgan, M. M. et al. (2021). A microphysiological approach to evaluate
513 effectors of intercellular hedgehog signaling in development. *Front Cell Dev Biol 9*, 621442.
514 doi:10.3389/fcell.2021.621442
515 Kim, C. H., Park, H. W., Kim, K. et al. (2004). Early development of the nose in human embryos: A
516 stereomicroscopic and histologic analysis. *Laryngoscope 114*, 1791-1800.
517 doi:10.1097/00005537-200410000-00022
518 Kim, J., Hsia, E. Y., Brigui, A. et al. (2015). The role of ciliary trafficking in hedgehog receptor signaling. *Sci*
519 *Signal 8*, ra55. doi:10.1126/scisignal.aaa5622
520 Kurosaka, H., Iulianella, A., Williams, T. et al. (2014). Disrupting hedgehog and wnt signaling interactions
521 promotes cleft lip pathogenesis. *J Clin Invest 124*, 1660-1671. doi:10.1172/jci72688
522 Kurosaka, H. (2015). The roles of hedgehog signaling in upper lip formation. *Biomed Res Int 2015*,
523 901041. doi:10.1155/2015/901041
524 Lan, Y. and Jiang, R. (2009). Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal
525 interactions controlling palatal outgrowth. *Development 136*, 1387-1396.
526 doi:10.1242/dev.028167
527 Lan, Y., Xu, J. and Jiang, R. (2015). Cellular and molecular mechanisms of palatogenesis. *Curr Top Dev Biol*
528 *115*, 59-84. doi:10.1016/bs.ctdb.2015.07.002
529 Lan, Y. and Jiang, R. (2022). Mouse models in palate development and orofacial cleft research:
530 Understanding the crucial role and regulation of epithelial integrity in facial and palate
531 morphogenesis. *Curr Top Dev Biol 148*, 13-50. doi:10.1016/bs.ctdb.2021.12.003

Lauth, M., Bergström, A., Shimokawa, T. et al. (2007). Inhibition of gli-mediated transcription and tumor cell growth by small-molecule antagonists. *Proc Natl Acad Sci U S A 104*, 8455-8460. doi:10.1073/pnas.0609699104

Lin, Y. C., Lo, L. J., Noordhoff, M. S. et al. (1999). Cleft of the lip and palate in twins. *Changgeng yi xue za zhi 22*, 61-67.

Lipinski, R. J., Dengler, E., Kiehn, M. et al. (2007). Identification and characterization of several dietary alkaloids as weak inhibitors of hedgehog signaling. *Toxicol Sci 100*, 456-463. doi:10.1093/toxsci/kfm222

Lipinski, R. J., Song, C., Sulik, K. K. et al. (2010a). Cleft lip and palate results from hedgehog signaling antagonism in the mouse: Phenotypic characterization and clinical implications. *Birth Defects Res A Clin Mol Teratol 88*, 232-240. doi:10.1002/bdra.20656

Lipinski, R. J., Song, C., Sulik, K. K. et al. (2010b). Cleft lip and palate results from hedgehog signaling antagonism in the mouse: Phenotypic characterization and clinical implications. *Birth defects research. Part A, Clinical and molecular teratology 88*, 232-240. doi:10.1002/bdra.20656

Milenkovic, L., Scott, M. P. and Rohatgi, R. (2009). Lateral transport of smoothened from the plasma membrane to the membrane of the cilium. *J Cell Biol 187*, 365-374. doi:10.1083/jcb.200907126

Milenkovic, L., Weiss, L. E., Yoon, J. et al. (2015). Single-molecule imaging of hedgehog pathway protein smoothened in primary cilia reveals binding events regulated by patched1. *Proc Natl Acad Sci U S A 112*, 8320-8325. doi:10.1073/pnas.1510094112

Mossey, P. A., Little, J., Munger, R. G. et al. (2009). Cleft lip and palate. *The Lancet 374*, 1773-1785. doi:https://doi.org/10.1016/S0140-6736(09)60695-4

OECD (2018). *Test no. 414: Prenatal developmental toxicity study*. Vol. https://www.oecd-ilibrary.org/content/publication/9789264070820-en doi:doi:https://doi.org/10.1787/9789264070820-en

Omnell, M. L., Sim, F. R., Keeler, R. F. et al. (1990). Expression of veratrum alkaloid teratogenicity in the mouse. *Teratology 42*, 105-119. doi:10.1002/tera.1420420202

Petrova, E., Rios-Esteves, J., Ouerfelli, O. et al. (2013a). Inhibitors of hedgehog acyltransferase block sonic hedgehog signaling. *Nature Chemical Biology 9*, 247-249. doi:10.1038/nchembio.1184

Petrova, E., Rios-Esteves, J., Ouerfelli, O. et al. (2013b). Inhibitors of hedgehog acyltransferase block sonic hedgehog signaling. *Nat Chem Biol 9*, 247-249. doi:10.1038/nchembio.1184

Reynolds, J. I., Vitek, R. A., Geiger, P. G. et al. (2022). Engineering epithelial–mesenchymal microtissues to study cell–cell interactions in development. In S. Dworkin (eds.), *Craniofacial development: Methods and protocols*. New York, NY: Springer US. https://doi.org/10.1007/978-1-0716-1847-9_13

https://link.springer.com/content/pdf/10.1007/978-1-0716-1847-9_13.pdf doi:10.1007/978-1-0716-1847-9_13

Reynolds, J. I., Choi, J. and Johnson, B. P. (2024). Crowdsourcing aop development: Leveraging the thesis literature review to identify knowledge gaps and facilitate research translation. *Curr Res Toxicol 7*, 100191. doi:10.1016/j.crtox.2024.100191

Rivera-González, K. S., Beames, T. G. and Lipinski, R. J. (2021). Examining the developmental toxicity of piperonyl butoxide as a sonic hedgehog pathway inhibitor. *Chemosphere 264*, N.PAG-N.PAG. doi:10.1016/j.chemosphere.2020.128414

Roessler, E., Belloni, E., Gaudenz, K. et al. (1996). Mutations in the human sonic hedgehog gene cause holoprosencephaly. *Nature Genetics 14*, 357-360. doi:10.1038/ng1196-357

Rohatgi, R., Milenkovic, L. and Scott, M. P. (2007). Patched1 regulates hedgehog signaling at the primary cilium. *Science 317*, 372-376. doi:10.1126/science.1139740

578 Rohatgi, R. and Scott, M. P. (2007). Patching the gaps in hedgehog signalling. *Nat Cell Biol 9*, 1005-1009.
579 doi:10.1038/ncb435
580 Rohatgi, R., Milenkovic, L., Corcoran, R. B. et al. (2009). Hedgehog signal transduction by smoothened:
581 Pharmacologic evidence for a 2-step activation process. *Proc Natl Acad Sci U S A 106*, 3196-
582 3201. doi:10.1073/pnas.0813373106
583 Rohatgi, R. and Snell, W. J. (2010). The ciliary membrane. *Curr Opin Cell Biol 22*, 541-546.
584 doi:10.1016/j.ceb.2010.03.010
585 Sasai, N., Toriyama, M. and Kondo, T. (2019). Hedgehog signal and genetic disorders. *Frontiers in*
586 *Genetics 10*, doi:10.3389/fgene.2019.01103
587 Scherz, P. J., McGlinn, E., Nissim, S. et al. (2007). Extended exposure to sonic hedgehog is required for
588 patterning the posterior digits of the vertebrate limb. *Dev Biol 308*, 343-354.
589 doi:10.1016/j.ydbio.2007.05.030
590 Som, P. M. and Naidich, T. P. (2013). Illustrated review of the embryology and development of the facial
591 region, part 1: Early face and lateral nasal cavities. *AJNR Am J Neuroradiol 34*, 2233-2240.
592 doi:10.3174/ajnr.A3415
593 Som, P. M. and Naidich, T. P. (2014). Illustrated review of the embryology and development of the facial
594 region, part 2: Late development of the fetal face and changes in the face from the newborn to
595 adulthood. *AJNR Am J Neuroradiol 35*, 10-18. doi:10.3174/ajnr.A3414
596 Sun, M. R., Chung, H. M., Matsuk, V. et al. (2020). Sonic hedgehog signaling in cranial neural crest cells
597 regulates microvascular morphogenesis in facial development. *Frontiers in Cell and*
598 *Developmental Biology 8*, doi:10.3389/fcell.2020.590539
599 Tickle, C. and Towers, M. (2017). Sonic hedgehog signaling in limb development. *Frontiers in Cell and*
600 *Developmental Biology 5*, doi:10.3389/fcell.2017.00014
601 Von Ohlen, T. and Hooper, J. E. (1997). Hedgehog signaling regulates transcription through gli/ci binding
602 sites in the wingless enhancer. *Mech Dev 68*, 149-156. doi:10.1016/s0925-4773(97)00150-0
603 Wang, J., Lu, J., Mook, R. A., Jr. et al. (2012a). The insecticide synergist piperonyl butoxide inhibits
604 hedgehog signaling: Assessing chemical risks. *Toxicol Sci 128*, 517-523.
605 doi:10.1093/toxsci/kfs165
606 Wang, Y., Arvanites, A. C., Davidow, L. et al. (2012b). Selective identification of hedgehog pathway
607 antagonists by direct analysis of smoothened ciliary translocation. *ACS Chem Biol 7*, 1040-1048.
608 doi:10.1021/cb300028a
609 Warbrick, J. G. (1960). The early development of the nasal cavity and upper lip in the human embryo. *J*
610 *Anat 94*, 351-362.
611 Wolf, C. J., Belair, D. G., Becker, C. M. et al. (2018). Development of an organotypic stem cell model for
612 the study of human embryonic palatal fusion. *Birth Defects Res 110*, 1322-1334.
613 doi:10.1002/bdr2.1394
614 Wolf, C. J., Fitzpatrick, H., Becker, C. et al. (2023). An improved multicellular human organoid model for
615 the study of chemical effects on palatal fusion. *Birth Defects Res 115*, 1513-1533.
616 doi:10.1002/bdr2.2229
617 Yaqoob, M., Mahmood, F., Hanif, G. et al. (2013). Etiology and genetic factors in clefts of lip and/or
618 palate reported at children's hospital, lahore, pakistan. *Indian J Hum Genet 19*, 136-143.
619 doi:10.4103/0971-6866.116103
620 Ye, X. and Ahmed, M. K. (2022). Genetic factors responsible for cleft lip and palate. In G. Q. Fayyaz (eds.),
621 *Surgical atlas of cleft palate and palatal fistulae*. Singapore: Springer Nature Singapore.
622 https://doi.org/10.1007/978-981-15-8124-3_95 doi:10.1007/978-981-15-8124-3_95

623

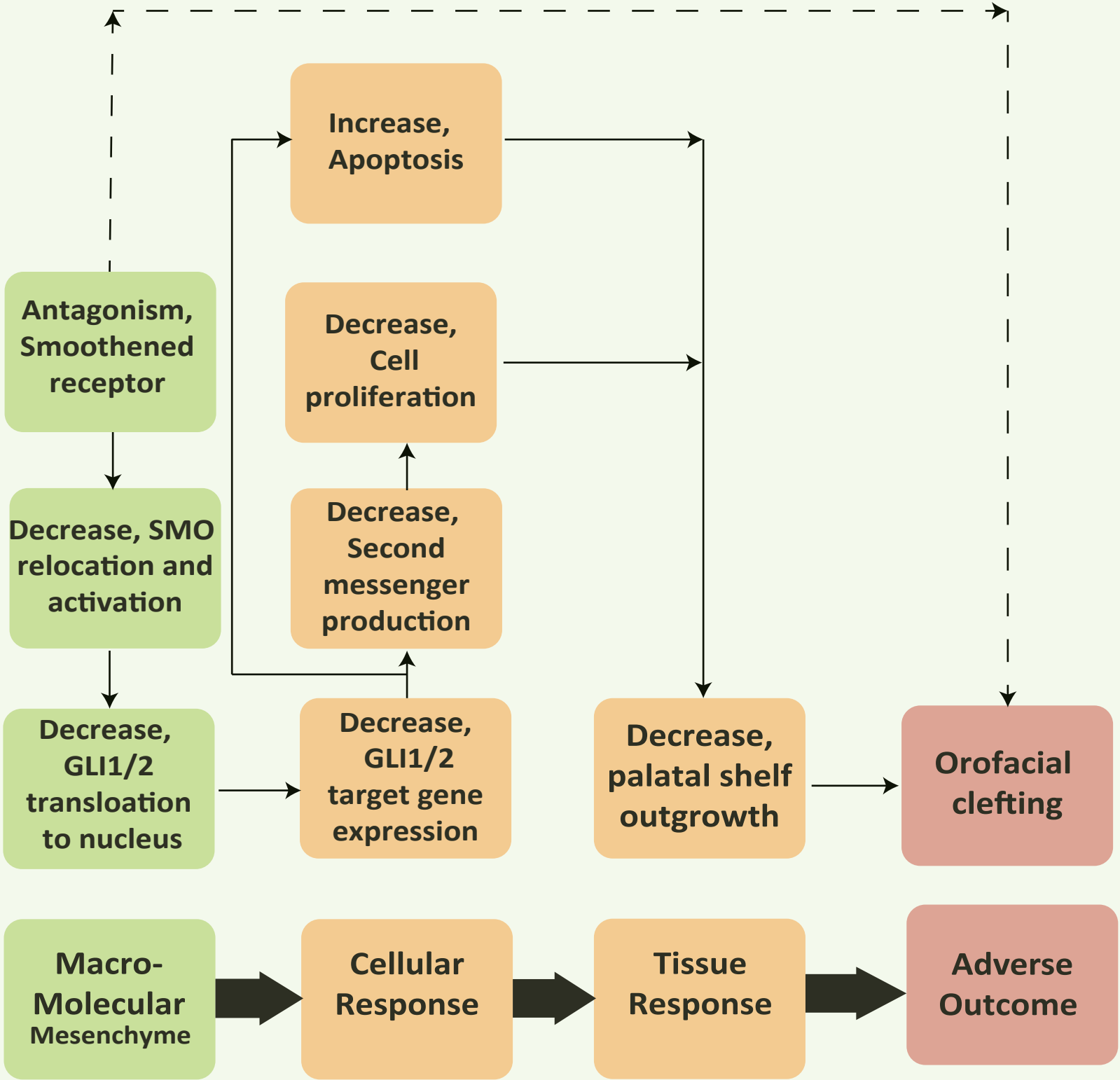

Increase, Apoptosis
Antagonism, Smoothened receptor
Decrease, Cell proliferation
Decrease, SMO relocation and activation
Decrease, Second messenger production
Decrease, GLI1/2 transloation to nucleus
Decrease, GLI1/2 target gene expression
Decrease, palatal shelf outgrowth
Orofacial clefting
Macro-Molecular
Mesenchyme
Cellular Response
Tissue Response
Adverse Outcome

Table 1: Assessment of the relative level of confidence in the overall AOP based on rank ordered weight of evidence elements.

Defining Question	High (Strong)	Moderate	Low (Weak)
1. Support for Biological Plausibility of KERS			
a) Is there a mechanistic relationship between KE_{up} and KE_{down} consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance.	KER is plausible based on analogy to accepted biological relationships, but scientific understanding is incomplete	Empirical support for association between KEs, but the structural or functional relationship between them is not understood.
Relationship 2734: Antagonism Smoothened (Event 2027) leads to Decrease, SMO relocation (Event 2044)		**STRONG** SMO signaling is well understood to be dependent upon its relocation to a subcellular location. This relocation occurs in the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005). It has been shown that SMO localization to the tip of the primary cilia is essential for the SHH signaling cascade in vertebrates (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Rohatgi, Milenkovic et al. 2009)	
Relationship 2735: Decrease, SMO relocation (Event 2044) leads to Decrease, GLI1/2 translocation (Event 2028)		**MODERATE** SMO causes the GLI family to become dislodged from their complex with the negative regulator of HH signaling, Suppressor of Fused (Sufu) (Kogerman, Grimm et al. 1999, Pearse, Collier et al. 1999, Stone, Murone et al. 1999, Tukachinsky, Lopez et al. 2010). The GLI-Sufu complex maintains retention of GLI in the cytosol allowing for exposure to phosphorylation via protein kinase A (PKA) which inhibits downstream signal transduction (Tuson, He et al. 2011). When SMO is activated, the GLI2/3-Sufu complex is dismantled allowing for retrograde transport of GLI back into the nucleus (Kim, Kato et al. 2009).	
Relationship 2721: Decrease, GLI1/2 translocation (Event 2028) leads to Decrease, GLI1/2 target gene expression (Event 2040)		**STRONG** It is well established that activation of the SHH pathway results in a downstream signaling cascade resulting in the relocation of GLI to the nucleus and subsequent gene transcription (Carballo, Honorato et al. 2018).	
Relationship 2731: Decrease GLI1/2 target gene expression (Event 2040) leads to Decrease, SHH second messenger production (Event 2043)		**WEAK** While it is understood that there is extensive crosstalk between SHH and other pathways during development there is an incomplete understanding of these interactions and their feedback and feed forward loops.	
Relationship 2732: Decrease SHH second messenger production (Event 2043) leads to Decrease, cell proliferation (Event 1821)		**STRONG** SHH is a known mitogen and known to regulate cellular proliferation.	
Relationship 2724: Decrease, Cell proliferation (Event 1821) leads to Decrease, outgrowth (Event 2041)		**MODERATE** The SHH pathway is well known to be associated with cellular proliferation and growth of the facial prominences.	
Relationship 2726: Decrease, outgrowth (Event 2041) leads to OFC (Event 2042)		**STRONG** OFCs caused by disruption to SHH are believed to be due to a reduction in epithelial induced proliferation and the subsequent decrease in tissue outgrowth and the failure of the facial processes to meet and fuse (Lipinski, Song et al. 2010, Heyne, Melberg et al. 2015).	
Relationship 2792: Apoptosis (Event 1262) leads to Decrease, outgrowth (Event 2041)		**WEAK** The SHH pathway is known to be associated with cell survival and that disruption of SHH signaling can lead to increased apoptosis. The understanding of this relationship is weak and further work is warranted to increase understanding.	
Relationship 2882: Decrease, GLI1/2 target gene expression (Event 2040) leads to Apoptosis (Event 1262)		**WEAK** The SHH pathway is well known to be associated with cellular proliferation and cell survival. Further investigation into how GLI1/2 gene expression regulates cellular survival is needed	

Relationship 2894: Antagonism Smoothened (Event 2027) leads to OFC (Event 2042)		**STRONG** The SHH pathway is well understood to be fundamental to proper embryonic development and that aberrant SHH signaling during embryonic development can cause birth defects including orofacial clefts (OFCs)	
Defining Question	**High (Strong)**	**Moderate**	**Low (Weak)**
2. Support for Essentiality of KEs			
Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.
Essentiality of the KEs was assessed for the AOP as a whole – rationale for the individual KE calls is provided.	To date, few studies have addressed the essentiality of the proposed sequence of key events. Evidence linking SHH disruption through a decrease in proliferation exists. The hypothesized sequence of events has a high temporal concordance for canonical SHH signaling pathway and orofacial development. • Studies have shown that SHH signaling is required for normal facial development and plays a critical role in the growth of the facial processes that form the upper palate and lip (Bush and Jiang 2012, Kurosaka 2015). • The epithelial derived SHH drives orofacial development through a high proximal low distal gradient of GLI activity in the underlying mesenchyme (Lan and Jiang 2009, Kurosaka 2015). This gradient of GLI induced transcription induces cellular proliferation and outgrowth of the mesenchyme (Lan and Jiang 2009). • OFCs caused by disruption to SHH are believed to be due to a reduction in epithelial induced proliferation and the subsequent decrease in tissue outgrowth and the failure of the facial processes to meet and fuse (Lipinski, Song et al. 2010, Heyne, Melberg et al. 2015).		
Defining Question	**High (Strong)**	**Moderate**	**Low (Weak)**
3. Empirical Support for KERs			
Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.
Relationship 2734: Antagonism Smoothened (Event 2027) leads to Decrease, SMO relocation (Event 2044)		**MODERATE** There is good evidence that the SANT compounds block the localization of SMO to the tip of the primary cilia. Contradictory in vivo data was found regarding whether cyclopamine blocks SMO relocation to the primary cilia. Further work is required to determine if SMO antagonism via cyclopamine results in decrease in SMO relocation. **Dose-response:** Data compiled thus far are insufficient to evaluate dose-response concordance for this KER. **Temporality:** There are currently no time-course studies addressing these events. **Uncertainties**: Contradictory data was found for whether or not cyclopamine causes a change in SMO relocation. Further investigation is needed to increase understanding of this discrepancy.	
Relationship 2735: Decrease, SMO relocation (Event 2044) leads to Decrease, GLI1/2 translocation (Event 2028)		**MODERATE** Moderate evidence is presented to support that a loss of the primary cilia leads to a significant decrease in GLI1. GLI1 requires activation prior to nuclear translocation. **Dose-response:** Data compiled thus far are insufficient to evaluate dose-response concordance for this KER. **Temporality:** There are currently no time-course studies addressing these events. **Uncertainties**: While we know that entry to the cilia is tightly controlled, the exact mechanism of SMO ciliary trafficking is not fully understood.	
Relationship 2721: Decrease, GLI1/2 translocation (Event 2028) leads to Decrease, GLI1/2 target gene expression (Event 2040)		**LOW** There is high biological plausibility of this relationship but to date few studies were found to explore the relationship. **Dose-response:** Data compiled thus far are insufficient to evaluate dose-response concordance for this KER. **Temporality:** There are currently no time-course studies addressing these events.	

Relationship 2731: Decrease GLI1/2 target gene expression (Event 2040) leads to Decrease, SHH second messenger production (Event 2043)	**LOW** Coordinated signaling is paramount for proper embryonic development and the GLI signaling cascade drives feedback/forward loops with FGF and BMP signaling pathways. Support was found for SHH having a feedforward loop with FGF10 and BMP4 however further investigation into the interaction of these pathways and their crosstalk is required. **Dose-response:** Data compiled thus far are insufficient to evaluate dose-response concordance for this KER. **Temporality:** There are currently no time-course studies addressing these events.
Relationship 2732: Decrease SHH second messenger production (Event 2043) leads to Decrease, cell proliferation (Event 1821)	**LOW** SHH was found to induce proliferation and FGF10 in vivo. In FGF10 deficient models SHH was found to be reduced. **Dose-response:** Data compiled thus far are insufficient to evaluate dose-response concordance for this KER. **Temporality:** There are currently no time-course studies addressing these events.
Relationship 2724: Decrease, Cell proliferation (Event 1821) leads to Decrease, outgrowth (Event 2041)	**LOW** SHH is a known mitogen that helps to drive the proper development of the face which includes the outgrowth of the facial prominences. To date, few studies have measured by outgrowth of the facial prominences and proliferation. Hypoplasia of pharyngeal arch 1 was found in SHH-/- embryos supporting that outgrowth is driven by proliferation and is reduced when proliferation is decreased. **Dose-response:** Data compiled thus far are insufficient to evaluate dose-response concordance for this KER. **Temporality:** There are currently no time-course studies addressing these events.
Relationship 2726: Decrease, outgrowth (Event 2041) leads to OFC (Event 2042)	**MODERATE** OFCs caused by disruption to SHH are believed to be due to a reduction in epithelial induced proliferation and the subsequent decrease in tissue outgrowth and the failure of the facial processes to meet and fuse (Lipinski, Song et al. 2010, Heyne, Melberg et al. 2015). Mice with disrupted SHH signaling are found to have palatal shelves that are spaced apart supporting that the cleft results from an EMi dependent, but epithelial-mesenchyme transition (Emt) independent manner. **Dose-response:** Data compiled thus far are insufficient to evaluate dose-response concordance for this KER. **Temporality:** There are currently no time-course studies addressing these events. However, critical periods of exposure for clefting have been identified.
Relationship 2792: Apoptosis (Event 1262) leads to Decrease, outgrowth (Event 2041)	**LOW** SHH signaling is known to be associated with cell survival and there is a high biological plausibility that increasing apoptosis would cause a decrease in outgrowth. Supporting evidence is offered with increases in apoptosis in the mandibular arch seen in SHH signaling disrupted mice that exhibit decreased outgrowth. **Dose-response:** Data compiled thus far are insufficient to evaluate dose-response concordance for this KER. **Temporality:** There are currently no time-course studies addressing these events.
Relationship 2882: Decrease, GLI1/2 target gene expression (Event 2040) leads to Apoptosis (Event 1262)	**LOW** To date few studies have examined the relationship of GLI1/2 target gene expression. There is a high biological plausibility that SHH plays a role in cell survival and death through GLI1/2 target gene expression. Decreased GLI1/2 target gene expression is seen in RA exposed dams alongside increased apoptosis on the CNCC. **Dose-response:** Data compiled thus far are insufficient to evaluate dose-response concordance for this KER. **Temporality:** There are currently no time-course studies addressing these events.
Relationship 2894: Antagonism Smoothened (Event 2027) leads to OFC (Event 2042)	**HIGH** Multiple studies have demonstrated *in vivo* that administration of SMO antagonists during critical windows of exposure leads to birth defects including OFC in a dose-dependent fashion.

	Dose-response: Multiple studies demonstrate a dose dependent incidence of clefting. It should be noted that a lack of studies investigating the dose concordance of this relationship were identified. **Temporality:** Critical exposure windows for OFC formation have been identified.

Bush, J. O. and R. Jiang (2012). "Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development." Development **139**(2): 231-243.
Carballo, G. B., J. R. Honorato, G. P. F. de Lopes and T. C. L. d. S. e. Spohr (2018). "A highlight on Sonic hedgehog pathway." Cell Communication and Signaling **16**(1): 11.
Corbit, K. C., P. Aanstad, V. Singla, A. R. Norman, D. Y. R. Stainier and J. F. Reiter (2005). "Vertebrate Smoothened functions at the primary cilium." Nature **437**(7061): 1018-1021.
Heyne, G. W., C. G. Melberg, P. Doroodchi, K. F. Parins, H. W. Kietzman, J. L. Everson, L. J. Ansen-Wilson and R. J. Lipinski (2015). "Definition of critical periods for Hedgehog pathway antagonist-induced holoprosencephaly, cleft lip, and cleft palate." PLoS One **10**(3): e0120517.
Huangfu, D. and K. V. Anderson (2005). "Cilia and Hedgehog responsiveness in the mouse." Proc Natl Acad Sci U S A **102**(32): 11325-11330.
Kim, J., M. Kato and P. A. Beachy (2009). "Gli2 trafficking links Hedgehog-dependent activation of Smoothened in the primary cilium to transcriptional activation in the nucleus." Proc Natl Acad Sci U S A **106**(51): 21666-21671.
Kogerman, P., T. Grimm, L. Kogerman, D. Krause, A. B. Undén, B. Sandstedt, R. Toftgård and P. G. Zaphiropoulos (1999). "Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1." Nat Cell Biol **1**(5): 312-319.
Kurosaka, H. (2015). "The Roles of Hedgehog Signaling in Upper Lip Formation." Biomed Res Int **2015**: 901041.
Lan, Y. and R. Jiang (2009). "Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth." Development **136**(8): 1387-1396.
Lipinski, R. J., C. Song, K. K. Sulik, J. L. Everson, J. J. Gipp, D. Yan, W. Bushman and I. J. Rowland (2010). "Cleft lip and palate results from Hedgehog signaling antagonism in the mouse: Phenotypic characterization and clinical implications." Birth Defects Res A Clin Mol Teratol **88**(4): 232-240.
Lipinski, R. J., C. Song, K. K. Sulik, J. L. Everson, J. J. Gipp, D. Yan, W. Bushman and I. J. Rowland (2010). "Cleft lip and palate results from Hedgehog signaling antagonism in the mouse: Phenotypic characterization and clinical implications." Birth defects research. Part A, Clinical and molecular teratology **88**(4): 232-240.
Pearse, R. V., 2nd, L. S. Collier, M. P. Scott and C. J. Tabin (1999). "Vertebrate homologs of Drosophila suppressor of fused interact with the gli family of transcriptional regulators." Dev Biol **212**(2): 323-336.
Rohatgi, R., L. Milenkovic, R. B. Corcoran and M. P. Scott (2009). "Hedgehog signal transduction by Smoothened: pharmacologic evidence for a 2-step activation process." Proc Natl Acad Sci U S A **106**(9): 3196-3201.
Rohatgi, R., L. Milenkovic and M. P. Scott (2007). "Patched1 regulates hedgehog signaling at the primary cilium." Science **317**(5836): 372-376.
Stone, D. M., M. Murone, S. Luoh, W. Ye, M. P. Armanini, A. Gurney, H. Phillips, J. Brush, A. Goddard, F. J. de Sauvage and A. Rosenthal (1999). "Characterization of the human suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli." J Cell Sci **112 (Pt 23)**: 4437-4448.
Tukachinsky, H., L. V. Lopez and A. Salic (2010). "A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes." J Cell Biol **191**(2): 415-428.
Tuson, M., M. He and K. V. Anderson (2011). "Protein kinase A acts at the basal body of the primary cilium to prevent Gli2 activation and ventralization of the mouse neural tube." Development **138**(22): 4921-4930.

Supplementary table 1: Organization of search terms and results for all KERs.

KER	Search date	Search terms	Number of search results	Title/ abstract review	Citations meeting title/abstract review	Number of citations in scope	Citations
2721	1/25/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("down regulation"[MeSH Terms] OR "down regulation"[All Fields] OR ("down"[All Fields] AND "regulation"[All Fields]) OR "down regulation"[All Fields]) AND "orofacial"[All Fields]	1	1	Everson et al 2017	1	Everson et al 2017
2721	1/25/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("trans activators"[MeSH Terms] OR "trans activators"[All Fields] OR ("trans"[All Fields] AND "activators"[All Fields]) OR "trans activators"[All Fields]) AND ("neural crest"[MeSH Terms] OR ("neural"[All Fields] AND "crest"[All Fields]) OR "neural crest"[All Fields]) AND ("face"[MeSH Terms] OR "face"[All Fields])	10	1	Jeong et al 2004	1	Jeong et al 2004
2721	1/27/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("gene	13	1	Lauth et al 2007	1	Lauth et al 2007

Supplementary table 1: Organization of search terms and results for all KERs.

		expression regulation"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields] AND "regulation"[All Fields]) OR "gene expression regulation"[All Fields]) AND ("zinc finger protein gli1"[MeSH Terms] OR ("zinc"[All Fields] AND "finger"[All Fields] AND "protein"[All Fields] AND "gli1"[All Fields]) OR "zinc finger protein gli1"[All Fields]) AND ("transcription, genetic"[MeSH Terms] OR ("transcription"[All Fields] AND "genetic"[All Fields]) OR "genetic transcription"[All Fields] OR ("transcription"[All Fields] AND "genetic"[All Fields]) OR "transcription genetic"[All Fields]) AND ("drug effects"[MeSH Subheading] OR ("drug"[All Fields] AND "effects"[All Fields]) OR "drug effects"[All Fields]) AND ("dna"[MeSH Terms] OR "dna"[All Fields])					
2721	1/27/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("cell proliferation"[MeSH Terms] OR ("cell"[All Fields] AND "proliferation"[All Fields]) OR "cell proliferation"[All Fields]) AND ("cell survival"[MeSH Terms] OR ("cell"[All Fields] AND "survival"[All Fields]) OR "cell survival"[All Fields]) AND ("gene expression regulation"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields] AND "regulation"[All Fields]) OR "gene expression regulation"[All Fields]) AND ("phenotype"[MeSH	27	4	Miyake et al 2005, Li et al 2017, Katoh et al 2009, Billmyre et al 2015	1	Katoh et al 2009

Supplementary table 1: Organization of search terms and results for all KERs.

		Terms] OR "phenotype"[All Fields] OR "phenotypes"[All Fields] OR "phenotyped"[All Fields] OR "phenotypic"[All Fields] OR "phenotypical"[All Fields] OR "phenotypically"[All Fields] OR "phenotyping"[All Fields] OR "phenotypings"[All Fields])					
2721	1/27/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("cell proliferation"[MeSH Terms] OR ("cell"[All Fields] AND "proliferation"[All Fields]) OR "cell proliferation"[All Fields]) AND ("mutate"[All Fields] OR "mutated"[All Fields] OR "mutates"[All Fields] OR "mutating"[All Fields] OR "mutation"[MeSH Terms] OR "mutation"[All Fields] OR "mutations"[All Fields] OR "mutation s"[All Fields] OR "mutational"[All Fields] OR "mutator"[All Fields] OR "mutators"[All Fields]) AND ("palatalization"[All Fields] OR "palatalized"[All Fields] OR "palatally"[All Fields] OR "palatals"[All Fields] OR "palate"[MeSH Terms] OR "palate"[All Fields] OR "palatal"[All Fields] OR "palates"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields])	15	4	Zhang et al 2002, thomason et al 2008, Lan et al 2009, Li et al 2018	1	Lan et al 2009
2724	1/27/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("cell proliferation"[MeSH Terms] OR ("cell"[All Fields] AND "proliferation"[All Fields]) OR "cell proliferation"[All Fields]) AND ("mutate"[All Fields]	15	4	Zhang et al 2002, thomason et al 2008, Lan et al	1	Lan et al 2009

Supplementary table 1: Organization of search terms and results for all KERs.

		OR "mutated"[All Fields] OR "mutates"[All Fields] OR "mutating"[All Fields] OR "mutation"[MeSH Terms] OR "mutation"[All Fields] OR "mutations"[All Fields] OR "mutation s"[All Fields] OR "mutational"[All Fields] OR "mutator"[All Fields] OR "mutators"[All Fields]) AND ("palatalization"[All Fields] OR "palatalized"[All Fields] OR "palatally"[All Fields] OR "palatals"[All Fields] OR "palate"[MeSH Terms] OR "palate"[All Fields] OR "palatal"[All Fields] OR "palates"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields])			2009, Li et al 2018		
2724	2/6/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("fibroblast growth factors"[MeSH Terms] OR ("fibroblast"[All Fields] AND "growth"[All Fields] AND "factors"[All Fields]) OR "fibroblast growth factors"[All Fields] OR ("fibroblast"[All Fields] AND "growth"[All Fields] AND "factor"[All Fields]) OR "fibroblast growth factor"[All Fields]) AND ("cleft palate"[MeSH Terms] OR ("cleft"[All Fields] AND "palate"[All Fields]) OR "cleft palate"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields])	15	1	Rice et al 2004,	1	Rice et al 2004
2724	3/10/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("embryology"[MeSH Subheading] OR	21	1	Yamagishi et al 2006,	1	Yamagishi et al 2006,

Supplementary table 1: Organization of search terms and results for all KERs.							
		"embryology"[All Fields] OR ("embryonic"[All Fields] AND "development"[All Fields]) OR "embryonic development"[All Fields] OR "embryonic development"[MeSH Terms] OR ("embryonic"[All Fields] AND "development"[All Fields])) AND ("branchial region"[MeSH Terms] OR ("branchial"[All Fields] AND "region"[All Fields]) OR "branchial region"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields])					
2724	3/10/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("mesoderm"[MeSH Terms] OR "mesoderm"[All Fields] OR "mesodermal"[All Fields] OR "mesodermalization"[All Fields] OR "mesodermalized"[All Fields] OR "mesodermalizing"[All Fields] OR "mesodermally"[All Fields] OR "mesodermic"[All Fields] OR "mesoderms"[All Fields]) AND ("neural crest"[MeSH Terms] OR ("neural"[All Fields] AND "crest"[All Fields]) OR "neural crest"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("membrane proteins"[MeSH Terms] OR ("membrane"[All Fields] AND "proteins"[All Fields]) OR "membrane proteins"[All Fields])	6		Jeong et al 2004, Hammond et al 2018	1	Jeong et al 2004
2726	1/27/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields])	15	4	Zhang et al 2002,	1	Lan et al 2009

Supplementary table 1: Organization of search terms and results for all KERs.

		OR "hedgehog proteins"[All Fields]) AND ("cell proliferation"[MeSH Terms] OR ("cell"[All Fields] AND "proliferation"[All Fields]) OR "cell proliferation"[All Fields]) AND ("mutate"[All Fields] OR "mutated"[All Fields] OR "mutates"[All Fields] OR "mutating"[All Fields] OR "mutation"[MeSH Terms] OR "mutation"[All Fields] OR "mutations"[All Fields] OR "mutation s"[All Fields] OR "mutational"[All Fields] OR "mutator"[All Fields] OR "mutators"[All Fields]) AND ("palatalization"[All Fields] OR "palatalized"[All Fields] OR "palatally"[All Fields] OR "palatals"[All Fields] OR "palate"[MeSH Terms] OR "palate"[All Fields] OR "palatal"[All Fields] OR "palates"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields])			thomason et al 2008, Lan et al 2009, Li et al 2018		
2726	2/6/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("fibroblast growth factors"[MeSH Terms] OR ("fibroblast"[All Fields] AND "growth"[All Fields] AND "factors"[All Fields]) OR "fibroblast growth factors"[All Fields] OR ("fibroblast"[All Fields] AND "growth"[All Fields] AND "factor"[All Fields]) OR "fibroblast growth factor"[All Fields]) AND ("cleft palate"[MeSH Terms] OR ("cleft"[All Fields] AND "palate"[All Fields]) OR "cleft palate"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields])	15	1	Rice et al 2004,	1	Rice et al 2004

Supplementary table 1: Organization of search terms and results for all KERs.

2731	2/2/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("cell proliferation"[MeSH Terms] OR ("cell"[All Fields] AND "proliferation"[All Fields]) OR "cell proliferation"[All Fields]) AND ("cell survival"[MeSH Terms] OR ("cell"[All Fields] AND "survival"[All Fields]) OR "cell survival"[All Fields]) AND ("gene expression regulation"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields] AND "regulation"[All Fields]) OR "gene expression regulation"[All Fields]) AND ("phenotype"[MeSH Terms] OR "phenotype"[All Fields] OR "phenotypes"[All Fields] OR "phenotyped"[All Fields] OR "phenotypic"[All Fields] OR "phenotypical"[All Fields] OR "phenotypically"[All Fields] OR "phenotyping"[All Fields] OR "phenotypings"[All Fields])	27	4	Miyake et al 2005, Li et al 2017, Katoh et al 2009, Billmyre et al 2015	1	Katoh et al 2009
2731	2/6/2023	("cleft palate"[MeSH Terms] OR ("cleft"[All Fields] AND "palate"[All Fields]) OR "cleft palate"[All Fields]) AND (("molecular"[All Fields] OR "moleculars"[All Fields]) AND ("aetiologie"[All Fields] OR "aetiologies"[All Fields] OR "aetiology"[All Fields] OR "etiologies"[All Fields] OR "etiology"[MeSH Subheading] OR "etiology"[All Fields] OR "causality"[MeSH Terms] OR "causality"[All Fields])) AND ("fibroblast growth factor 10"[MeSH Terms] OR "fibroblast growth	1	1	Alappat et al 2005	1	Alappat et al 2005

Supplementary table 1: Organization of search terms and results for all KERs.

		factor 10"[All Fields]) AND ("transforming growth factor beta"[MeSH Terms] OR ("transforming"[All Fields] AND "growth"[All Fields] AND "factor"[All Fields] AND "beta"[All Fields]) OR "transforming growth factor beta"[All Fields])					
2731	2/6/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("cell proliferation"[MeSH Terms] OR ("cell"[All Fields] AND "proliferation"[All Fields]) OR "cell proliferation"[All Fields]) AND ("mutate"[All Fields] OR "mutated"[All Fields] OR "mutates"[All Fields] OR "mutating"[All Fields] OR "mutation"[MeSH Terms] OR "mutation"[All Fields] OR "mutations"[All Fields] OR "mutation s"[All Fields] OR "mutational"[All Fields] OR "mutator"[All Fields] OR "mutators"[All Fields]) AND ("palatalization"[All Fields] OR "palatalized"[All Fields] OR "palatally"[All Fields] OR "palatals"[All Fields] OR "palate"[MeSH Terms] OR "palate"[All Fields] OR "palatal"[All Fields] OR "palates"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields])	15	4	Zhang et al 2002, thomason et al 2008, Lan et al 2009, Li et al 2018	1	Lan et al 2009
2731	2/6/2023	("gene expression regulation"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields] AND "regulation"[All Fields]) OR "gene expression regulation"[All Fields]) AND ("fibroblast growth factor 10"[MeSH Terms] OR "fibroblast growth factor 10"[All Fields]) AND ("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields]	8	2	Ohuchi et al 1997, Rice et al 2001	1	Ohuchi et al 1997

Supplementary table 1: Organization of search terms and results for all KERs.

		AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("fibroblast growth factor 8"[MeSH Terms] OR "fibroblast growth factor 8"[All Fields])					
2731	2/6/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("fibroblast growth factors"[MeSH Terms] OR ("fibroblast"[All Fields] AND "growth"[All Fields] AND "factors"[All Fields]) OR "fibroblast growth factors"[All Fields] OR ("fibroblast"[All Fields] AND "growth"[All Fields] AND "factor"[All Fields]) OR "fibroblast growth factor"[All Fields]) AND ("cleft palate"[MeSH Terms] OR ("cleft"[All Fields] AND "palate"[All Fields]) OR "cleft palate"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields])	15	1	Rice et al 2004,	1	Rice et al 2004
2732	1/27/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("cell proliferation"[MeSH Terms] OR ("cell"[All Fields] AND "proliferation"[All Fields]) OR "cell proliferation"[All Fields]) AND ("mutate"[All Fields] OR "mutated"[All Fields] OR "mutates"[All Fields] OR "mutating"[All Fields] OR "mutation"[MeSH Terms] OR "mutation"[All Fields] OR "mutations"[All Fields] OR "mutation s"[All Fields] OR "mutational"[All Fields] OR "mutator"[All Fields] OR "mutators"[All Fields]) AND ("palatalization"[All Fields] OR "palatalized"[All Fields] OR "palatally"[All	15	4	Zhang et al 2002, thomason et al 2008, Lan et al 2009, Li et al 2018	2	Lan et al 2009, Zhang et al 2002

Supplementary table 1: Organization of search terms and results for all KERs.

		Fields] OR "palatals"[All Fields] OR "palate"[MeSH Terms] OR "palate"[All Fields] OR "palatal"[All Fields] OR "palates"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields])					
2732	2/6/2023	("gene expression regulation"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields] AND "regulation"[All Fields]) OR "gene expression regulation"[All Fields]) AND ("fibroblast growth factor 10"[MeSH Terms] OR "fibroblast growth factor 10"[All Fields]) AND ("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("fibroblast growth factor 8"[MeSH Terms] OR "fibroblast growth factor 8"[All Fields])	8	2	Ohuchi et al 1997, Rice et al 2001	1	Ohuchi et al 1997
2732	2/6/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("fibroblast growth factors"[MeSH Terms] OR ("fibroblast"[All Fields] AND "growth"[All Fields] AND "factors"[All Fields]) OR "fibroblast growth factors"[All Fields] OR ("fibroblast"[All Fields] AND "growth"[All Fields] AND "factor"[All Fields]) OR "fibroblast growth factor"[All Fields]) AND ("cleft palate"[MeSH Terms] OR ("cleft"[All Fields] AND "palate"[All Fields]) OR "cleft palate"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields])	15	1	Rice et al 2004,	1	Rice et al 2004

Supplementary table 1: Organization of search terms and results for all KERs.

2732	2/6/2023	("cleft palate"[MeSH Terms] OR ("cleft"[All Fields] AND "palate"[All Fields]) OR "cleft palate"[All Fields]) AND (("molecular"[All Fields] OR "moleculars"[All Fields]) AND ("aetiologie"[All Fields] OR "aetiologies"[All Fields] OR "aetiology"[All Fields] OR "etiologies"[All Fields] OR "etiology"[MeSH Subheading] OR "etiology"[All Fields] OR "causality"[MeSH Terms] OR "causality"[All Fields])) AND ("fibroblast growth factor 10"[MeSH Terms] OR "fibroblast growth factor 10"[All Fields]) AND ("transforming growth factor beta"[MeSH Terms] OR ("transforming"[All Fields] AND "growth"[All Fields] AND "factor"[All Fields] AND "beta"[All Fields]) OR "transforming growth factor beta"[All Fields])	1	1	Alappat et al 2005	1	Alappat et al 2005
2732	2/10/2023	("cyclin d1"[MeSH Terms] OR ("cyclin"[All Fields] AND "d1"[All Fields]) OR "cyclin d1"[All Fields]) AND ("cyclin d2"[MeSH Terms] OR ("cyclin"[All Fields] AND "d2"[All Fields]) OR "cyclin d2"[All Fields]) AND ("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("mitogene"[All Fields] OR "mitogenes"[All Fields] OR "mitogenic"[All Fields] OR "mitogenically"[All Fields] OR "mitogenicity"[All Fields] OR "mitogens"[Pharmacological Action] OR "mitogens"[MeSH Terms] OR "mitogens"[All Fields] OR "mitogen"[All Fields])	2	1	Kenney et al 2000	1	Kenney et al 2000
2732	2/10/2023	("cyclin d1"[MeSH Terms] OR ("cyclin"[All Fields] AND "d1"[All Fields]) OR "cyclin d1"[All Fields]) AND ("cycline"[All Fields] OR "cyclines"[All Fields] OR	4	2	Lan et al 2009,	2	Lan et al 2009,

Supplementary table 1: Organization of search terms and results for all KERs.

		"cyclins"[MeSH Terms] OR "cyclins"[All Fields] OR "cyclin"[All Fields]) AND ("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("fibroblast growth factors"[MeSH Terms] OR ("fibroblast"[All Fields] AND "growth"[All Fields] AND "factors"[All Fields]) OR "fibroblast growth factors"[All Fields] OR ("fibroblast"[All Fields] AND "growth"[All Fields] AND "factor"[All Fields]) OR "fibroblast growth factor"[All Fields])			Lobjois et al 2004		Lobjois et al 2004
2734	1/18/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("receptor"[All Fields] OR "receptor s"[All Fields] OR "receptors"[All Fields]) AND "g-protein-coupled"[All Fields] AND ("smoothened receptor"[MeSH Terms] OR ("smoothened"[All Fields] AND "receptor"[All Fields]) OR "smoothened receptor"[All Fields]) AND ("binding sites"[MeSH Terms] OR ("binding"[All Fields] AND "sites"[All Fields]) OR "binding sites"[All Fields]) AND ("trans activators"[MeSH Terms] OR "trans activators"[All Fields] OR ("trans"[All Fields] AND "activators"[All Fields]) OR "trans activators"[All Fields])	8	3	Frank-Kamenetsky et al 2002, Chen et al 2002a, Chen et al 2002b	2	Chen et al 2002a, Chen et al 2002b
2734	1/23/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields] OR ("hedgehog"[All Fields] AND "protein"[All Fields]) OR "hedgehog protein"[All Fields]) AND ("smoothened receptor"[MeSH Terms] OR	10	2	Frank-Kamenetsky et al 2002, Taipale et al 2000,	1	Taipale et al 2000

Supplementary table 1: Organization of search terms and results for all KERs.

		("smoothened"[All Fields] AND "receptor"[All Fields]) OR "smoothened receptor"[All Fields]) AND ("receptors, g protein coupled"[MeSH Terms] OR ("receptors"[All Fields] AND "g protein coupled"[All Fields]) OR "g-protein-coupled receptors"[All Fields] OR "receptors g protein coupled"[All Fields]) AND "patch*"[All Fields] AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("trans activators"[MeSH Terms] OR "trans activators"[All Fields] OR ("trans"[All Fields] AND "activators"[All Fields]) OR "trans activators"[All Fields]) AND ("drug effects"[MeSH Subheading] OR ("drug"[All Fields] AND "effects"[All Fields]) OR "drug effects"[All Fields]) AND (("cytoplasm"[MeSH Terms] OR "cytoplasm"[All Fields] OR "intracellular"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields] OR "signaling"[All Fields] OR "signal"[All Fields] OR "signal s"[All Fields] OR "signaled"[All Fields] OR "signaler"[All Fields] OR "signaler s"[All Fields] OR "signalers"[All Fields] OR "signalings"[All Fields] OR "signalization"[All Fields] OR "signalled"[All Fields] OR "signaller"[All Fields] OR "signaller s"[All Fields] OR "signallers"[All Fields] OR "signalling"[All Fields] OR "signallings"[All Fields] OR "signals"[All Fields]))					
2734	1/23/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("human	2	2	Taipale et al 2000,	2	Taipale et al 2000,

Supplementary table 1: Organization of search terms and results for all KERs.

		s"[All Fields] OR "humans"[MeSH Terms] OR "humans"[All Fields] OR "human"[All Fields]) AND "neoplasms/metabolism"[MeSH Terms] AND ("therapeutics"[MeSH Terms] OR "therapeutics"[All Fields] OR "therapies"[All Fields] OR "therapy"[MeSH Subheading] OR "therapy"[All Fields] OR "therapy s"[All Fields] OR "therapys"[All Fields]) AND ("patched receptors"[MeSH Terms] OR ("patched"[All Fields] AND "Receptors"[All Fields]) OR "patched receptors"[All Fields]) AND ("proto oncogene mas"[MeSH Terms] OR ("proto oncogene"[All Fields] AND "mas"[All Fields]) OR "proto oncogene mas"[All Fields] OR ("proto"[All Fields] AND "oncogene"[All Fields] AND "mas"[All Fields]) OR "proto oncogene mas"[All Fields]) AND "receptors, cell surface/metabolism"[MeSH Terms] AND ("receptors, g protein coupled"[MeSH Terms] OR ("Receptors"[All Fields] AND "g protein coupled"[All Fields]) OR "g-protein-coupled receptors"[All Fields] OR "receptors g protein coupled"[All Fields]) AND ("metabolic"[All Fields] OR "metabolical"[All Fields] OR "metabolically"[All Fields] OR "metabolics"[All Fields] OR "metabolism"[MeSH Terms] OR "metabolism"[All Fields] OR "metabolisms"[All Fields] OR "metabolism"[MeSH Subheading] OR "metabolities"[All Fields] OR "metabolization"[All Fields] OR "metabolize"[All Fields] OR "metabolized"[All Fields] OR "metabolizer"[All Fields] OR "metabolizers"[All Fields] OR "metabolizes"[All Fields] OR "metabolizing"[All			Rohatgi et al 2007		Rohatgi et al 2007

Supplementary table 1: Organization of search terms and results for all KERs.

		Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("smoothened receptor"[MeSH Terms] OR ("smoothened"[All Fields] AND "receptor"[All Fields]) OR "smoothened receptor"[All Fields])					
2734	1/23/2023	"cleft lip/chemically induced"[MeSH Terms] AND ("metabolic"[All Fields] OR "metabolical"[All Fields] OR "metabolically"[All Fields] OR "metabolics"[All Fields] OR "metabolism"[MeSH Terms] OR "metabolism"[All Fields] OR "metabolisms"[All Fields] OR "metabolism"[MeSH Subheading] OR "metabolities"[All Fields] OR "metabolization"[All Fields] OR "metabolize"[All Fields] OR "metabolized"[All Fields] OR "metabolizer"[All Fields] OR "metabolizers"[All Fields] OR "metabolizes"[All Fields] OR "metabolizing"[All Fields]) AND ("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("cleft palate"[MeSH Terms] OR ("Cleft"[All Fields] AND "palate"[All Fields]) OR "cleft palate"[All Fields])	2	2	Heyne et al 2015, Lipinski et al 2008	1	Heyne et al 2015
2734	1/23/2023	("cholesterol"[MeSH Terms] OR "cholesterol"[All Fields] OR "cholesterol s"[All Fields] OR "cholesterole"[All Fields] OR "cholesterols"[All Fields]) AND ("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND	8	1	Incardona et al 1998	1	Incardona et al 1998

Supplementary table 1: Organization of search terms and results for all KERs.

		("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("trans activators"[MeSH Terms] OR "trans activators"[All Fields] OR ("trans"[All Fields] AND "activator"[All Fields]) OR "trans activator"[All Fields]) AND ("alkaloidal"[All Fields] OR "alkaloide"[All Fields] OR "alkaloidic"[All Fields] OR "alkaloids"[MeSH Terms] OR "alkaloids"[All Fields] OR "alkaloid"[All Fields])					
2734	1/23/2023	("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("cilia"[MeSH Terms] OR "cilia"[All Fields] OR "eyelashes"[MeSH Terms] OR "eyelashes"[All Fields] OR "cilias"[All Fields]) AND ("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("sonic"[All Fields] OR "sonically"[All Fields] OR "sonicate"[All Fields] OR "sonicated"[All Fields] OR "sonicates"[All Fields] OR "sonicating"[All Fields] OR "sonication"[MeSH Terms] OR "sonication"[All Fields] OR "sonications"[All Fields] OR "sonicator"[All Fields] OR "sonicators"[All Fields] OR "sonics"[All Fields]) AND ("craniofacial"[All Fields] OR "craniofacies"[All Fields])	13	2	Niida et al 2021, Millington et al 2017	1	Millington et al 2017
2734	1/23/2023	"cilia/drug effects"[MeSH Terms] AND ("metabolic"[All Fields] OR "metabolical"[All Fields] OR "metabolically"[All Fields] OR "metabolics"[All Fields] OR "metabolism"[MeSH Terms] OR "metabolism"[All Fields] OR "metabolisms"[All Fields] OR "metabolism"[MeSH Subheading] OR	7	3	Peluso et al 2014, Maurya et al 2017, Wang et al 2012	2	Maurya et al 2017, Wang et al 2012

Supplementary table 1: Organization of search terms and results for all KERs.

		"metabolities"[All Fields] OR "metabolization"[All Fields] OR "metabolize"[All Fields] OR "metabolized"[All Fields] OR "metabolizer"[All Fields] OR "metabolizers"[All Fields] OR "metabolizes"[All Fields] OR "metabolizing"[All Fields]) AND "hedgehog proteins/metabolism"[MeSH Terms] AND ("Cilia"[MeSH Terms] OR "Cilia"[All Fields] OR "eyelashes"[MeSH Terms] OR "eyelashes"[All Fields] OR "cilias"[All Fields]) AND ("drug effects"[MeSH Subheading] OR ("drug"[All Fields] AND "effects"[All Fields]) OR "drug effects"[All Fields]) AND ("smoothened receptor"[MeSH Terms] OR ("smoothened"[All Fields] AND "receptor"[All Fields]) OR "smoothened receptor"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields])					
2735	1/12/2023	(("smoothened receptor"[MeSH Terms] OR ("smoothened"[All Fields] AND "receptor"[All Fields]) OR "smoothened receptor"[All Fields]) AND ("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields] OR ("hedgehog"[All Fields] AND "protein"[All Fields]) OR "hedgehog protein"[All Fields]) AND ("cilia"[MeSH Terms] OR "cilia"[All Fields] OR "eyelashes"[MeSH Terms] OR "eyelashes"[All Fields] OR "cilias"[All Fields]) AND ("zinc fingers"[MeSH Terms] OR ("zinc"[All Fields] AND "fingers"[All Fields]) OR "zinc fingers"[All Fields] OR	17	4	Kim et al 2009, quin et al 2011, tukachinsky et al 2010, may et al 2005	1	Kim et al 2009

Supplementary table 1: Organization of search terms and results for all KERs.

		("zinc"[All Fields] AND "finger"[All Fields]) OR "zinc finger"[All Fields]) AND ("protein transport"[MeSH Terms] OR ("protein"[All Fields] AND "transport"[All Fields]) OR "protein transport"[All Fields])) NOT (neuro*)					
2735	1/12/2023	("smoothened receptor"[MeSH Terms] OR ("smoothened"[All Fields] AND "receptor"[All Fields]) OR "smoothened receptor"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("patched receptors"[MeSH Terms] OR ("patched"[All Fields] AND "receptors"[All Fields]) OR "patched receptors"[All Fields]) AND ("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("gene expression regulation"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields] AND "regulation"[All Fields]) OR "gene expression regulation"[All Fields]) AND ("receptors, cell surface"[MeSH Terms] OR ("receptors"[All Fields] AND "cell"[All Fields] AND "surface"[All Fields]) OR "cell surface receptors"[All Fields] OR ("receptors"[All Fields] AND "cell"[All Fields] AND "surface"[All Fields]) OR "receptors cell surface"[All Fields]) AND ("canonic"[All Fields] OR "canonical"[All Fields] OR "canonically"[All Fields] OR "canonicals"[All Fields])	5	2	Doheny et al 2020, Blotta et al 2012	1	Blotta et al 2012
2735	1/12/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND	6	2	rhogati et al 2007,	1	Huang et al 2016

Supplementary table 1: Organization of search terms and results for all KERs.

		("cholesterol"[MeSH Terms] OR "cholesterol"[All Fields] OR "cholesterol s"[All Fields] OR "cholesterole"[All Fields] OR "cholesterols"[All Fields]) AND ("smoothened receptor"[MeSH Terms] OR ("smoothened"[All Fields] AND "receptor"[All Fields]) OR "smoothened receptor"[All Fields]) AND ("protein binding"[MeSH Terms] OR ("protein"[All Fields] AND "binding"[All Fields]) OR "protein binding"[All Fields]) AND ("oxysterols"[MeSH Terms] OR "oxysterols"[All Fields] OR "oxysterol"[All Fields])			Huang et al 2016		
2735	1/13/2023	("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("cilia"[MeSH Terms] OR "cilia"[All Fields] OR "eyelashes"[MeSH Terms] OR "eyelashes"[All Fields] OR "cilias"[All Fields]) AND ("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("sonic"[All Fields] OR "sonically"[All Fields] OR "sonicate"[All Fields] OR "sonicated"[All Fields] OR "sonicates"[All Fields] OR "sonicating"[All Fields] OR "sonication"[MeSH Terms] OR "sonication"[All Fields] OR "sonications"[All Fields] OR "sonicator"[All Fields] OR "sonicators"[All Fields] OR "sonics"[All Fields]) AND ("craniofacial"[All Fields] OR "craniofacies"[All Fields])	13	2	Niida et al 2021, Millington et al 2017	1	Millington et al 2017
2735	1/13/2023	("gene expression regulation"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields] AND "regulation"[All Fields]) OR "gene expression regulation"[All Fields]) AND ("transcription	11	2	Zhang et al 2013, Kogerman et al 1999	1	Kogerman et al 1999

Supplementary table 1: Organization of search terms and results for all KERs.

		factors"[MeSH Terms] OR ("transcription"[All Fields] AND "factors"[All Fields]) OR "transcription factors"[All Fields]) AND "gli1"[All Fields] AND (("zinc fingers"[MeSH Terms] OR ("zinc"[All Fields] AND "fingers"[All Fields]) OR "zinc fingers"[All Fields] OR ("zinc"[All Fields] AND "finger"[All Fields]) OR "zinc finger"[All Fields]) AND ("protein s"[All Fields] OR "proteinous"[All Fields] OR "proteins"[MeSH Terms] OR "proteins"[All Fields] OR "protein"[All Fields])) AND ("embryo s"[All Fields] OR "embryoes"[All Fields] OR "embryonic structures"[MeSH Terms] OR ("embryonic"[All Fields] AND "structures"[All Fields]) OR "embryonic structures"[All Fields] OR "embryo"[All Fields] OR "embryos"[All Fields]) AND (("suppressor"[All Fields] OR "suppressors"[All Fields]) AND ("fused"[All Fields] OR "fuses"[All Fields] OR "fusing"[All Fields]))					
2882	4/10/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("cell death"[MeSH Terms] OR ("cell"[All Fields] AND "death"[All Fields]) OR "cell death"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("smoothened receptor"[MeSH Terms] OR ("smoothened"[All Fields] AND "receptor"[All Fields]) OR "smoothened receptor"[All Fields]) AND ("gene expression"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields]) OR "gene	17	1	Ahlgren et al 2002	1	Ahlgren et 2002

Supplementary table 1: Organization of search terms and results for all KERs.

		expression"[All Fields]) AND ("patched receptors"[MeSH Terms] OR ("patched"[All Fields] AND "receptors"[All Fields]) OR "patched receptors"[All Fields])					
2882	5/1/2023	("apoptosis"[MeSH Terms] OR "apoptosis"[All Fields]) AND ("embryology"[MeSH Subheading] OR "embryology"[All Fields] OR ("embryonic"[All Fields] AND "development"[All Fields]) OR "embryonic development"[All Fields] OR "embryonic development"[MeSH Terms] OR ("embryonic"[All Fields] AND "development"[All Fields])) AND (("hedgehogs"[MeSH Terms] OR "hedgehogs"[All Fields] OR "hedgehog"[All Fields]) AND "protiens"[All Fields]) AND ("embryonic development"[MeSH Terms] OR ("embryonic"[All Fields] AND "development"[All Fields]) OR "embryonic development"[All Fields] OR "embryogenesis"[All Fields]) AND ("cleft"[All Fields] OR "clefted"[All Fields] OR "clefting"[All Fields] OR "clefts"[All Fields]) AND ("n n bis 2 chloroethyl nitrosocarbamoyl cystamine"[Supplementary Concept] OR "n n bis 2 chloroethyl nitrosocarbamoyl cystamine"[All Fields] OR "cncc"[All Fields])	1	1	Kurosaka et al 2019	1	Kurosaka et al 2019
2894	1/23/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields] OR ("hedgehog"[All Fields] AND "protein"[All Fields]) OR "hedgehog protein"[All Fields]) AND ("smoothened receptor"[MeSH Terms] OR ("smoothened"[All Fields] AND "receptor"[All	10	2	Frank-Kamenetsky et al 2002, Taipale et al 2000,	1	Taipale et al 2000

Supplementary table 1: Organization of search terms and results for all KERs.

		Fields]) OR "smoothened receptor"[All Fields]) AND ("receptors, g protein coupled"[MeSH Terms] OR ("receptors"[All Fields] AND "g protein coupled"[All Fields]) OR "g-protein-coupled receptors"[All Fields] OR "receptors g protein coupled"[All Fields]) AND "patch*"[All Fields] AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("trans activators"[MeSH Terms] OR "trans activators"[All Fields] OR ("trans"[All Fields] AND "activators"[All Fields]) OR "trans activators"[All Fields]) AND ("drug effects"[MeSH Subheading] OR ("drug"[All Fields] AND "effects"[All Fields]) OR "drug effects"[All Fields]) AND (("cytoplasm"[MeSH Terms] OR "cytoplasm"[All Fields] OR "intracellular"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields] OR "signaling"[All Fields] OR "signal"[All Fields] OR "signal s"[All Fields] OR "signaled"[All Fields] OR "signaler"[All Fields] OR "signaler s"[All Fields] OR "signalers"[All Fields] OR "signalings"[All Fields] OR "signalization"[All Fields] OR "signalled"[All Fields] OR "signaller"[All Fields] OR "signaller s"[All Fields] OR "signallers"[All Fields] OR "signalling"[All Fields] OR "signallings"[All Fields] OR "signals"[All Fields]))					
2894	1/18/2023	("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("receptor"[All Fields] OR "receptor s"[All Fields] OR	8	3	Frank-Kamenetsky et al 2002, Chen et al	1	Chen et al 2002

Supplementary table 1: Organization of search terms and results for all KERs.

		"receptors"[All Fields]) AND "g-protein-coupled"[All Fields] AND ("smoothened receptor"[MeSH Terms] OR ("smoothened"[All Fields] AND "receptor"[All Fields]) OR "smoothened receptor"[All Fields]) AND ("binding sites"[MeSH Terms] OR ("binding"[All Fields] AND "sites"[All Fields]) OR "binding sites"[All Fields]) AND ("trans activators"[MeSH Terms] OR "trans activators"[All Fields] OR ("trans"[All Fields] AND "activators"[All Fields]) OR "trans activators"[All Fields])			2002a, Chen et al 2002b		
2894	1/23/2023	"cleft lip/chemically induced"[MeSH Terms] AND ("metabolic"[All Fields] OR "metabolical"[All Fields] OR "metabolically"[All Fields] OR "metabolics"[All Fields] OR "metabolism"[MeSH Terms] OR "metabolism"[All Fields] OR "metabolisms"[All Fields] OR "metabolism"[MeSH Subheading] OR "metabolities"[All Fields] OR "metabolization"[All Fields] OR "metabolize"[All Fields] OR "metabolized"[All Fields] OR "metabolizer"[All Fields] OR "metabolizers"[All Fields] OR "metabolizes"[All Fields] OR "metabolizing"[All Fields]) AND ("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal transduction"[All Fields]) AND ("cleft palate"[MeSH Terms] OR ("Cleft"[All Fields] AND "palate"[All Fields]) OR "cleft palate"[All Fields])	2	2	Heyne et al 2015, Lipinski et al 2008	1	Heyne et al 2015
2894	1/23/2023	("signal transduction"[MeSH Terms] OR ("signal"[All Fields] AND "transduction"[All Fields]) OR "signal	13	2	Niida et al 2021,	1	Millington et al 2017

Supplementary table 1: Organization of search terms and results for all KERs.

		transduction"[All Fields]) AND ("cilia"[MeSH Terms] OR "cilia"[All Fields] OR "eyelashes"[MeSH Terms] OR "eyelashes"[All Fields] OR "cilias"[All Fields]) AND ("hedgehog proteins"[MeSH Terms] OR ("hedgehog"[All Fields] AND "proteins"[All Fields]) OR "hedgehog proteins"[All Fields]) AND ("sonic"[All Fields] OR "sonically"[All Fields] OR "sonicate"[All Fields] OR "sonicated"[All Fields] OR "sonicates"[All Fields] OR "sonicating"[All Fields] OR "sonication"[MeSH Terms] OR "sonication"[All Fields] OR "sonications"[All Fields] OR "sonicator"[All Fields] OR "sonicators"[All Fields] OR "sonics"[All Fields]) AND ("craniofacial"[All Fields] OR "craniofacies"[All Fields])			Millington et al 2017		
2894	5/2/2023	("cyclopamine"[Supplementary Concept] OR "cyclopamine"[All Fields]) AND ("cleft"[All Fields] OR "clefted"[All Fields] OR "clefting"[All Fields] OR "clefts"[All Fields])	14	2	Lipinski et al 2010, Lipinski et al 2014	2	Lipinski et al 2010, Lipinski et al 2014

Supplementary table 2: Dose concordance for AOP. Studies were recorded for dose and any indication of any of the KEs in the AOP were noted.

Reference	Taxonomic Applicability	Life stage/ exposure period	Type	Concentration	Additional exposure details	MIE 2027	KE 2044	KE 2028	KE 2040	KE 1262	KE 2043	KE 1821	KE 2041	AO 2042	Notes
Heyne et al 2015	mouse- C57BL/6J	embryonic GD 7.0-10.0	vismodegib (GDC-0449)	40mg/kg	oral gavage									X	
Heyne et al 2015	mouse- C57BL/6J	embryonic GD 8.25-9.9375	cyclopamine	120mg/kg/d	subcutaneous									X	
Lipinski, Song et al 2010	mouse- C57BL/6J	embryonic GD 8.25	cyclopamine	120mg/kg/d	subcutaneous micro osmotic pump									X	25/45 pups had cleft
Millington et al 2017	mouse- Kif3af/f Wnt1-Cre	embryonic							X					X	GLI1 downregulation evidenced via RNA-seq
Maurya et al 2017	mouse- C57BL/6J	postnatal day 12-14	vismodegib (GDC-0449)	40mg/kg	5 doses 12h apart subcutatneous		X								Evidenced using decreased ciliary immunofluorescence for M71/M72
Maurya et al 2018	mouse- Smofl/fl; CRE	E18.5					X								Evidenced via BrdU that exogenous SHH induced proliferation in palatal mesenchyme explants
Incardona et al 1998	white leghorn chick- neural plate explant	stage 9-10	cyclopamine	120nm		X									disruption of SHH signaling evidenced via immunostianing for pax7
Kogerman, Grimm et al 1999	mouse	GD 8.5-15.5						X							disruption of SHH signaling evidenced via immunostianing for pax7
Everson, Fink et al 2017	mouse- C57BL/6J	GD 8.25-9.375	cyclopamine	120mg/kg/d	subcutaneous micro osmotic pump				X			X		X	Evidenced via RT-PCR showing down regulation of GLI1 and PTCH1 as well as nine Fox members: Foxa2, Foxb2, Foxc1, Foxc2, Foxd1, Foxe1, Foxf1, Foxf2, Foxl1 Evidenced via ki-67 staining showing decrease proliferation in for GD 10.25 embryos in medial protion of the MNP
Lan and Jiang 2009	mouse- Osr2-IresCre;Smoc/c (SMO inactive)	embryonic							X		X	X	X	X	Evidenced by down regualtion of PTCH1 and GLI1 in the palatal shelves Evidenced via in situ hybridization showing decrease in FGF10 correlated with down regulation of PTCH1 Evidenced by reduced ccnd1 and ccnd2 mRNA expression in palatal mesenchyme of mutants Evidenced by mutant E 14.5 palatal shelves having retarded growth and not making contact
Jeong, Mao et al 2005	mouse- Wnt1-Cre;Smon/c (SMO inactive)	embryonic -GD 9.5, 10.5, 12.5)						X		X	X		X		Evidenced via in situ hybridization of facial primordia showing down regulation of PTCH1, FOXC2, FOXD1, FOXD2, FOXF1, FOXF2 Evidenced for CNCCs in MNAs at E9.5, 10.5 via staining using rabit anti-cleaved- caspase 3 antibody Evidenced in MNAs at E11.5 via staining using anti-phospho-histone H3 antibody Evidenced via decrease in mandibular arch in both PD and DV axes
Ohuchi et al 1997	chick	embryonic									X				Evidenced through observation of rudimentary palatal shelves that are spaced apart without contact
Rice, Spencer-Dene et al 2004	mouse- K-14Cre;Shhc/n (shh null epithelium)												X	X	Evidenced through observation of rudimentary palatal shelves that are spaced apart without contact
Rice, Spencer-Dene et al 2005	mouse- K-14Cre;Smoc/n (SMO inactive epithelium)														clefting not observed suggesting SHH exerts its' effect on adjacent mesenchyme

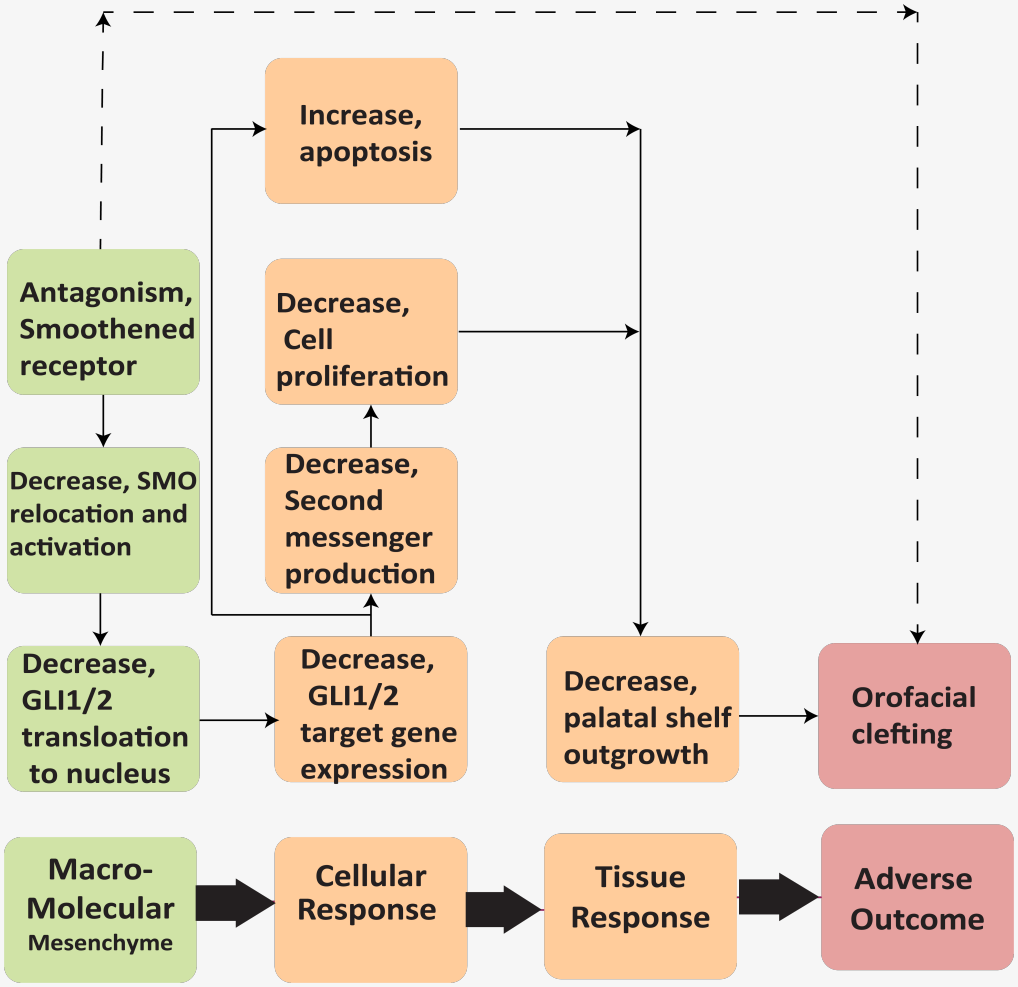
Supplementary table 2: Dose concordance for AOP. Studies were recorded for dose and any indication of any of the KEs in the AOP were noted.

Reference	Taxonomic Applicability	Life stage/ exposure period	Type	Concentration	Additional exposure details	MIE 2027	KE 2044	KE 2028	KE 2040	KE 1262	KE 2043	KE 1821	KE 2041	AO 2042	Notes
Rice, Spencer-Dene et al 2006	mouse-Fgf10–/–										X				Evidenced via BrdU that exogenous SHH induced proliferation in palatal mesenchyme explants. Exogenous FGF10 also induced proliferation. Evidenced via in situ hybridization showing decrease in SHH in palatal epithelium
Rice, Spencer-Dene et al 2007	mouse-Fgfr2b-/-										X	X			Evidenced via in situ hybridization showing decrease in SHH in palatal epithelium Evidenced via BrdU that exogenous SHH induced proliferation in palatal mesenchyme explants
Zhang, Song et al 2002	Mouse- CD-1	Embryonic- E13.5										X			Evidenced through SHH beads inducing proliferation in palatal shelves as measured through BrdU
Zhang, Song et al 2002	Mouse-MSX1-/-	Embryonic- E13.5										X			Evidenced through SHH beads inducing proliferation in palatal shelves as measured through BrdU
Yamagishi, Yamagishi et al 2006	mouse-SHH-/-	embryonic									X		X		Evidenced via TUNNEL assay at E9.0 and E9.5 Evidenced via hypoplasia in PA1 at E9.5
Keeler et al 1975	Rat-sprague Dawley	embryonic GD 6-9	cyclopamine	240mg/kg/day	oral gavage									X	
Keeler et al 1975	golden hamster	embryonic GD 6-9	cyclopamine	170mg/kg/day	oral gavage									X	
Omnell, Sim et al 1990	mouse-C57BL/6J	embryonic GD 8	jervine	70, 150, 300mg/kg	single dose gavage									X	dose response pattern for CLP
Omnell, Sim et al 1990	mouse- A/J	embryonic GD 8	jervine	70, 150, 300mg/kg	single dose gavage									X	dose response pattern for CLP

AOP ID and Title:

AOP 460: Antagonism of Smoothened receptor leading to orofacial clefting
Short Title: Antagonism SMO leads to OFC

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.101	Included in OECD Work Plan

Abstract

The Sonic Hedgehog (SHH) is a major signaling pathway of intercellular signaling during embryonic development. Disruption of SHH during critical periods of development can lead to orofacial clefts (OFCs). In canonical SHH signaling, the SHH ligand binds to the Patched1 (PTCH1) receptor and relieves its' suppression of Smoothened (SMO) receptor. Antagonism of SMO results in disruption of the downstream SHH signaling cascade. Disruption to the signaling cascade causes a decrease in the translocation of the GLI1/2 transcription factors to the nucleus resulting in a decrease in expression of the GLI1/2 target genes. This decrease in gene expression causes a reduction in production of SHH secondary messengers, namely Fgf10 and members of the BMP family. This reduction in secondary messengers leads to a decrease in cellular proliferation in the palatal shelves. This reduction in cellular proliferation leads to a decrease in palatal shelf outgrowth which ultimately results in a cleft. This AOP is intended to serve as a tool

for risk assessment for drug and chemical exposures during embryonic development when disruption to SHH through antagonism of SMO occurs.

Background

This AOP was developed as part of a larger network of AOPs linking disruption of SHH signaling with OFCs (OECD Advisory Group on Emerging Science in Chemicals Assessment (ESCA) workplan project 1.101.). This was the first AOP of the network to be developed and was selected due most stressors of the SHH pathway being believed to work at the level of SMO. Development was led by the Johnson lab at Michigan State University and coached by Dr. Judy Choi. This AOP serves as the primary literature for graduate student Jacob Reynolds' dissertation project. This work was supported by the National Institutes of Health R00-ES028744 and the National Institute of Environmental Health Sciences P42ES004911.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	2027	Antagonism, Smoothened receptor	Antagonism Smoothened
	KE	2044	Decrease, Smoothend relocation and activation	Decrease, SMO relocation
2	KE	2028	Decrease, GLI1/2 translocation to nucleus	Decrease, GLI1/2 translocation
	KE	2040	Decrease, GLI1/2 target gene expression	Decrease, GLI1/2 target gene expression
	KE	1262	Apoptosis	Apoptosis
	KE	2043	Decrease, Sonic Hedgehog second messenger production	Decrease, SHH second messenger production
	KE	1821	Decrease, Cell proliferation	Decrease, Cell proliferation
	KE	2041	Decrease, facial prominence outgrowth	Decrease, facial prominence outgrowth
	AO	2042	Increase, Orofacial clefting	orofacial cleft

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Antagonism, Smoothened receptor	adjacent	Decrease, Smoothend relocation and activation	Moderate	Low
Decrease, Smoothend relocation and activation	adjacent	Decrease, GLI1/2 translocation to nucleus	Moderate	Low
Decrease, GLI1/2 translocation to nucleus	adjacent	Decrease, GLI1/2 target gene expression	Low	Low
Decrease, GLI1/2 target gene expression	adjacent	Decrease, Sonic Hedgehog second messenger production	Low	Low
Decrease, Sonic Hedgehog second messenger production	adjacent	Decrease, Cell proliferation	Low	Low
Decrease, Cell proliferation	adjacent	Decrease, facial prominence outgrowth	Low	Low
Decrease, facial prominence outgrowth	adjacent	Increase, Orofacial clefting	Moderate	Low
Apoptosis	adjacent	Decrease, facial prominence outgrowth	Low	Low
Decrease, GLI1/2 target gene expression	adjacent	Apoptosis	Low	Low

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Antagonism, Smoothened receptor	non-adjacent	Increase, Orofacial clefting	High	Moderate

Stressors

Name	Evidence
Vismodegib	High
SANT-1	
SANT-2	
SANT-3	
SANT-4	

Vismodegib

Vismodegib (GDC-0449) is small molecule modulator of the sonic hedgehog (shh) pathway. It functions as an antagonist by binding to Smoothened (SMO) blockings its' activation and subsequent downstream signalling cascade. Vismodegib became the first agent approved to target the shh pathway in Jan. 2012 by the US FDA. It was approved by the European Medicines Agency (EMA) in July 2012 (Meiss, Andrlová et al. 2018). It has been used to identify critical periods of development for the shh pathway. Pregnant C57BL/6J mice dosed with 40mg/kg of Vismodegib between E7 and E10.0 had a peak incidence of CPO (34.38%) at E9.5(Heyne, Melberg et al. 2015). Pregnant C57/BL6J mice treated with 100mg/kg vismodegib via oral gavage at E10.5 and E12.5 displayed a 100% penetrance of complete cleft palate (Zhang, Wang et al. 2017). In a HWJSC/HPEKp spheroid fusion model 10µm vismodegib did not affect HPEKp viability or migration, did not affect *in vitro* fusion (Belair, Wolf et al. 2018).

Overall Assessment of the AOP

Annex 1 Table, Assessment of the relative level of confidence in the overall AOP based on rank ordered weight of evidence elements is attached in PDF format.

Annex 1

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus		NCBI

Sex Applicability

Sex	Evidence
Unspecific	High

Chemical: This AOP applies to antagonists of the SMO receptor. Chemical modulators of the SHH pathway have been identified including the natural alkaloid cyclopamine, both natural and synthetic pharmaceuticals (e.g. Vismodegib) , the widely used pesticide syergist piperonyl butoxide (PBO) with established human exposures (Lipinski, Dengler et al. 2007, Lipinski, Song et al. 2010, Wang, Lu et al. 2012, Everson, Sun et al. 2019, Rivera-González, Beames et al. 2021).

Sex: This AOP is unspecific to sex.

Life Stages: The relevant life stage for this AOP is embryonic development. More specifically, the development of the craniofacial region which occurs between GD 10.0 and GD 14.0 in the mouse and week 4-12 in human.

Taxonomic: At present, the empirical taxonomic applicability domain of this AOP is mouse (mus musculus). Most of the toxicological data that this AOP is based on has used mice as their model organism. Mice are a good analog of human craniofacial development and undergo similar signaling by SHH. The plausible domain of applicability for this AOP is mammals due to the largely conserved mechanisms of orofacial development and embryonic pathway

signaling.

Essentiality of the Key Events

To date, few studies have addressed the essentiality of the proposed sequence of key events. Evidence linking SHH disruption through a decrease in proliferation exists. The hypothesized sequence of events has a high temporal concordance for canonical SHH signaling pathway and orofacial development.
• Studies have shown that SHH signaling is required for normal facial development and plays a critical role in the growth of the facial processes that form the upper palate and lip (Bush and Jiang 2012, Kurosaka 2015).
•The epithelial derived SHH drives orofacial development through an induced gradient in the underlying mesenchyme (Lan and Jiang 2009, Kurosaka 2015). This gradient of SHH induces cellular proliferation and outgrowth of the mesenchyme (Lan and Jiang 2009).
• OFCs caused by disruption to SHH are believed to be due to a reduction in epithelial induced proliferation and the subsequent decrease in tissue outgrowth and the failure of the facial processes to meet and fuse (Lipinski, Song et al. 2010, Heyne, Melberg et al. 2015).

Weight of Evidence Summary

Evidence Assessment
- **KER ID**-Title-[Adjacency], [Evidence], [Quantitative Understanding]

- **Relationship 2734:** Antagonism Smoothened (Event 2027) leads to Decrease, SMO relocation (Event 2044)-[Adjacent], [Moderate], [Low]-There is a high biological plausibility of this relationship and SMO localization to the primary cilia is essential for proper SHH signaling in vertebrates (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Rohatgi, Milenkovic et al. 2009). There is good evidence that the SANT compounds block the localization of SMO to the tip of the primary cilia. Contradictory in vivo data was found regarding whether cyclopamine blocks SMO relocation to the primary cilia. Further work is required to determine if SMO antagonism via cyclopamine results in decrease in SMO relocation.

- **Relationship 2735:** Decrease, SMO relocation (Event 2044) leads to Decrease, GLI1/2 translocation (Event 2028)-[Adjacent], [Moderate], [Low]- Moderate evidence is presented to support that a loss of SMO relocation to the primary cilia leads to a significant decrease in GLI1. GLI1 requires activation prior to nuclear translocation.

- **Relationship 2721:** Decrease, GLI1/2 translocation (Event 2028) leads to Decrease, GLI1/2 target gene expression (Event 2040)-[Adjacent], [Low], [Low]- There is high biological plausibility of this relationship but to date few studies were found to explore the relationship.

- **Relationship 2731:** Decrease GLI1/2 target gene expression (Event 2040) leads to Decrease, SHH second messenger production (Event 2043)-[Adjacent], [Low], [Low]-Coordinated signaling is paramount for proper embryonic development and the GLI signaling cascade drives feedback/forward loops with FGF and BMP signaling pathways. Support was found for SHH having a feedforward loop with FGF10 and BMP4 however further investigation into the interaction of these pathways and their crosstalk is required.

- **Relationship 2732**: Decrease SHH second messenger production (Event 2043) leads to Decrease, cell proliferation (Event 1821)-[Adjacent], [Low], [Low]- SHH is a known mitogen and drives proliferation through its' secondary messengers. SHH was found to induce proliferation and FGF10 in vivo.

- **Relationship 2724:** Decrease, Cell proliferation (Event 1821) leads to Decrease, outgrowth (Event 2041)-[Adjacent], [Low], [Low]-SHH is a known mitogen that helps to drive the proper development of the face which includes the outgrowth of the facial prominences. To date, few studies have measured by outgrowth of the facial prominences and proliferation. Hypoplasia of pharyngeal arch 1 was found in SHH-/- embryos supporting that outgrowth is driven by proliferation and is reduced when proliferation is decreased.

- **Relationship 2726**: Decrease, outgrowth (Event 2041) leads to OFC (Event 2042)-[Adjacent], [Moderate], [Low]-OFCs caused by disruption to SHH are believed to be due to a reduction in epithelial induced mesenchymal proliferation and the subsequent decrease in tissue outgrowth and the failure of the facial processes to meet and fuse (Lipinski, Song et al. 2010, Heyne, Melberg et al. 2015). Mice with disrupted SHH signaling are found to have palatal shelves that are spaced apart supporting that the cleft results from an EMi dependent, but epithelial-mesenchyme transition (Emt) independent manner.

- **Relationship 2792**: Apoptosis (Event 1262) leads to Decrease, outgrowth (Event 2041)-[Adjacent], [Low], [Low]-

SHH signaling is known to be associated with cell survival and there is a high biological plausibility that increasing apoptosis would cause a decrease in outgrowth. Supporting evidence is offered with increases in apoptosis in the mandibular arch seen in SHH signaling disrupted mice that exhibit decreased outgrowth.

- **Relationship 2882**: Decrease, GLI1/2 target gene expression (Event 2040) leads to Apoptosis (Event 1262) - [Adjacent], [Low], [Low]- To date few studies have examined the relationship of GLI1/2 target gene expression. There is a high biological plausibility that SHH plays a role in cell survival and death through GLI1/2 target gene expression. Decreased GLI1/2 target gene expression is seen in RA exposed dams alongside increased apoptosis on the cranial neural crest cells (CNCC).

- **Relationship 2894:** Antagonism Smoothened (Event 2027) leads to OFC (Event 2042)-[Non-adjacent], [High], [Moderate]- multiple studies have demonstrated in vivo that administration of SMO antagonists during critical windows of exposure leads to birth defects including OFC in a dose-dependent fashion.

Biological Plausibility

Biological plausibility refers to the structural and/or functional relationship that exists between the key events based on our understanding of normal biology. SHH signaling is largely conserved in mammals and is required for normal facial development and plays a critical role in the growth of the facial processes that form the upper palate and lip (Bush and Jiang 2012, Kurosaka 2015). Multiple antagonists of the SMO receptor have been identified through binding studies. Identified SMO antagonists include cyclopamine, vismodegib, PBO, and the SANT compounds (Lipinski, Dengler et al. 2007, Lipinski, Song et al. 2010, Wang, Lu et al. 2012, Everson, Sun et al. 2019, Rivera-González, Beames et al. 2021). While the level of support for most of the KERs is low, there is high support for the non-adjacent relationship linking antagonism of SMO and OFC.
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Concordance of dose-response relationships

Agreed, Wiki updated- There are a limited number of studies in which multiple key events were assessed in the same study following exposure to known SMO antagonists. These studies form the basis of the dose-response concordance of this AOP. A summary of the dose-concordance can be found in Supplementary Table 2. Many of the studies identified while researching this AOP were performed using a single dose of antagonist making the study not suited for dose response concordance. This AOP would benefit greatly from increased studies designed to explore the dose-response concordance of the proposed relationships. The concentration-dependence of the key event responses regarding concentration of known in vitro and/or in vivo for some of the KEs in this AOP is summarized below.
- Concentration dependent clefting with cyclopamine exposure (Omnell, Sim et al. 1990)
- Dose dependent binding to SMO (Chen, Taipale et al. 2002)
- Concentration dependent decrease in SMO-ciliary accumulation in vitro for vismodegib exposure (Wang, Arvanites et al. 2012)

Temporal concordance
The hypothesized sequence of events is supported by the existing data and follow the field’s current understanding of the canonical SHH signaling pathway.

Consistency
The AO is not specific to this AOP. Many of the events is this AOP will overlap with AOPs linking disruption of SHH to OFC and some are expected to overlap with AOPs linking other developmental signaling pathways to OFCs.

Uncertainties, inconsistencies, and data gaps
This AOP would be strengthened by studies examining the dose-response and time-course relationships for these KERs. The main data gaps for this AOP exist in the lack of studies that have examined the relationship in the context of dose response or time course. Additional studies using the mice would help to strengthen this AOP.

Data gaps:
- Dose response and time course studies relating a Decrease, SMO relocation leads to Decrease, GLI1/2 translocation
- Dose response and time course studies relating a decrease GLI translocation leads to decrease GLI target gene expression
- Dose response and time course studies relating a Decrease, GLI1/2 target gene expression leads to Decrease, SHH second messenger production
- Dose response and time course studies relating a Decrease, SHH second messenger production leads to Decrease, Cell proliferation
- Dose response and time course studies relating a Decrease, Cell proliferation leads to Decrease, outgrowth
- Dose response and time course studies relating a Decrease, outgrowth leads to OFC
- Dose response and time course studies relating a Apoptosis leads to Decrease, Outgrowth
- Dose response and time course studies relating a Decrease, GLI1/2 target gene expression leads to Apoptosis

Inconsistencies:
- While it is well understood that cyclopamine is an antagonist of SMO, contradictory in vivo data was found regarding whether cyclopamine blocks SMO relocation to the primary cilia. Rohatgi et al used NIH 3T3s cell and found

that cyclopamine did not inhibit the accumulation of SMO in the cilia even when dosed at 5-10um (>10 fold above kd). All three antagonists inhibited SHH pathway transduction and target gene expression (Rohatgi, Milenkovic et al. 2009). Corbit et al used a renal epithelial MDCK (Madin-Darby canine kidney) line was engineered to express Myc-tagged SMO. Following culture for 1hr in SHH conditioned media SMO presence in the primary cilium is upregulated while cells cultured in the presence of cyclopamine see a downregulation of SMO in the primary cilia (Corbit, Aanstad et al. 2005). Further work is required to determine if SMO antagonism via cyclopamine results in decrease in SMO relocation.

Uncertainties:

- While we know that entry to the cilia is tightly controlled, the exact mechanism of SMO ciliary trafficking is not fully understood. The primary cilia (PC) is separated from the plasma membrane by the ciliary pockets and the transition zone which function together to regulate the movement of lipids and proteins in and out of the organelle (Goetz, Ocbina et al. 2009, Rohatgi and Snell 2010). The SHH receptor PTCH contains a ciliary localization sequence in its' carboxy tail. Localization of PTCH to the PC is essential for inhibition of SMO as deletion of the CLS in PTCH prevents PTCH localization as well as inhibition of SMO (Kim, Hsia et al. 2015) (53). SMO also contains a CLS, but only accumulates in the PC upon ligand binding (Corbit, Aanstad et al. 2005). The entry of SMO into the PC is thought to occur either laterally through the ciliary pockets or internally via recycling endosomes (Milenkovic, Scott et al. 2009). Once inside the PC, SMO can diffuse freely, however it will usually accumulate in specific locations depending upon its' activation state. Inactive SMO will accumulate more at the base of the PC while active SMO will accumulate in the tip of the PC (Milenkovic, Weiss et al. 2015).
- The relationships and feedback/feedforward loops that exist between SHH and its' secondary messengers primarily FGF10 and BMP4 are not well understood. More investigation into these relationships is warranted.
- The exact mechanism through which SHH promotes cell survival is not well understood. Further studies are needed to illuminate the mechanism that links SHH signaling with cell survival.
- The relationship between GLI1/2 target gene expression and increased apoptosis has a high biological plausibility although there is currently lack of studies that address this relationship.

Quantitative Consideration

Assessment of quantitative understanding of the AOP:

The quantitative understanding for this AOP with the exception of the non-adjacent relationship between Antagonism Smoothened leads to OFC is low. Most of the data found through the literature search was obtained from studies that employed a single dose and were not conducted with dose-response or time-course in mind. For Antagonism Smoothend leads to OFC several studies with dose response data showing a dose-dependent incidence of clefting were found. This AOP would benefit from the generation of additional data that addresses these relationships in a dose response and time course methodology to allow for an increased quantitative understanding of the linkage.

Considerations for Potential Applications of the AOP (optional)

Considerations for potential applications of the AOP

The intended use of this AOP from a regulatory standpoint is to improve predictive potential of developmental hazards as they relate to the SHH pathway and OFCs. It is hoped that this AOP can be applied to data from in silico and in vitro high-throughput screening assays (HTS) to guide selection of agents for further investigation in more representative models of orofacial development. Disruption of the Sonic Hedgehog pathway has broader outcomes than just OFCs and SHH is known to play a role in many aspects of embryonic development including patterning of many systems and limb and digit development. This AOP can be used as part of an integrated assessment of toxicity and can help to guide risk assessment for potential exposures during development.

There is a need for development of New Approach Methodologies (NAMs) to increase understanding of the relationships that exist within this AOP to provide facilitate screenings abilities. Humans are exposed to upwards of 80,000 industrial chemicals and natural products, the majority of which have not undergone any type of toxicity testing either alone or in mixtures. Even highly regulated drugs are typically not tested for safety in pregnant women for obvious reasons despite the medical need in this population (Wise 2022). To help address this, we have engineered an in vitro microphysiological model (MPM) model of orofacial development to facilitate the study of both normal and abnormal orofacial development including disruption of SHH (Johnson, Vitek et al. 2021, Reynolds, Vitek et al. 2022). Traditional high throughput screening (HTS) assays are optimized for one pathway: one readout. This oversimplifies toxicant metabolism, intercellular pathway interactions, and ultimately makes the assay not representative of real-life exposures. Problems with HTS in drug discovery have been identified including missing intercellular interactions, co-exposures, and off target safety (Macarron, Banks et al. 2011). We can learn from these identified problems and engineer in vitro systems to more accurately recapitulate the biology to give a more thorough assessment of chemical and drug exposure.

References

Bush, J. O. and R. Jiang (2012). "Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development." Development **139**(2): 231-243.

Chen, J. K., J. Taipale, M. K. Cooper and P. A. Beachy (2002). "Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened." Genes Dev **16**(21): 2743-2748.

Corbit, K. C., P. Aanstad, V. Singla, A. R. Norman, D. Y. R. Stainier and J. F. Reiter (2005). "Vertebrate Smoothened functions at the primary cilium." Nature **437**(7061): 1018-1021.

Everson, J. L., M. R. Sun, D. M. Fink, G. W. Heyne, C. G. Melberg, K. F. Nelson, P. Doroodchi, L. J. Colopy, C. M. Ulschmid, A. A. Martin, M. T. McLaughlin and R. J. Lipinski (2019). "Developmental Toxicity Assessment of Piperonyl Butoxide Exposure Targeting Sonic Hedgehog Signaling and Forebrain and Face Morphogenesis in the Mouse: An in Vitro and in Vivo Study." Environ Health Perspect **127**(10): 107006.

Goetz, S. C., P. J. Ocbina and K. V. Anderson (2009). "The primary cilium as a Hedgehog signal transduction machine."Methods Cell Biol **94**: 199-222.

Heyne, G. W., C. G. Melberg, P. Doroodchi, K. F. Parins, H. W. Kietzman, J. L. Everson, L. J. Ansen-Wilson and R. J. Lipinski (2015). "Definition of critical periods for Hedgehog pathway antagonist-induced holoprosencephaly, cleft lip, and cleft palate." PLoS One **10**(3): e0120517.

IPDTOC Working Group(2011). "Prevalence at birth of cleft lip with or without cleft palate: data from the International Perinatal Database of Typical Oral Clefts (IPDTOC)." Cleft Palate Craniofac J **48**(1): 66-81.

Jeong, J. and A. P. McMahon (2002). "Cholesterol modification of Hedgehog family proteins." The Journal of Clinical Investigation **110**(5): 591-596.

Johnson, B. P., R. A. Vitek, M. M. Morgan, D. M. Fink, T. G. Beames, P. G. Geiger, D. J. Beebe and R. J. Lipinski (2021). "A Microphysiological Approach to Evaluate Effectors of Intercellular Hedgehog Signaling in Development." Front Cell Dev Biol **9**: 621442.

Kim, J., E. Y. Hsia, A. Brigui, A. Plessis, P. A. Beachy and X. Zheng (2015). "The role of ciliary trafficking in Hedgehog receptor signaling." Sci Signal **8**(379): ra55.

Kurosaka, H. (2015). "The Roles of Hedgehog Signaling in Upper Lip Formation."Biomed Res Int **2015**: 901041.

Lan, Y. and R. Jiang (2009). "Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth." Development **136**(8): 1387-1396.

Lauth, M., A. Bergström, T. Shimokawa and R. Toftgård (2007). "Inhibition of GLI-mediated transcription and tumor cell growth by small-molecule antagonists." Proc Natl Acad Sci U S A **104**(20): 8455-8460.

Lidral, A. C., L. M. Moreno and S. A. Bullard (2008). "Genetic Factors and Orofacial Clefting."Semin Orthod **14**(2): 103-114.

Lipinski, R. J. and W. Bushman (2010). "Identification of Hedgehog signaling inhibitors with relevant human exposure by small molecule screening." Toxicol In Vitro **24**(5): 1404-1409.

Lipinski, R. J., E. Dengler, M. Kiehn, R. E. Peterson and W. Bushman (2007). "Identification and characterization of several dietary alkaloids as weak inhibitors of hedgehog signaling." Toxicol Sci **100**(2): 456-463.

Lipinski, R. J., C. Song, K. K. Sulik, J. L. Everson, J. J. Gipp, D. Yan, W. Bushman and I. J. Rowland (2010). "Cleft lip and palate results from Hedgehog signaling antagonism in the mouse: Phenotypic characterization and clinical implications." Birth Defects Res A Clin Mol Teratol **88**(4): 232-240.

Lipinski, R. J., C. Song, K. K. Sulik, J. L. Everson, J. J. Gipp, D. Yan, W. Bushman and I. J. Rowland (2010). "Cleft lip and palate results from Hedgehog signaling antagonism in the mouse: Phenotypic characterization and clinical implications." Birth defects research. Part A, Clinical and molecular teratology **88**(4): 232-240.

Macarron, R., M. N. Banks, D. Bojanic, D. J. Burns, D. A. Cirovic, T. Garyantes, D. V. Green, R. P. Hertzberg, W. P. Janzen, J. W. Paslay, U. Schopfer and G. S. Sittampalam (2011). Impact of high-throughput screening in biomedical research. Nat Rev Drug Discov. England. **10:** 188-195.

Milenkovic, L., M. P. Scott and R. Rohatgi (2009). "Lateral transport of Smoothened from the plasma membrane to the membrane of the cilium." J Cell Biol **187**(3): 365-374.

Milenkovic, L., L. E. Weiss, J. Yoon, T. L. Roth, Y. S. Su, S. J. Sahl, M. P. Scott and W. E. Moerner (2015). "Single-molecule imaging of Hedgehog pathway protein Smoothened in primary cilia reveals binding events regulated by Patched1." Proc Natl Acad Sci U S A **112**(27): 8320-8325.

Omnell, M. L., F. R. Sim, R. F. Keeler, L. C. Harne and K. S. Brown (1990). "Expression of Veratrum alkaloid teratogenicity in the mouse." Teratology **42**(2): 105-119.

Petrova, E., J. Rios-Esteves, O. Ouerfelli, J. F. Glickman and M. D. Resh (2013). "Inhibitors of Hedgehog acyltransferase block Sonic Hedgehog signaling." Nat Chem Biol **9**(4): 247-249.

Reynolds, J. I., R. A. Vitek, P. G. Geiger and B. P. Johnson (2022). Engineering Epithelial–Mesenchymal Microtissues to Study Cell–Cell Interactions in Development. Craniofacial Development: Methods and Protocols. S. Dworkin. New York, NY, Springer US: 201-213.

Rivera-González, K. S., T. G. Beames and R. J. Lipinski (2021). "Examining the developmental toxicity of piperonyl butoxide as a Sonic hedgehog pathway inhibitor." Chemosphere **264**: N.PAG-N.PAG.

Rohatgi, R., L. Milenkovic, R. B. Corcoran and M. P. Scott (2009). "Hedgehog signal transduction by Smoothened: pharmacologic evidence for a 2-step activation process." Proc Natl Acad Sci U S A **106**(9): 3196-3201.

Rohatgi, R., L. Milenkovic and M. P. Scott (2007). "Patched1 regulates hedgehog signaling at the primary cilium." Science **317**(5836): 372-376.

Rohatgi, R. and W. J. Snell (2010). "The ciliary membrane." Curr Opin Cell Biol **22**(4): 541-546.

Wang, J., J. Lu, R. A. Mook, Jr., M. Zhang, S. Zhao, L. S. Barak, J. H. Freedman, H. K. Lyerly and W. Chen (2012). "The insecticide synergist piperonyl butoxide inhibits hedgehog signaling: assessing chemical risks." Toxicol Sci **128**(2): 517-523.

Wang, Y., A. C. Arvanites, L. Davidow, J. Blanchard, K. Lam, J. W. Yoo, S. Coy, L. L. Rubin and A. P. McMahon (2012). "Selective identification of hedgehog pathway antagonists by direct analysis of smoothened ciliary translocation." ACS Chem Biol **7**(6): 1040-1048.

Wise, J. (2022). "Pregnant women should be included in clinical trials to improve outcomes, says commission." Bmj **377**: o1193.

Appendix 1

List of MIEs in this AOP

Event: 2027: Antagonism, Smoothened receptor

Short Name: Antagonism Smoothened

Key Event Component

Process	Object	Action
regulation of receptor activity	smoothened	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term
mesenchymal cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates		NCBI
Invertebrates	Invertebrates		NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- SMO is present in both male and females and differences in activation or antagonism between sex have not been demonstrated.
- Life stages- The Hedgehog pathway is a major pathway in embryonic development. While the pathway is largely inactive following development, aberrant activation of SHH signaling is known to cause cancer (Dahmane, Lee et al. 1997, Kimura, Stephen et al. 2005). For these reasons all stages of life are of relevance.
- Taxonomic- SMO is conserved in both vertebrates and invertebrates. SMO signaling is dependent upon its relocation to a subcellular location. This occurs in the plasma membrane for flies (Denef, Neubüser et al. 2000) and the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005).

Key Event Description

The Smoothened (SMO) receptor is Class F G protein coupled receptor involved in signal transduction of the Sonic Hedgehog (SHH) pathway. It includes distinct functional groups including ligand binding pockets, cysteine rich domain (CRD), transmembrane helix (TM), extracellular loop (ECL), intracellular loop (ICL), and a carboxyl-terminal tail (C-term tail) (Arensdorf, Marada et al. 2016). SMO signaling is dependent upon its relocation to a subcellular location. This occurs in the plasma membrane for flies (Denef, Neubüser et al. 2000) and the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005).

In the absence of Hedgehog (HH) ligand, the Patched (PTCH) receptor suppresses the activation of SMO. When HH ligand binds to PTCH, suppression on SMO is released and SMO is able to relocate, accumulate, and signal to intracellular effectors (Denef, Neubüser et al. 2000). This signaling to effectors results in the activation of the GLI transcription factors and the subsequent induction of HH target gene expression(Alexandre, Jacinto et al. 1996, Von Ohlen and Hooper 1997). The exact mechanism through which PTCH and SMO interact is not known.

An endogenous ligand for SMO has not been discovered although evidence for one exists and that PTCH controls SMO by controlling its' availability or accessibility. To support this, it has been shown that PTCH and SMO do not physically interact (Chen and Struhl 1998). PTCH acts catalytically with SMO with one PTCH receptor capable of controlling many (~50) SMO receptors (Taipale, Cooper et al. 2002). Since PTCH includes a sterol sensing domain and shares characteristics of ancient bacterial transporters, a model of PTCH functioning by pumping a sterol-like MSO regulator has been proposed (Mukhopadhyay and Rohatgi 2014). SMO is constitutively active in the absence of PTCH suggesting that the elusive molecule is an agonist (Rohatgi and Scott 2007). Conversely, the discovery that oxysterols bind to the CRD binding domain acting as positive modulators suggest that the molecule could be an agonist with PTCH functioning to sequester away or limit cellular concentration (Corcoran and Scott 2006, Nachtergaele, Mydock et al. 2012)

The activity of SMO is controlled by ligand binding (Kobilka 2007). Two separate binding pockets, one in the groove of the extracellular CRD and the other in the helices of the TMD have been identified (Nachtergaele, Mydock et al. 2012, Rana, Carroll et al. 2013, Wang, Wu et al. 2013, Byrne, Sircar et al. 2016, Huang, Zheng et al. 2018). These two binding pockets have been shown to interact in an allosteric manner (Nachtergaele, Mydock et al. 2012). The binding pocket in the helices of the TMD binds several SMO agonists including SAG as well as antagonists Vismodegib and Sonidegib. The CRD binding pocket binds cholesterol and its' oxidized derivates (Byrne, Luchetti et al. 2018). The antagonist cyclopamine binds to the TMD binding pocket and inhibits SHH signal transduction. However, in mSMO carrying the mutations D477G/E552K that disable the TMD binding pocket, cyclopamine binds to the CRD pocket and activates the pathway (Huang, Nedelcu et al. 2016). To date several oxysterols including 20(S)-hydroxylcholesterol, 22(S)-hydroxylcholesterol, 7-keto-25-hydroxylcholesterol and 7-keto-27-hydroxylcholesterol have been identified as activators of SMO (Dwyer, Sever et al. 2007, Nachtergaele, Mydock et al. 2012, Myers, Sever et al. 2013). A binding site for 24(S),25-epoxycholesterol has been identified in the TMD pocket using cryo-EM of SMO in complex with 24(S),25-epoxycholesterol (Qi, Liu et al. 2019).

How it is Measured or Detected

Verification of binding and affinity for SMO can be measured using fluorescence binding assays and photoaffinity labeling respectively (Chen, Taipale et al. 2002).

References

Alexandre, C., A. Jacinto and P. W. Ingham (1996). "Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins." Genes Dev **10**(16): 2003-2013.

Arensdorf, A. M., S. Marada and S. K. Ogden (2016). "Smoothened Regulation: A Tale of Two Signals." Trends Pharmacol Sci **37**(1): 62-72.

Byrne, E. F. X., G. Luchetti, R. Rohatgi and C. Siebold (2018). "Multiple ligand binding sites regulate the Hedgehog signal transducer Smoothened in vertebrates." Current Opinion in Cell Biology **51**: 81-88.

Byrne, E. F. X., R. Sircar, P. S. Miller, G. Hedger, G. Luchetti, S. Nachtergaele, M. D. Tully, L. Mydock-McGrane, D. F. Covey, R. P. Rambo, M. S. P. Sansom, S. Newstead, R. Rohatgi and C. Siebold (2016). "Structural basis of Smoothened regulation by its extracellular domains." Nature **535**(7613): 517-522.

Chen, J. K., J. Taipale, M. K. Cooper and P. A. Beachy (2002). "Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened." Genes Dev **16**(21): 2743-2748.

Chen, J. K., J. Taipale, K. E. Young, T. Maiti and P. A. Beachy (2002). "Small molecule modulation of Smoothened activity." Proc Natl Acad Sci U S A **99**(22): 14071-14076.

Chen, Y. and G. Struhl (1998). "In vivo evidence that Patched and Smoothened constitute distinct binding and transducing components of a Hedgehog receptor complex." Development **125**(24): 4943-4948.

Corcoran, R. B. and M. P. Scott (2006). "Oxysterols stimulate Sonic hedgehog signal transduction and proliferation of medulloblastoma cells." Proc Natl Acad Sci U S A **103**(22): 8408-8413.

Dahmane, N., J. Lee, P. Robins, P. Heller and A. Ruiz i Altaba (1997). "Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours." Nature **389**(6653): 876-881.

Denef, N., D. Neubüser, L. Perez and S. M. Cohen (2000). "Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened." Cell **102**(4): 521-531.

Dwyer, J. R., N. Sever, M. Carlson, S. F. Nelson, P. A. Beachy and F. Parhami (2007). "Oxysterols are novel activators of the hedgehog signaling pathway in pluripotent mesenchymal cells." J Biol Chem **282**(12): 8959-8968.

Heyne, G. W., C. G. Melberg, P. Doroodchi, K. F. Parins, H. W. Kietzman, J. L. Everson, L. J. Ansen-Wilson and R. J. Lipinski (2015). "Definition of critical periods for Hedgehog pathway antagonist-induced holoprosencephaly, cleft lip, and cleft palate." PLoS One **10**(3): e0120517.

Huang, P., D. Nedelcu, M. Watanabe, C. Jao, Y. Kim, J. Liu and A. Salic (2016). "Cellular Cholesterol Directly Activates Smoothened in Hedgehog Signaling." Cell **166**(5): 1176-1187.e1114.

Huang, P., S. Zheng, B. M. Wierbowski, Y. Kim, D. Nedelcu, L. Aravena, J. Liu, A. C. Kruse and A. Salic (2018). "Structural Basis of Smoothened Activation in Hedgehog Signaling." Cell **174**(2): 312-324.e316.

Huangfu, D. and K. V. Anderson (2005). "Cilia and Hedgehog responsiveness in the mouse." Proc Natl Acad Sci U S A **102**(32): 11325-11330.

Incardona, J. P., W. Gaffield, R. P. Kapur and H. Roelink (1998). "The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction." Development **125**(18): 3553-3562.

Kimura, H., D. Stephen, A. Joyner and T. Curran (2005). "Gli1 is important for medulloblastoma formation in Ptc1+/- mice." Oncogene **24**(25): 4026-4036.

Kobilka, B. K. (2007). "G protein coupled receptor structure and activation." Biochimica et Biophysica Acta (BBA) - Biomembranes **1768**(4): 794-807.

Lou, H., H. Li, A. R. Huehn, N. I. Tarasova, B. Saleh, S. K. Anderson and M. Dean (2020). "Genetic and Epigenetic Regulation of the Smoothened Gene (SMO) in Cancer Cells." Cancers (Basel) **12**(8).

Meiss, F., H. Andrlová and R. Zeiser (2018). "Vismodegib." Recent Results Cancer Res **211**: 125-139.

Mukhopadhyay, S. and R. Rohatgi (2014). "G-protein-coupled receptors, Hedgehog signaling and primary cilia." Semin Cell Dev Biol **33**: 63-72.

Myers, Benjamin R., N. Sever, Yong C. Chong, J. Kim, Jitendra D. Belani, S. Rychnovsky, J. F. Bazan and Philip A. Beachy (2013). "Hedgehog Pathway Modulation by Multiple Lipid Binding Sites on the Smoothened Effector of Signal Response." Developmental Cell **26**(4): 346-357.

Nachtergaele, S., L. K. Mydock, K. Krishnan, J. Rammohan, P. H. Schlesinger, D. F. Covey and R. Rohatgi (2012). "Oxysterols are allosteric activators of the oncoprotein Smoothened." Nature Chemical Biology **8**(2): 211-220.

Nachtergaele, S., L. K. Mydock, K. Krishnan, J. Rammohan, P. H. Schlesinger, D. F. Covey and R. Rohatgi (2012). "Oxysterols are allosteric activators of the oncoprotein Smoothened." Nat Chem Biol **8**(2): 211-220.

Qi, X., H. Liu, B. Thompson, J. McDonald, C. Zhang and X. Li (2019). "Cryo-EM structure of oxysterol-bound human Smoothened coupled to a heterotrimeric Gi." Nature **571**(7764): 279-283.

Rana, R., C. E. Carroll, H.-J. Lee, J. Bao, S. Marada, C. R. R. Grace, C. D. Guibao, S. K. Ogden and J. J. Zheng (2013). "Structural insights into the role of the Smoothened cysteine-rich domain in Hedgehog signalling." Nature Communications **4**(1): 2965.

Rohatgi, R. and M. P. Scott (2007). "Patching the gaps in Hedgehog signalling."Nat Cell Biol **9**(9): 1005-1009.

Sharpe, H. J., W. Wang, R. N. Hannoush and F. J. de Sauvage (2015). "Regulation of the oncoprotein Smoothened by small molecules." Nat Chem Biol **11**(4): 246-255.

Sinha, S. and J. K. Chen (2006). "Purmorphamine activates the Hedgehog pathway by targeting Smoothened."Nat Chem Biol **2**(1): 29-30.

Taipale, J., M. K. Cooper, T. Maiti and P. A. Beachy (2002). "Patched acts catalytically to suppress the activity of Smoothened." Nature **418**(6900): 892-896.

Von Ohlen, T. and J. E. Hooper (1997). "Hedgehog signaling regulates transcription through Gli/Ci binding sites in the wingless enhancer." Mech Dev **68**(1-2): 149-156.

Wang, C., H. Wu, T. Evron, E. Vardy, G. W. Han, X. P. Huang, S. J. Hufeisen, T. J. Mangano, D. J. Urban, V. Katritch, V. Cherezov, M. G. Caron, B. L. Roth and R. C. Stevens (2014). "Structural basis for Smoothened receptor modulation and chemoresistance to anticancer drugs." Nat Commun **5**: 4355.

Wang, C., H. Wu, V. Katritch, G. W. Han, X. P. Huang, W. Liu, F. Y. Siu, B. L. Roth, V. Cherezov and R. C. Stevens (2013). "Structure of the human smoothened receptor bound to an antitumour agent." Nature **497**(7449): 338-343.

List of Key Events in the AOP

Event: 2044: Decrease, Smoothend relocation and activation

Short Name: Decrease, SMO relocation

Key Event Component

Process	Object	Action
protein localization to cilium	smoothened	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates		NCBI

Life Stage Applicability

Life Stage	Evidence

Life Stage	Evidence
All life stages	
Embryo	

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- SMO and cilia are present in both male and females and differences in gene expression has not been demonstrated.

- Life stages- The Hedgehog pathway is a major pathway in embryonic development.
- Taxonomic-SMO relocation to the tip of primary cilia occurs in vertebrates Huangfu and Anderson 2005)

Key Event Description

The Smoothened (SMO) receptor is Class F G protein coupled receptor involved in signal transduction of the Sonic Hedgehog (SHH) pathway. It includes distinct functional groups including ligand binding pockets, cysteine rich domain (CRD), transmembrane helix (TM), extracellular loop (ECL), intracellular loop (ICL), and a carboxyl-terminal tail (C-term tail) (Arensdorf, Marada et al. 2016). SMO signaling is dependent upon its relocation to a subcellular location. This relocation occurs in the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005). Relocation of SMO to the PC typically occurs within ~20 minutes of agonist stimulation (Arensdorf, Marada et al. 2016).

In the absence of SHH ligand, the Patched (PTCH) receptor suppresses the activation of SMO. When HH ligand binds to PTCH, suppression on SMO is released and SMO can relocate, accumulate, and signal to intracellular effectors (Denef, Neubüser et al. 2000, Rohatgi and Scott 2007). It has been shown that SMO localization to the tip of the primary cilia is essential for the SHH signaling cascade in vertebrates (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Rohatgi, Milenkovic et al. 2009). This relocation then leads to signaling to effectors resulting in the activation of the GLI transcription factors and the subsequent induction of HH target gene expression (Alexandre, Jacinto et al. 1996, Von Ohlen and Hooper 1997). The exact mechanism through which PTCH and SMO interact is not known.

While we know that entry to the cilia is tightly controlled, the exact mechanism of SMO ciliary trafficking is not fully understood. The PC is separated from the plasma membrane by the ciliary pockets and the transition zone which function together to regulate the movement of lipids and proteins in and out of the organelle (Goetz, Ocbina et al. 2009, Rohatgi and Snell 2010). The SHH receptor PTCH contains a ciliary localization sequence in its' carboxy tail. Localization of PTCH to the PC is essential for inhibition of SMO as deletion of the CLS in PTCH prevents PTCH localization as well as inhibition of SMO (Kim, Hsia et al. 2015) (53). SMO also contains a CLS, but only accumulates in the PC upon ligand binding (Corbit, Aanstad et al. 2005). The entry of SMO into the PC is thought to occur either laterally through the ciliary pockets or internally via recycling endosomes (Milenkovic, Scott et al. 2009). Once inside the PC, SMO can diffuse freely, however it will usually accumulate in specific locations depending upon its' activation state. Inactive SMO will accumulate more at the base of the PC while active SMO will accumulate in the tip of the PC (Milenkovic, Weiss et al. 2015).

How it is Measured or Detected

- Fluorescent proteins can be used tag SMO, cilia and the plasma membrane to determine if SMO has relocated to the cilia (Filipova, Diaz Garcia et al. 2020).
- Fluorescent binding assay can be used to verify if a compound binds to SMO (Chen, Taipale et al. 2002).
- Cell lines can be engineered to express Myc-tagged SMO. This gives a user friendly readout of SMO activation. (Corbit, Aanstad et al. 2005).

References

Alexandre, C., A. Jacinto and P. W. Ingham (1996). "Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins." Genes Dev **10**(16): 2003-2013.

Arensdorf, A. M., S. Marada and S. K. Ogden (2016). "Smoothened Regulation: A Tale of Two Signals."Trends Pharmacol Sci **37**(1): 62-72.

Chen, J. K., J. Taipale, M. K. Cooper and P. A. Beachy (2002). "Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened." Genes Dev **16**(21): 2743-2748.

Corbit, K. C., P. Aanstad, V. Singla, A. R. Norman, D. Y. R. Stainier and J. F. Reiter (2005). "Vertebrate Smoothened functions at the primary cilium." Nature **437**(7061): 1018-1021.

Denef, N., D. Neubüser, L. Perez and S. M. Cohen (2000). "Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened." Cell **102**(4): 521-531.

Filipova, A., D. Diaz Garcia, J. Dvorak, S. Filip, M. Jelicova and Z. Sinkorova (2020). "Simple Detection of Primary Cilia by Immunofluorescence." J Vis Exp(159).

Goetz, S. C., P. J. Ocbina and K. V. Anderson (2009). "The primary cilium as a Hedgehog signal transduction machine."Methods Cell Biol **94**: 199-222.

Huangfu, D. and K. V. Anderson (2005). "Cilia and Hedgehog responsiveness in the mouse."Proc Natl Acad Sci U S A **102**(32): 11325-11330.

Kim, J., E. Y. Hsia, A. Brigui, A. Plessis, P. A. Beachy and X. Zheng (2015). "The role of ciliary trafficking in Hedgehog receptor signaling." Sci Signal **8**(379): ra55.

Milenkovic, L., M. P. Scott and R. Rohatgi (2009). "Lateral transport of Smoothened from the plasma membrane to the membrane of the cilium." J Cell Biol **187**(3): 365-374.

Milenkovic, L., L. E. Weiss, J. Yoon, T. L. Roth, Y. S. Su, S. J. Sahl, M. P. Scott and W. E. Moerner (2015). "Single-molecule imaging of Hedgehog pathway protein Smoothened in primary cilia reveals binding events regulated by Patched1." Proc Natl Acad Sci U S A **112**(27): 8320-8325.

Rohatgi, R., L. Milenkovic, R. B. Corcoran and M. P. Scott (2009). "Hedgehog signal transduction by Smoothened: pharmacologic evidence for a 2-step activation process." Proc Natl Acad Sci U S A **106**(9): 3196-3201.

Rohatgi, R., L. Milenkovic and M. P. Scott (2007). "Patched1 regulates hedgehog signaling at the primary cilium."Science **317**(5836): 372-376.

Rohatgi, R. and M. P. Scott (2007). "Patching the gaps in Hedgehog signalling."Nat Cell Biol **9**(9): 1005-1009.

Rohatgi, R. and W. J. Snell (2010). "The ciliary membrane."Curr Opin Cell Biol **22**(4): 541-546.

Von Ohlen, T. and J. E. Hooper (1997). "Hedgehog signaling regulates transcription through Gli/Ci binding sites in the wingless enhancer." Mech Dev **68**(1-2): 149-156.

Event: 2028: Decrease, GLI1/2 translocation to nucleus

Short Name: Decrease, GLI1/2 translocation

Key Event Component

Process	Object	Action
protein import into nucleus, translocation	zinc finger protein GLI1	decreased
protein import into nucleus, translocation	zinc finger protein GLI2	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent
Aop:502 - Decrease, cholesterol synthesis leads to orofacial clefting	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

cell

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Embryo	High
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- The Gli family of transcription factors is present in both male and females and differences in activation or antagonism between sex have not been demonstrated.
- Life stages- The Hedgehog pathway is a major pathway in embryonic development. Aberrant activation of HH signalling is known to cause cancer (Dahmane, Lee et al. 1997, Kimura, Stephen et al. 2005). For these reasons all stages of life are of relevance.
- Taxonomic-HH signalling including the Gli transcription factors is present in vertebrates and some invertebrates inclubind flies (Denef, Neubüser et al. 2000, Huangfu and Anderson 2005)

Key Event Description

The Glioma-associated onocogene (Gli) family of zinc finger transcription factors (Gli1, Gli2, Gli3) are the primarily downstream effectors of the Hedgehog (HH) signaling cascade. When HH ligand binds to Patched (PTCH), its' inhibition on SMO is relieved. SMO this then able to accumulate to the tip of primary cilium in its' active form (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Kim, Kato et al. 2009). SMO causes the GLI family to become dislodged from their complex with the negative regulator of HH signaling, Suppressor of Fused (Sufu) (Kogerman, Grimm et al. 1999, Pearse, Collier et al. 1999, Stone, Murone et al. 1999, Tukachinsky, Lopez et al. 2010). The GLI-Sufu complex maintains retention of Gli in the cytosol allowing for exposure to phosphorylation via protein kinase A (PKA) which inhibits downstream signal transduction (Tuson, He et al. 2011). When SMO is activated the GLI2/3-Sufu complex is dismantled allowing for retrograde transport of GLI back into the nucleus (Kim, Kato et al. 2009).

The GLI family is found in both a long activator form (GliA) or a proteolytically cleaved repressor form (GliR). Current understanding is that Gli3 functions primarily as a repressor while Gli1 and Gli2 function mainly as activators of the pathway and that recruitment of SMO to the cilium leads to a increase in the ratio of GliA:GliR (Hui and Angers 2011, Liu 2016).

How it is Measured or Detected

- A nuclear translocation assay (NTA) can be applied to determine the amount of protein that translocate into the nucleus (Dixon and Lim 2010).
- Nuclear protein extracts can be analysed to determine if the protein of interest (GLI1/2) translocated to the nucleus (Kim, Kato et al. 2009).
- Immunofluorescence and microscopy can be used to determine how much of a protein has translocated to the nucleus. Primary antibodies can be used to tag GLI in combination with a secondary stain for the nucleus (Blotta, Jakubikova et al. 2012).

References

Blotta, S., J. Jakubikova, T. Calimeri, A. M. Roccaro, N. Amodio, A. K. Azab, U. Foresta, C. S. Mitsiades, M. Rossi, K. Todoerti, S. Molica, F. Morabito, A. Neri, P. Tagliaferri, P. Tassone, K. C. Anderson and N. C. Munshi (2012). "Canonical and noncanonical Hedgehog pathway in the pathogenesis of multiple myeloma." Blood **120**(25): 5002-5013.

Corbit, K. C., P. Aanstad, V. Singla, A. R. Norman, D. Y. R. Stainier and J. F. Reiter (2005). "Vertebrate Smoothened functions at the primary cilium." Nature **437**(7061): 1018-1021.

Dahmane, N., J. Lee, P. Robins, P. Heller and A. Ruiz i Altaba (1997). "Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours." Nature **389**(6653): 876-881.

Denef, N., D. Neubüser, L. Perez and S. M. Cohen (2000). "Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened." Cell **102**(4): 521-531.

Dixon, A. S. and C. S. Lim (2010). "The nuclear translocation assay for intracellular protein-protein interactions and its application to the Bcr coiled-coil domain." Biotechniques **49**(1): 519-524.

Huangfu, D. and K. V. Anderson (2005). "Cilia and Hedgehog responsiveness in the mouse." Proc Natl Acad Sci U S A **102**(32): 11325-11330.

Hui, C. C. and S. Angers (2011). "Gli proteins in development and disease."Annu Rev Cell Dev Biol **27**: 513-537.

Kim, J., M. Kato and P. A. Beachy (2009). "Gli2 trafficking links Hedgehog-dependent activation of Smoothened in the primary cilium to transcriptional activation in the nucleus." Proc Natl Acad Sci U S A **106**(51): 21666-21671.

Kimura, H., D. Stephen, A. Joyner and T. Curran (2005). "Gli1 is important for medulloblastoma formation in Ptc1+/- mice." Oncogene **24**(25): 4026-4036.

Kogerman, P., T. Grimm, L. Kogerman, D. Krause, A. B. Undén, B. Sandstedt, R. Toftgård and P. G. Zaphiropoulos (1999). "Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1." Nat Cell Biol **1**(5): 312-319.

Liu, K. J. (2016). "Craniofacial Ciliopathies and the Interpretation of Hedgehog Signal Transduction." PLoS Genet **12**(12): e1006460.

Pearse, R. V., 2nd, L. S. Collier, M. P. Scott and C. J. Tabin (1999). "Vertebrate homologs of Drosophila suppressor of fused interact with the gli family of transcriptional regulators." Dev Biol **212**(2): 323-336.

Rohatgi, R., L. Milenkovic and M. P. Scott (2007). "Patched1 regulates hedgehog signaling at the primary cilium." Science **317**(5836): 372-376.

Stone, D. M., M. Murone, S. Luoh, W. Ye, M. P. Armanini, A. Gurney, H. Phillips, J. Brush, A. Goddard, F. J. de Sauvage and A. Rosenthal (1999). "Characterization of the human suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli." J Cell Sci **112 (Pt 23)**: 4437-4448.

Tukachinsky, H., L. V. Lopez and A. Salic (2010). "A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes." J Cell Biol **191**(2): 415-428.

Tuson, M., M. He and K. V. Anderson (2011). "Protein kinase A acts at the basal body of the primary cilium to prevent Gli2 activation and ventralization of the mouse neural tube." Development **138**(22): 4921-4930.

Event: 2040: Decrease, GLI1/2 target gene expression

Short Name: Decrease, GLI1/2 target gene expression

Key Event Component

Process	Object	Action
gene expression	zinc finger protein GLI1	decreased
gene expression	zinc finger protein GLI2	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent
Aop:491 - Decrease, GLI1/2 target gene expression leads to orofacial clefting	MolecularInitiatingEvent
Aop:502 - Decrease, cholesterol synthesis leads to orofacial clefting	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- The GLI family of transcription factors is present in both male and females and differences in gene expression have not been demonstrated.
- Life stages- The Hedgehog pathway with the main transcription factors of GLI1/2 can be active during all stages of life. It is a major pathway in embryonic development. Aberrant activation of HH signaling is known to cause cancer (Dahmane, Lee et al. 1997, Kimura, Stephen et al. 2005). For these reasons all stages of life are of relevance.
- Taxonomic-HH signaling including the GLI transcription factors is present in vertebrates and some invertebrates including flies (Denef, Neubüser et al. 2000, Huangfu and Anderson 2005)

Key Event Description

The Glioma-associated onocogene (GLI) family of zinc finger transcription factors (Gli1, Gli2, Gli3) are the primarily downstream effectors of the Hedgehog (HH) signaling cascade. When HH ligand binds to Patched (PTCH), its' inhibition on SMO is relieved. SMO is then able to accumulate to the tip of primary cilium in its' active form (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Kim, Kato et al. 2009). SMO causes the GLI family to become dislodged from their complex with the negative regulator of HH signaling, Suppressor of Fused (Sufu) (Kogerman, Grimm et al. 1999, Pearse, Collier et al. 1999, Stone, Murone et al. 1999, Tukachinsky, Lopez et al. 2010). The GLI-Sufu complex maintains retention of Gli in the cytosol allowing for exposure to phosphorylation via protein kinase A (PKA) which inhibits downstream signal transduction (Tuson, He et al. 2011). When SMO is activated the GLI2/3-Sufu complex is dismantled allowing for retrograde transport of GLI back into the nucleus (Kim, Kato et al. 2009). Following translocation into the nucleus, the GLI family of transcription factors initiates transcription of a variety of genes. The genes transcribed by activation of the SHH pathway are cell type dependent but commonly include GLI1 and PTCH1 (Stamataki, Ulloa et al. 2005, Cohen, Kicheva et al. 2015, Tickle and Towers 2017). During development of the neural tube SHH is associated with NKX6.1, OLIG2, NKX2.2 and the FOXA2 genes (Vokes, Ji et al. 2007, Kutejova, Sasai et al. 2016). Other genes have are known targets of GLI transcription include PTCH2, HHIP1, MYCN, CCND1, CCND2, BCL2, CFLA, FOXF1, FOXFL1, PRDM1, JAG2, GREM1, FOXB2, FOXA2, FOXB2, FOXC1, FOXC2, FOXD1, FOXE1, FOXF1, FOXF2, FOXL1 and follistatin (Katoh and Katoh 2009, Everson, Fink et al. 2017).

How it is Measured or Detected

- Changes in gene expression can be measured using serial analysis of gene expression (SAGE), rapid analysis of gene expression (RAGE), RT-PCR, Northern/Southern blotting, differential display, and DNA microarray assay (Kirby, Heath et al. 2007).

References

Cohen, M., A. Kicheva, A. Ribeiro, R. Blassberg, K. M. Page, C. P. Barnes and J. Briscoe (2015). "Ptch1 and Gli regulate Shh signalling dynamics via multiple mechanisms." Nature Communications **6**(1): 6709.

Corbit, K. C., P. Aanstad, V. Singla, A. R. Norman, D. Y. R. Stainier and J. F. Reiter (2005). "Vertebrate Smoothened functions at the primary cilium." Nature **437**(7061): 1018-1021.

Dahmane, N., J. Lee, P. Robins, P. Heller and A. Ruiz i Altaba (1997). "Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours." Nature **389**(6653): 876-881.

Denef, N., D. Neubüser, L. Perez and S. M. Cohen (2000). "Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened." Cell **102**(4): 521-531.

Everson, J. L., D. M. Fink, J. W. Yoon, E. J. Leslie, H. W. Kietzman, L. J. Ansen-Wilson, H. M. Chung, D. O. Walterhouse, M. L. Marazita and R. J. Lipinski (2017). "Sonic hedgehog regulation of Foxf2 promotes cranial neural crest mesenchyme proliferation and is disrupted in cleft lip morphogenesis." Development **144**(11): 2082-2091.

Huangfu, D. and K. V. Anderson (2005). "Cilia and Hedgehog responsiveness in the mouse." Proc Natl Acad Sci U S A **102**(32): 11325-11330.

Katoh, Y. and M. Katoh (2009). "Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation." Curr Mol Med **9**(7): 873-886.

Kim, J., M. Kato and P. A. Beachy (2009). "Gli2 trafficking links Hedgehog-dependent activation of Smoothened in the primary cilium to transcriptional activation in the nucleus." Proc Natl Acad Sci U S A **106**(51): 21666-21671.

Kimura, H., D. Stephen, A. Joyner and T. Curran (2005). "Gli1 is important for medulloblastoma formation in Ptc1+/- mice." Oncogene **24**(25): 4026-4036.

Kirby, J., P. R. Heath, P. J. Shaw and F. C. Hamdy (2007). Gene Expression Assays. Advances in Clinical Chemistry, Elsevier. **44:** 247-292.

Kogerman, P., T. Grimm, L. Kogerman, D. Krause, A. B. Undén, B. Sandstedt, R. Toftgård and P. G. Zaphiropoulos (1999). "Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1." Nat Cell Biol **1**(5): 312-319.

Kutejova, E., N. Sasai, A. Shah, M. Gouti and J. Briscoe (2016). "Neural Progenitors Adopt Specific Identities by Directly Repressing All Alternative Progenitor Transcriptional Programs." Dev Cell **36**(6): 639-653.

Pearse, R. V., 2nd, L. S. Collier, M. P. Scott and C. J. Tabin (1999). "Vertebrate homologs of Drosophila suppressor of fused interact with the gli family of transcriptional regulators." Dev Biol **212**(2): 323-336.

Rohatgi, R., L. Milenkovic and M. P. Scott (2007). "Patched1 regulates hedgehog signaling at the primary cilium." Science **317**(5836): 372-376.

Stamataki, D., F. Ulloa, S. V. Tsoni, A. Mynett and J. Briscoe (2005). "A gradient of Gli activity mediates graded Sonic Hedgehog signaling in the neural tube." Genes Dev **19**(5): 626-641.

Stone, D. M., M. Murone, S. Luoh, W. Ye, M. P. Armanini, A. Gurney, H. Phillips, J. Brush, A. Goddard, F. J. de Sauvage and A. Rosenthal (1999). "Characterization of the human suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli." J Cell Sci **112 (Pt 23)**: 4437-4448.

Tickle, C. and M. Towers (2017). "Sonic Hedgehog Signaling in Limb Development." Front Cell Dev Biol **5**: 14.

Tukachinsky, H., L. V. Lopez and A. Salic (2010). "A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes." J Cell Biol **191**(2): 415-428.

Tuson, M., M. He and K. V. Anderson (2011). "Protein kinase A acts at the basal body of the primary cilium to prevent Gli2 activation and ventralization of the mouse neural tube." Development **138**(22): 4921-4930.

Vokes, S. A., H. Ji, S. McCuine, T. Tenzen, S. Giles, S. Zhong, W. J. Longabaugh, E. H. Davidson, W. H. Wong and A. P. McMahon (2007). "Genomic characterization of Gli-activator targets in sonic hedgehog-mediated neural patterning." Development **134**(10): 1977-1989.

Event: 1262: Apoptosis

Short Name: Apoptosis

Key Event Component

Process	Object	Action
apoptotic process		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:205 - AOP from chemical insult to cell death	AdverseOutcome
Aop:207 - NADPH oxidase and P38 MAPK activation leading to reproductive failure in Caenorhabditis elegans	KeyEvent
Aop:212 - Histone deacetylase inhibition leading to testicular atrophy	KeyEvent
Aop:285 - Inhibition of N-linked glycosylation leads to liver injury	KeyEvent
Aop:419 - Aryl hydrocarbon receptor activation leading to impaired lung function through P53 toxicity pathway	KeyEvent
Aop:439 - Activation of the AhR leading to metastatic breast cancer	KeyEvent
Aop:452 - Adverse outcome pathway of PM-induced respiratory toxicity	KeyEvent
Aop:393 - AOP for thyroid disorder caused by triphenyl phosphate via TRβ activation	KeyEvent
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	KeyEvent
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent

AOP ID and Name	Event Type
Aop:491 - Decrease, GLI1/2 target gene expression leads to orofacial clefting	KeyEvent
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	KeyEvent
Aop:502 - Decrease, cholesterol synthesis leads to orofacial clefting	KeyEvent
Aop:441 - Ionizing radiation-induced DNA damage leads to microcephaly via apoptosis and premature cell differentiation	KeyEvent
Aop:535 - Binding and activation of GPER leading to learning and memory impairments	KeyEvent
Aop:540 - Oxidative Stress in the Fish Ovary Leads to Reproductive Impairment via Reduced Vitellogenin Production	KeyEvent
Aop:563 - Aryl hydrocarbon Receptor (AHR) activation causes Premature Ovarian Insufficiency via Bax mediated apoptosis	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Organ term

Organ term

organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Caenorhabditis elegans	Caenorhabditis elegans	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Sex Applicability

Sex	Evidence
Unspecific	High

Apoptosis is induced in human prostate cancer cell lines (*Homo sapiens*) [Parajuli et al., 2014].

Apoptosis occurs in B6C3F1 mouse (*Mus musculus*) [Elmore, 2007].

Apoptosis occurs in Sprague-Dawley rat (*Rattus norvegicus*) [Elmore, 2007].

Apoptosis occurs in the nematode (*Caenorhabditis elegans*) [Elmore, 2007].

- Apoptosis occurs in breast cancer cells, human and mouse (Parton)

Key Event Description

Apoptosis, the process of programmed cell death, is characterized by distinct morphology with DNA fragmentation and energy dependency [Elmore, 2007]. Apoptosis, also called “physiological cell death”, is involved in cell turnover, physiological involution, and atrophy of various tissues and organs [Kerr et al., 1972]. The formation of apoptotic bodies involves marked condensation of both nucleus and cytoplasm, nuclear fragmentation, and separation of protuberances [Kerr et al., 1972]. Apoptosis is characterized by DNA ladder and chromatin condensation. Several stimuli such as hypoxia, nucleotides deprivation, chemotherapeutical drugs, DNA damage, and mitotic spindle damage induce p53 activation, leading to p21 activation and cell cycle arrest [Pucci et al., 2000]. The SAHA or TSA treatment on neonatal human dermal fibroblasts (NHDFs) for 24 or 72 hrs inhibited proliferation of the NHDF cells [Glaser et al., 2003]. Considering that the acetylation of histone H4 was increased by the treatment of SAHA for 4 hrs, histone deacetylase inhibition may be involved in the inhibition of the cell proliferation [Glaser et al., 2003]. The impaired proliferation was observed in HDAC1$^{-/-}$ ES cells, which was rescued with the reintroduction of HDAC1 [Zupkovitz et al., 2010]. An AOP focuses existes on p21 pathway leading to apoptosis, however, alternative pathways such as NF-kappaB signaling pathways may be involved in the apoptosis of spermatocytes [Wang et al., 2017].

Apoptosis is defined as a programmed cell death. A decrease in apoptosis or a resistance to cell death is noted is described as a hallmark of cancer by Hanahan et al. It is widely admitted as an essential step in tumor proliferation (Adams, Lowe). Apoptosis occurs after activation of a number of intrinsic and extrinsic signals which activate the protease caspase system which in turn activates the destruction of the cell.

The Bcl-2 is a protein family suppressing apoptosis bybinding and inhibiting two proapoptotic proteins (Bax and Bak) and transferring them to the mitochondrial outer membrane. In the absence of inhibition by Bcl2, Bax and Bak destroy the mitochondrial membrane and releases proapoptotic signaling proteins, such as cytochrome *c which activated the caspase system.* An increased expression of these antiapoptotic proteins (Bcl-2, Bcl-x_L) *occurs in cancer (Hanahan, Adams, Lowe). Several others pathways such as the* loss of TP53 tumor suppressor function, or the increase of survival signals (Igf1/2), or decrease of proapoptotic factors (Bax, Bim, Puma) can also increase tumor growth *(Hanahan, Juntilla).*

In breast cancer a decrease in apoptosis and a resistance to cell death has been described thoroughly, especially using a dysregulation of the Bcl2 system or TP53 (Parton, Williams, Shahbandi).

How it is Measured or Detected

Apoptosis is characterized by many morphological and biochemical changessuch as homogenous condensation of chromatin to one side or the periphery of the nuclei, membrane blebbing and formation of apoptotic bodies with fragmented nuclei, DNA fragmentation, enzymatic activation of pro-caspases, or phosphatidylserine translocation that can be measured using electron and cytochemical optical microscopy, proteomic and genomic methods, and spectroscopic techniques [Archana et al., 2013; Martinez et al., 2010; Taatjes et al., 2008; Yasuhara et al., 2003].

DNA fragmentation can be quantified with comet assay using electrophoresis, where the tail length, head size, tail intensity, and head intensity of the comet are measured [Yasuhara et al., 2003].

The apoptosis is detected with the expression alteration of procaspases 7 and 3 by Western blotting using antibodies [Parajuli et al., 2014].

The apoptosis is measured with down-regulation of anti-apoptotic gene baculoviral inhibitor of apoptosis protein repeat containing 2 (BIRC2, or cIAP1) [Parajuli et al., 2014].

Apoptotic nucleosomes are detected using Cell Death Detection ELISA kit, which was calculated as absorbance subtraction at 405 nm and 490 nm [Parajuli et al., 2014].

Cleavage of PARP is detected with Western blotting [Parajuliet al., 2014].

Caspase-3 and caspase-9 activity is measured with the enzyme-catalyzed release of p-nitroanilide (pNA) and quantified at 405 nm [Wu et al., 2016].

Apoptosis is measured with Annexin V-FITC probes, and the relative percentage of Annexin V-FITC-positive/PI-negative cells is analyzed by flow cytometry [Wu et al., 2016].

Apoptosis is detected with the Terminal dUTP Nick End-Labeling (TUNEL) method to assay the endonuclease cleavage products by enzymatically end-labeling the DNA strand breaks [Kressel and Groscurth, 1994].

For the detection of apoptosis, the testes are fixed in neutral buffered formalin and embedded in paraffin. Germ cell death is visualized in testis sections by Terminal dUTP Nick End-Labeling (TUNEL) staining method [Wade et al., 2008]. The incidence of TUNEL-positive cells is expressed as the number of positive cells per tubule examined for one entire testis section per animal [Wade et al., 2008]

References

Archana, M. et al. (2013), "Various methods available for detection of apoptotic cells", Indian J Cancer 50:274-283

Elmore, S. (2007), "Apoptosis: a review of programmed cell death", Toxicol Pathol 35:495-516

Glaser, K.B. et al. (2003), "Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines", Mol Cancer Ther 2:151-163

Kerr, J.F.R. et al. (1972), "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics", Br J Cancer 26:239-257

Kressel, M. and Groscurth, P. (1994), "Distinction of apoptotic and necrotic cell death by in situ labelling of fragmented DNA", Cell Tissue Res 278:549-556

Martinez, M.M. et al. (2010), "Detection of apoptosis: A review of conventioinal and novel techniques", Anal Methods 2:996-1004

Parajuli, K.R. et al. (2014), "Methoxyacetic acid suppresses prostate cancer cell growth by inducing growth arrest and apoptosis", Am J Clin Exp Urol 2:300-313

Pucci, B. et al. (2000), "Cell cycle and apoptosis", Neoplasia 2:291-299

Taatjes, D.J. et al. (2008), "Morphological and cytochemical determination of cell death by apoptosis", Histochem Cell Biol 129:33-43

Wade, M.G. et al. (2008), "Methoxyacetic acid-induced spermatocyte death is associated with histone hyperacetylation in rats", Biol Reprod 78:822-831

Wang, C. et al. (2017), "CD147 regulates extrinsic apoptosis in spermatocytes by modulating NFkB signaling pathways", Oncotarget 8:3132-3143

Wu, R. et al. (2016), "microRNA-497 induces apoptosis and suppressed proliferation via the Bcl-2/Bax-caspase9-caspase 3 pathway and cyclin D2 protein in HUVECs", PLoS One 11:e0167052

Yasuhara, S. et al. (2003),"Comparison of comet assay, electron microscopy, and flow cytometry for detection of apoptosis", J Histochem Cytochem 51:873-885

Zupkovitz, G. et al. (2010), "The cyclin-dependent kinase inhibitor p21 is a crucial target for histone deacetylase 1 as a regulator of cellular proliferation", Mol Cell Biol 30:1171-1181

Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74. doi: 10.1016/j.cell.2011.02.013. PMID: 21376230

Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene. 2007 Feb 26;26(9):1324-37. doi: 10.1038/sj.onc.1210220. PMID: 17322918; PMCID: PMC2930981.

Lowe, S., Cepero, E. & Evan, G. Intrinsic tumour suppression.*Nature* **432**, 307–315 (2004). https://doi.org/10.1038/nature03098

Parton M, Dowsett M, Smith I. Studies of apoptosis in breast cancer. BMJ. 2001 Jun 23;322(7301):1528-32. doi: 10.1136/bmj.322.7301.1528. PMID: 11420276; PMCID: PMC1120573.

Junttila MR, Evan GI. p53--a Jack of all trades but master of none. Nat Rev Cancer. 2009 Nov;9(11):821-9. doi: 10.1038/nrc2728. Epub 2009 Sep 24. PMID: 19776747.

Williams MM, Cook RS. Bcl-2 family proteins in breast development and cancer: could Mcl-1 targeting overcome therapeutic resistance? Oncotarget. 2015 Feb 28;6(6):3519-30. doi: 10.18632/oncotarget.2792. PMID: 25784482; PMCID: PMC4414133.

Shahbandi A, Nguyen HD, Jackson JG. TP53 Mutations and Outcomes in Breast Cancer: Reading beyond the Headlines. Trends Cancer. 2020 Feb;6(2):98-110. doi: 10.1016/j.trecan.2020.01.007. Epub 2020 Feb 5. PMID: 32061310; PMCID: PMC7931175.

Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74. doi: 10.1016/j.cell.2011.02.013. PMID: 21376230

Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene. 2007 Feb 26;26(9):1324-37. doi: 10.1038/sj.onc.1210220. PMID: 17322918; PMCID: PMC2930981.

Lowe, S., Cepero, E. & Evan, G. Intrinsic tumour suppression.*Nature* **432**, 307–315 (2004). https://doi.org/10.1038/nature03098

Parton M, Dowsett M, Smith I. Studies of apoptosis in breast cancer. BMJ. 2001 Jun 23;322(7301):1528-32. doi: 10.1136/bmj.322.7301.1528. PMID: 11420276; PMCID: PMC1120573.

Junttila MR, Evan GI. p53--a Jack of all trades but master of none. Nat Rev Cancer. 2009 Nov;9(11):821-9. doi:

10.1038/nrc2728. Epub 2009 Sep 24. PMID: 19776747.

Williams MM, Cook RS. Bcl-2 family proteins in breast development and cancer: could Mcl-1 targeting overcome therapeutic resistance? Oncotarget. 2015 Feb 28;6(6):3519-30. doi: 10.18632/oncotarget.2792. PMID: 25784482; PMCID: PMC4414133.

Shahbandi A, Nguyen HD, Jackson JG. TP53 Mutations and Outcomes in Breast Cancer: Reading beyond the Headlines. Trends Cancer. 2020 Feb;6(2):98-110. doi: 10.1016/j.trecan.2020.01.007. Epub 2020 Feb 5. PMID: 32061310; PMCID: PMC7931175.

Parton M, Dowsett M, Smith I. Studies of apoptosis in breast cancer. BMJ. 2001 Jun 23;322(7301):1528-32. doi: 10.1136/bmj.322.7301.1528. PMID: 11420276; PMCID: PMC1120573.

Event: 2043: Decrease, Sonic Hedgehog second messenger production

Short Name: Decrease, SHH second messenger production

Key Event Component

Process	Object	Action
second-messenger-mediated signaling	sonic hedgehog protein	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent
Aop:491 - Decrease, GLI1/2 target gene expression leads to orofacial clefting	KeyEvent
Aop:502 - Decrease, cholesterol synthesis leads to orofacial clefting	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates		NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- Secondary messenger production of the SHH pathway is present in both male and females and differences in gene expression has not been demonstrated.
- Life stages- The Hedgehog pathway is a major pathway in embryonic development.

- Taxonomic-HH signalling, and its' secondary messenger production is present in vertebrates and some invertebrates including flies (Denef, Neubüser et al. 2000, Huangfu and Anderson 2005)

Key Event Description

During normal Sonic Hedgehog (SHH) signaling, GLI target gene expression regulates several other signaling pathways. Expression of FOXF1 and FOXL1 upregulate BMP4, BMP 2, and FGF10 in the mesenchyme (Katoh and Katoh 2009, Lan and Jiang 2009). Induction of FGF10 in the mesenchyme is able to induce SHH in the adjacent epithelium via a positive feedback loop with FGFR2 (Cobourne and Green 2012). SHH signaling also upregulates BCL2 and CFLAR to promote cell survival (Katoh and Katoh 2009).

How it is Measured or Detected

- Changes in gene expression can be measured using serial analysis of gene expression (SAGE), rapid analysis of gene expression (RAGE), RT-PCR, Northern/Southern blotting, differential display, and DNA microarray assay (Kirby, Heath et al. 2007).
- RNA in situ hybridization can be used to determine sites of gene expression (Nouri-Aria 2008, Abler, Mansour et al. 2009)
- Antibody staining of tissue sections can be used to determine location and amounts of BMP4, BMP2, FGF10

References

Abler, L. L., S. L. Mansour and X. Sun (2009). "Conditional gene inactivation reveals roles for Fgf10 and Fgfr2 in establishing a normal pattern of epithelial branching in the mouse lung." Dev Dyn **238**(8): 1999-2013.

Cobourne, M. T. and J. B. Green (2012). "Hedgehog signalling in development of the secondary palate." Front Oral Biol **16**: 52-59.

Denef, N., D. Neubüser, L. Perez and S. M. Cohen (2000). "Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened." Cell **102**(4): 521-531.

Huangfu, D. and K. V. Anderson (2005). "Cilia and Hedgehog responsiveness in the mouse." Proc Natl Acad Sci U S A **102**(32): 11325-11330.

Katoh, Y. and M. Katoh (2009). "Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation." Curr Mol Med **9**(7): 873-886.

Kirby, J., P. R. Heath, P. J. Shaw and F. C. Hamdy (2007). Gene Expression Assays. Advances in Clinical Chemistry, Elsevier. **44:** 247-292.

Lan, Y. and R. Jiang (2009). "Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth." Development **136**(8): 1387-1396.

Nouri-Aria, K. T. (2008). "In situ Hybridization." Methods Mol Med **138**: 331-347.

Event: 1821: Decrease, Cell proliferation

Short Name: Decrease, Cell proliferation

Key Event Component

Process	Object	Action
cell proliferation	cell	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:263 - Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased cell proliferation	KeyEvent
Aop:290 - Mitochondrial ATP synthase antagonism leading to growth inhibition (1)	KeyEvent
Aop:286 - Mitochondrial complex III antagonism leading to growth inhibition (1)	KeyEvent

AOP ID and Name	Event Type
Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	KeyEvent
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent
Aop:267 - Uncoupling of oxidative phosphorylation leading to growth inhibition via glucose depletion	KeyEvent
Aop:491 - Decrease, GLI1/2 target gene expression leads to orofacial clefting	KeyEvent
Aop:502 - Decrease, cholesterol synthesis leads to orofacial clefting	KeyEvent
Aop:331 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage and reduced cell proliferation	KeyEvent
Aop:332 - Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and reduced cell proliferation	KeyEvent
Aop:333 - Excessive reactive oxygen species leading to growth inhibition via uncoupling of oxidative phosphorylation	KeyEvent

Stressors

Name
2,4-Dinitrophenol
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
Carbonyl cyanide m-chlorophenyl hydrazone
Pentachlorophenol
Triclosan
Emodin
Malonoben

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Juvenile	High

Sex Applicability

Sex	Evidence

Sex	Evidence
Unspecific	High

Taxonomic applicability domain

This key event is in general applicable to all eukaryotes, as most organisms are known to use cell proliferation to achieve growth.

Life stage applicability domain

This key event is in general applicable to all life stages. As cell proliferation not only occurs in developing organisms, but also in adults.

Sex applicability domain

This key event is sex-unspecific, as both genders use the same cell proliferation mechanisms.

Key Event Description

Decreased cell proliferation describes the outcome of reduced cell division and cell growth. Cell proliferation is considered the main mechanism of tissue and organismal growth (Conlon 1999). Decreased cell proliferation has been associated with abnormal growth-factor signaling and cellular energy depletion (DeBerardinis 2008).

How it is Measured or Detected

Multiple types of *in vitro* bioassays can be used to measure this key event:

- ToxCast high-throughput screening bioassays such as “BSK_3C_Proliferation”, “BSK_CASM3C_Proliferation” and “BSK_SAg_Proliferation” can be used to measure cell proliferation status.
- Commercially available methods such as the well-established 5-bromo-2’-deoxyuridine (BrdU) (Raza 1985; Muir 1990) or 5-ethynyl-2’-deoxyuridine (EdU) assay. Both assays measure DNA synthesis in dividing cells to indicate proliferation status.

References

Conlon I, Raff M. 1999. Size control in animal development. *Cell* 96:235-244. DOI: 10.1016/s0092-8674(00)80563-2.

DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. 2008. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metabolism* 7:11-20. DOI: https://doi.org/10.1016/j.cmet.2007.10.002.

Muir D, Varon S, Manthorpe M. 1990. An enzyme-linked immunosorbent assay for bromodeoxyuridine incorporation using fixed microcultures. *Analytical Biochemistry* 185:377-382. DOI: https://doi.org/10.1016/0003-2697(90)90310-6.

Raza A, Spiridonidis C, Ucar K, Mayers G, Bankert R, Preisler HD. 1985. Double labeling of S-phase murine cells with bromodeoxyuridine and a second DNA-specific probe. *Cancer Research* 45:2283-2287.

Event: 2041: Decrease, facial prominence outgrowth

Short Name: Decrease, facial prominence outgrowth

Key Event Component

Process	Object	Action
palatal shelves fail to meet at midline	primary palate	increased
palatal shelves fail to meet at midline	secondary palate	increased

AOPs Including This Key Event

AOP ID and Name	Event Type

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent
Aop:491 - Decrease, GLI1/2 target gene expression leads to orofacial clefting	KeyEvent
Aop:502 - Decrease, cholesterol synthesis leads to orofacial clefting	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- There are no known differences in palatal outgrowth in terms of sex.
- Life stages- The palate develops early in embryonic development. This begins between the 6^{th} and 12^{th} week of pregnancy in humans and between day 10.0 and 15 in mice (Okuhara and Iseki 2012).
- Taxonomic- Palatal outgrowth is required for proper palate formation in all vertebrates.

Key Event Description

For humans and other mammals, the palate serves as a barrier between the mouth and nasal cavity allowing for simultaneous breathing and eating. The palate consists of an anterior bony hard palate and a posterior muscular soft palate that closes the nasal airways for swallowing and directs airflow to help in generation of speech (Li, Lan et al. 2017). The palate is divided into primary and secondary portions. The primary palate contains the philtrum and the upper incisor region anterior to the incisive foramen while the secondary palate encompasses the remainder of the hard and soft palate (Bush and Jiang 2012). The secondary palate arises during embryonic development as bilateral outgrowths from the maxillary processes. In mammals, these shelves grow first vertically down the tongue before elevating to a position above the dorsum of the tongue where the two shelves meet and fuse to form an intact palate (Ferguson 1988).

How it is Measured or Detected

- Palatal shelf outgrowth can be quantified using imaging techniques such as 3D CT scans during development. Insufficient palatal outgrowth will result in cleft palate. The distance between palatal shelves corelating with outgrowth can be measured and quantified for these individuals.
- Embryos can be dissected and the facial prominences measured (Rice, Connor et al. 2006).

References

Bush, J. O. and R. Jiang (2012). "Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development." Development **139**(2): 231-243.

Ferguson, M. W. (1988). "Palate development." Development **103 Suppl**: 41-60.

Li, C., Y. Lan and R. Jiang (2017). "Molecular and Cellular Mechanisms of Palate Development." J Dent Res **96**(11): 1184-1191.

Okuhara, S. and S. Iseki (2012). "Epithelial integrity in palatal shelf elevation." Japanese Dental Science Review **48**(1): 18-22.

Rice, R., E. Connor and D. P. C. Rice (2006). "Expression patterns of Hedgehog signalling pathway members during mouse palate development." Gene Expression Patterns **6**(2): 206-212.

List of Adverse Outcomes in this AOP

Event: 2042: Increase, Orofacial clefting

Short Name: orofacial cleft

Key Event Component

Process	Object	Action
Cleft palate		increased
cleft upper lip		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	AdverseOutcome
Aop:491 - Decrease, GLI1/2 target gene expression leads to orofacial clefting	AdverseOutcome
Aop:502 - Decrease, cholesterol synthesis leads to orofacial clefting	AdverseOutcome

Biological Context

Level of Biological Organization

Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates		NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- OFC can occur for all sexes. Differences in incidence between males and females have been found however a clear understanding of what causes this difference is not understood. Cleft lip with or without cleft palate is more common in males while cleft palate only is more common for females (Barbosa Martelli, Machado et al. 2012).
- Life stages- Orofacial development and any disruption leading to clefting occurs early in embryonic development. This begins between the 6^{th} and 12^{th} week of pregnancy in humans and between day 10.0 and 15 in mice (Okuhara and Iseki 2012).
- Taxonomic- Orofacial development occurs in all vertebrates.

Key Event Description

Orofacial clefts (OFC) are one of the most common birth defects. Orofacial clefts are commonly divided on the anatomy they affect by clefts of the lip and/or palate (CL/P) and those of the palate only (CPO) (Murray 2002). Clefts can also be classified as either syndromic when they occur with other physical or developmental anomalies or nonsydromic in the absence of other symptoms (Stanier and Moore 2004). Like most births, the etiology of OFCs are complex and include a combination of genetic and chemical factors (Lipinski and Bushman 2010, Heyne, Melberg et al. 2015). Orofacial development is tightly regulated by multiple signaling pathways and genes including: fibroblast growth factors (Fgfs), Sonic Hedgehog (shh), bone morphogenic protein (Bmp), transforming growth factor beta (Tgf- β) and transcription factors including Dlx, Pitx, Hox, Gli and T-box (Stanier and Moore 2004). Orofacial development requires precise cell migration, growth, differentiation and apoptosis to create the needed orofacial structures from the oropharyngeal membrane (Jugessur and Murray 2005). During the sixth week of human embryogenesis the medial nasal prominences merge to form the primary palate and the upper lip. The mandibular prominences merge across the midline to produce the lower jaw and lip. Development of the secondary palate begins in the

sixth week where the palatal shelves extend internally to the maxillary processes. The shelves then elevate above the tongue and grow towards each other until contact occurs. During weeks 7-8 the medial edges of the palatal shelves fuse through as series of epithelial-mesenchyme transition (EMT) and apoptosis(Jugessur and Murray 2005, Zhang, Tian et al. 2016). Disruption to the complex processes required for proper orofacial development can occur both through genetic factors and environmental (i.e. chemical) exposure by causing disruption to one or multiple steps of orofacial development resulting in OFC.

How it is Measured or Detected

- OFC can be visually observed both in humans and in animals. It can be classified by which tissues (e.g.cleft lip and palate) are effected and its' severity (complete/incomplete, unilateral/bilateral). Techniques such as the revised Smith-modified Kernahan 'Y' classification can be used describe the type, location, and extent of OFC deformities (Khan, Ullah et al. 2013).

Regulatory Significance of the AO

OFC is one of the most common birth defects occurring in approximately 1 in 700 live births. The etiology of OFC is poorly understood and is believed to be a combination of genetic and environmental factors. Understanding the genetic and environmental factors that can lead to OFC is the first step in preventing this birth defect.

References

Barbosa Martelli, D. R., R. A. Machado, M. S. Oliveira Swerts, L. A. Mendes Rodrigues, S. N. de Aquino and H. M. Júnior (2012). "Non sindromic cleft lip and palate: relationship between sex and clinical extension." Brazilian Journal of Otorhinolaryngology **78**(5): 116-120.

Heyne, G. W., C. G. Melberg, P. Doroodchi, K. F. Parins, H. W. Kietzman, J. L. Everson, L. J. Ansen-Wilson and R. J. Lipinski (2015). "Definition of critical periods for Hedgehog pathway antagonist-induced holoprosencephaly, cleft lip, and cleft palate." PLoS One **10**(3): e0120517.

Jugessur, A. and J. C. Murray (2005). "Orofacial clefting: recent insights into a complex trait." Curr Opin Genet Dev **15**(3): 270-278.

Khan, M., H. Ullah, S. Naz, T. Iqbal, T. Ullah, M. Tahir and O. Ullah (2013). "A revised classification of the cleft lip and palate." Can J Plast Surg **21**(1): 48-50.

Lipinski, R. J. and W. Bushman (2010). "Identification of Hedgehog signaling inhibitors with relevant human exposure by small molecule screening." Toxicol In Vitro **24**(5): 1404-1409.

Murray, J. C. (2002). "Gene/environment causes of cleft lip and/or palate." Clin Genet **61**(4): 248-256.

Okuhara, S. and S. Iseki (2012). "Epithelial integrity in palatal shelf elevation." Japanese Dental Science Review **48**(1): 18-22.

Stanier, P. and G. E. Moore (2004). "Genetics of cleft lip and palate: syndromic genes contribute to the incidence of non-syndromic clefts." Hum Mol Genet **13 Spec No 1**: R73-81.

Zhang, J., X.-J. Tian and J. Xing (2016). "Signal Transduction Pathways of EMT Induced by TGF-β, SHH, and WNT and Their Crosstalks." Journal of clinical medicine **5**(4): 41.

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2734: Antagonism Smoothened leads to Decrease, SMO relocation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Low	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

The relationship between antagonism of SMO and a decrease in SMO relocation and activation has been shown repeatedly in mice models as detailed in the empirical evidence section. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question. The SHH pathway is well understood to be fundamental to proper embryonic development and that aberrant SHH signaling during embryonic development can cause birth defects indculding orofacial clefts (OFCs). For this reason, this KER is applicable to the embryonic stage with a high level of confidence.

Key Event Relationship Description

The Smoothened (SMO) receptor is Class F G protein coupled receptor involved in signal transduction of the Sonic Hedgehog (SHH) pathway. It includes distinct functional groups including ligand binding pockets, cysteine rich domain (CRD), transmembrane helix (TM), extracellular loop (ECL), intracellular loop (ICL), and a carboxyl-terminal tail (C-term tail) (Arensdorf, Marada et al. 2016). SMO signaling is dependent upon its relocation to a subcellular location. This relocation occurs in the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005). Relocation of SMO to the PC typically occurs within ~20 minutes of agonist stimulation (Arensdorf, Marada et al. 2016).

In the absence of SHH ligand, the Patched (PTCH) receptor suppresses the activation of SMO. When HH ligand binds to PTCH, suppression on SMO is released and SMO can relocate, accumulate, and signal to intracellular effectors (Denef, Neubüser et al. 2000, Rohatgi and Scott 2007). It has been shown that SMO localization to the tip of the primary cilia is essential for the SHH signaling cascade in vertebrates (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Rohatgi, Milenkovic et al. 2009). The exact mechanism through which PTCH and SMO interact is not known.

Evidence Supporting this KER

Biological Plausibility

SMO signaling is dependent upon its relocation to a subcellular location. This relocation occurs in the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005). It has been shown that SMO localization to the tip of the primary cilia is essential for the SHH signaling cascade in vertebrates (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Rohatgi, Milenkovic et al. 2009)

Empirical Evidence

- In vitro
  - NIH 3t3 (murine fibroblast) were used to study the effects of three SHH pathway antagonists, SANT 1, SANT2, and cyclopamine on SMO localization using fluorescent microscopy. Cells were treated with increasing concentrations of the antagonists in the presence of SHH ligand. SANT1 and SANT2 both blocked SMO localization in the cilia with IC50 values of 5 and 13nM respectively. Cyclopamine did not inhibit the accumulation of SMO in the cilia even when dosed at 5-10µm (>10 fold above kd). All three antagonists inhibited SHH pathway transduction and target gene expression (Rohatgi, Milenkovic et al. 2009).
  - A small molecule screen of 10,000 compounds identified six inhibitors of SHH signaling, four of which bind directly to SMO (SANT1-4). Screening was conducted using NIH 3T3 SHH LightII cells cultured in media conditioned from HEK 293 transfected to stably express Shh-N. Cells were dosed with the compound library at 0.714ug/ml and SHH activity was quantified at 30h using Renilla luciferase activity. A fluorescent binding assay using BODIPY-cyclopamine was used to verify binding to SMO for the SANT compounds. Dose response reported as IC50 for the inhibition of SHH signaling was conducting in NIH 3T3 SHH light2, NIH 3T3 SmoA1-Light2, P2 Ptch1-/- (mouse embryonic fibroblasts) (Chen, Taipale et al. 2002).

Compound/Cell	SHH-Light2 (nM)	SmoA1-Light2 (nM)	Ptch1-/- (nM)
SANT-1	20	30	20
SANT-2	30	70	50
SANT-3	100	80	80
SANT-4	200	300	300

    - Direct binding of cyclopamine to SMO was verified using a photoaffinity form of cyclopamine (PA-cyclopamine). PA-cyclopamine had previously been shown to inhibit SHH signaling in NIH 3T3 Shh-LightII cells with similar IC50 values to cyclopamine (300nm and 150nm respectively) (Taipale, Chen et al. 2000). Binding to SMO was verified using a COS-1 (fibroblast, monkey) line transfected to over express SMO. The location of cyclopamine binding was further investigated using BODIPY- cyclopamine and COS-1 cells modified to lack either a N-terminal, extracellular cysteine-rich domain, or the cytoplasmic C terminal of SMO. The findings support that cyclopamine does not require these domains and instead binds directly to the heptahelical domain (Chen, Taipale et al. 2002).
    - To investigate whether SMO localization is regulated by SHH, a renal epithelial MDCK (Madin-Darby canine kidney) line was engineered to express Myc-tagged SMO. Following culture for 1hr in SHH conditioned media SMO presence in the primary cilium is upregulated while cells cultured in the presence of cyclopamine see a downregulation of SMO in the primary cilia (Corbit, Aanstad et al. 2005)
    - To determine whether PTCH1 regulates localization of SMO MEFs from PTCH1$^{-/-}$ mice were used. These showed SHH activity and SMO localization in the primary cilium in the absence of SHH ligand or SAG. Reintroduction of PTCH1 via a retrovirus suppressed SHH activity and prevented SMO accumulation in primary cilia (Rohatgi and Scott 2007)
    - A high content assay to detect compounds that block SMO accumulation to the primary cilia in the presence of SHH was used to screen a library of ~5600 compounds. This screen identified 26 hits with DY131 and its analog GSK4716 further investigated as potent hits. These compounds inhibited SHH induced accumulation of SMO::EGFP with IC50s of 0.8um and 2um respectively. DY131 and GSK4716 both inhibited the activation of a Glireporter with IC50s of 2um and 10um respectively (Wang, Arvanites et al. 2012).
- In vivo
    - Two-week-old mice were dosed with 40mg/kg vismodegib (GDC-0449) via ip injection twice a day for 3 consecutive days. Quantification of immunofluorescence and ciliary length showed that like SMO$^{fl/+}$ mice, ciliary M71/M72 OR was reduced while cilia lengths were not changed. To determine if SMO regulates ciliary localization an OMP-CRE mouse line was used. It was found that immunofluorescence of M71/M72 was reduced in both SMO$^{fl/+}$, SMO$^{fl/fl}$, as compared to SMO$^{+/+}$ control (Maurya, Bohm et al. 2017).
    - Cyclopamine was found to inhibit SHH signaling in White leghorn neural plate explants. Explants were dissected from stage 9-10 embryo chicks and cultured in collagen gels. Tissues were cultured in Shh-N media from COS-1 cells. Cyclopamine was dissolved in ethanol and added to test tissues. Tissues were fixed at 24-29hr and processed for immunofluorescence. 120nm cyclopamine was found to repress SHH induction as determined by Pax7 repression and the blockage of floor plate and motor neuron induction (Incardona, Gaffield et al. 1998).
        - Multiple ciliopathies associated with clefting in humans including Meckel-Gruber syndrome (OMIM 249000) and Ellis-van Creveld syndrome (OMIM 225500)(Brugmann, Cordero et al. 2010)

Uncertainties and Inconsistencies

While we know that entry to the cilia is tightly controlled, the exact mechanism of SMO ciliary trafficking is not fully understood. The PC is separated from the plasma membrane by the ciliary pockets and the transition zone which function together to regulate the movement of lipids and proteins in and out of the organelle (Goetz, Ocbina et al. 2009, Rohatgi and Snell 2010). The SHH receptor PTCH contains a ciliary localization sequence in its' carboxy tail. Localization of PTCH to the PC is essential for inhibition of SMO as deletion of the CLS in PTCH prevents PTCH localization as well as inhibition of SMO (Kim, Hsia et al. 2015) (53). SMO also contains a CLS, but only accumulates in the PC upon ligand binding (Corbit, Aanstad et al. 2005). The entry of SMO into the PC is thought to occur either laterally through the ciliary pockets or internally via recycling endosomes (Milenkovic, Scott et al. 2009). Once inside the PC, SMO can diffuse freely, however it will usually accumulate in specific locations depending upon its' activation state. Inactive SMO will accumulate more at the base of the PC while active SMO will accumulate in the tip of the PC (Milenkovic, Weiss et al. 2015).

An endogenous ligand for SMO has not been discovered although evidence for one exists and that PTCH controls SMO by controlling its' availability or accessibility. To support this, it has been shown that PTCH and SMO do not physically interact (Chen and Struhl 1998). PTCH acts catalytically with SMO with one PTCH receptor capable of controlling many (~50) SMO receptors (Taipale, Cooper et al. 2002). Since PTCH includes a sterol sensing domain and shares characteristics of ancient bacterial transporters, a model of PTCH functioning by pumping a sterol-like MSO regulator has been proposed (Mukhopadhyay and Rohatgi 2014). SMO is constitutively active in the absence of PTCH suggesting that the elusive molecule is an agonist (Rohatgi and Scott 2007). Conversely, the discovery that oxysterols bind to the CRD binding domain acting as positive modulators suggest that the molecule could be an agonist with PTCH functioning to sequester away or limit cellular concentration (Corcoran and Scott 2006, Nachtergaele, Mydock et al. 2012)

The activity of SMO is controlled by ligand binding(Kobilka 2007). Two separate binding pockets, one in the groove of the extracellular CRD and the other in the helices of the TMD have been identified (Nachtergaele, Mydock et al. 2012, Rana, Carroll et al. 2013, Wang, Wu et al. 2013, Byrne, Sircar et al. 2016, Huang, Zheng et al. 2018). These two binding pockets have been shown to interact in an allosteric manner (Nachtergaele, Mydock et al. 2012). The binding pocket in the helices of the TMD binds several SMO agonists including SAG as well as antagonists Vismodegib and Sonidegib. The CRD binding pocket binds cholesterol and its' oxidized derivates (Byrne, Luchetti et al. 2018). The antagonist cyclopamine binds to the TMD binding pocket and inhibits SHH signal transduction. However, in mSMO carrying the mutations D477G/E552K that disable the TMD binding pocket, cyclopamine binds to the CRD pocket and

activates the pathway (Huang, Nedelcu et al. 2016). To date several oxysterols including 20(S)-hydroxylcholesterol, 22(S)-hydroxylcholesterol, 7-keto-25-hydroxylcholesterol and 7-keto-27-hydroxylcholesterol have been identified as activators of SMO (Dwyer, Sever et al. 2007, Nachtergaele, Mydock et al. 2012, Myers, Sever et al. 2013). A binding site for 24(S),25-epoxycholesterol has been identified in the TMD pocket using cryo-EM of SMO in complex with 24(S),25-epoxycholesterol (Qi, Liu et al. 2019).

While it is well understood that cyclopamine is an antagonist of SMO, contradictory in vivo data was found regarding whether cyclopamine blocks SMO relocation to the primary cilia. Rohatgi et al used NIH 3T3s cell and found that cyclopamine did not inhibit the accumulation of SMO in the cilia even when dosed at 5-10um (>10 fold above kd). All three antagonists inhibited SHH pathway transduction and target gene expression (Rohatgi, Milenkovic et al. 2009). Corbit et al used a renal epithelial MDCK (Madin-Darby canine kidney) line was engineered to express Myc-tagged SMO. Following culture for 1hr in SHH conditioned media SMO presence in the primary cilium is upregulated while cells cultured in the presence of cyclopamine see a downregulation of SMO in the primary cilia (Corbit, Aanstad et al. 2005). Further work is required to determine if SMO antagonism via cyclopamine results in decrease in SMO relocation.

Quantitative Understanding of the Linkage

The data presented in support of this KER includes both in vitro and in vivo studies. The in vivo work identifies multiple antagonists of SMO and validates that they directly bind to SMO. These studies also offer data to show that antagonism of SMO causes a down regulation in SMO relocation the primary cilia. Dose dependent SMO localization is seen in the studies performed by Rohtagi et al 2009 and Chen et al 2002.The response time of SMO antagonism and subsequent time for a decrease in SMO relocation and activation has not been reported. No dose dependent in vivo data for antagonism of SMO and relocation to the cilia was found and all in vivo evidence is conducted under steady state exposure. Dose response data for disruption of SHH using the antagonists exists and is well charactered however quantification of ciliary relocation is lacking. Further studies are needed to expand our quantitative understanding of this linkage.

Response-response relationship

No studies identified

Time-scale

Relocation of SMO to the PC typically occurs within ~20 minutes of agonist stimulation(Arensdorf, Marada et al. 2016). No data was found on how fast antagonism of SMO will stop its' relocation to the primary cilia.

Known Feedforward/Feedback loops influencing this KER

None identified

References

Alexandre, C., A. Jacinto and P. W. Ingham (1996). "Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins." Genes Dev **10**(16): 2003-2013.

Arensdorf, A. M., S. Marada and S. K. Ogden (2016). "Smoothened Regulation: A Tale of Two Signals." Trends Pharmacol Sci **37**(1): 62-72.

Brugmann, S. A., D. R. Cordero and J. A. Helms (2010). "Craniofacial ciliopathies: A new classification for craniofacial disorders." Am J Med Genet A **152A**(12): 2995-3006.

Byrne, E. F. X., G. Luchetti, R. Rohatgi and C. Siebold (2018). "Multiple ligand binding sites regulate the Hedgehog signal transducer Smoothened in vertebrates." Current Opinion in Cell Biology **51**: 81-88.

Byrne, E. F. X., R. Sircar, P. S. Miller, G. Hedger, G. Luchetti, S. Nachtergaele, M. D. Tully, L. Mydock-McGrane, D. F. Covey, R. P. Rambo, M. S. P. Sansom, S. Newstead, R. Rohatgi and C. Siebold (2016). "Structural basis of Smoothened regulation by its extracellular domains." Nature **535**(7613): 517-522.

Chen, J. K., J. Taipale, M. K. Cooper and P. A. Beachy (2002). "Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened." Genes Dev **16**(21): 2743-2748.

Chen, J. K., J. Taipale, K. E. Young, T. Maiti and P. A. Beachy (2002). "Small molecule modulation of Smoothened activity." Proc Natl Acad Sci U S A **99**(22): 14071-14076.

Chen, Y. and G. Struhl (1998). "In vivo evidence that Patched and Smoothened constitute distinct binding and transducing components of a Hedgehog receptor complex." Development **125**(24): 4943-4948.

Corbit, K. C., P. Aanstad, V. Singla, A. R. Norman, D. Y. R. Stainier and J. F. Reiter (2005). "Vertebrate Smoothened functions at

the primary cilium." Nature **437**(7061): 1018-1021.

Corcoran, R. B. and M. P. Scott (2006). "Oxysterols stimulate Sonic hedgehog signal transduction and proliferation of medulloblastoma cells." Proc Natl Acad Sci U S A **103**(22): 8408-8413.

Denef, N., D. Neubüser, L. Perez and S. M. Cohen (2000). "Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened." Cell **102**(4): 521-531.

Dwyer, J. R., N. Sever, M. Carlson, S. F. Nelson, P. A. Beachy and F. Parhami (2007). "Oxysterols are novel activators of the hedgehog signaling pathway in pluripotent mesenchymal cells." J Biol Chem **282**(12): 8959-8968.

Goetz, S. C., P. J. Ocbina and K. V. Anderson (2009). "The primary cilium as a Hedgehog signal transduction machine."Methods Cell Biol **94**: 199-222.

Heyne, G. W., C. G. Melberg, P. Doroodchi, K. F. Parins, H. W. Kietzman, J. L. Everson, L. J. Ansen-Wilson and R. J. Lipinski (2015). "Definition of critical periods for Hedgehog pathway antagonist-induced holoprosencephaly, cleft lip, and cleft palate." PLoS One **10**(3): e0120517.

Huang, P., D. Nedelcu, M. Watanabe, C. Jao, Y. Kim, J. Liu and A. Salic (2016). "Cellular Cholesterol Directly Activates Smoothened in Hedgehog Signaling." Cell **166**(5): 1176-1187.e1114.

Huang, P., S. Zheng, B. M. Wierbowski, Y. Kim, D. Nedelcu, L. Aravena, J. Liu, A. C. Kruse and A. Salic (2018). "Structural Basis of Smoothened Activation in Hedgehog Signaling." Cell **174**(2): 312-324.e316.

Huangfu, D. and K. V. Anderson (2005). "Cilia and Hedgehog responsiveness in the mouse."Proc Natl Acad Sci U S A **102**(32): 11325-11330.

Incardona, J. P., W. Gaffield, R. P. Kapur and H. Roelink (1998). "The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction." Development **125**(18): 3553-3562.

Kim, J., E. Y. Hsia, A. Brigui, A. Plessis, P. A. Beachy and X. Zheng (2015). "The role of ciliary trafficking in Hedgehog receptor signaling." Sci Signal **8**(379): ra55.

Kobilka, B. K. (2007). "G protein coupled receptor structure and activation."Biochimica et Biophysica Acta (BBA) - Biomembranes **1768**(4): 794-807.

Maurya, D. K., S. Bohm and M. Alenius (2017). "Hedgehog signaling regulates ciliary localization of mouse odorant receptors." Proc Natl Acad Sci U S A **114**(44): E9386-e9394.

Milenkovic, L., M. P. Scott and R. Rohatgi (2009). "Lateral transport of Smoothened from the plasma membrane to the membrane of the cilium." J Cell Biol **187**(3): 365-374.

Milenkovic, L., L. E. Weiss, J. Yoon, T. L. Roth, Y. S. Su, S. J. Sahl, M. P. Scott and W. E. Moerner (2015). "Single-molecule imaging of Hedgehog pathway protein Smoothened in primary cilia reveals binding events regulated by Patched1." Proc Natl Acad Sci U S A **112**(27): 8320-8325.

Millington, G., K. H. Elliott, Y. T. Chang, C. F. Chang, A. Dlugosz and S. A. Brugmann (2017). "Cilia-dependent GLI processing in neural crest cells is required for tongue development." Dev Biol **424**(2): 124-137.

Mukhopadhyay, S. and R. Rohatgi (2014). "G-protein-coupled receptors, Hedgehog signaling and primary cilia."Semin Cell Dev Biol **33**: 63-72.

Myers, B. R., L. Neahring, Y. Zhang, K. J. Roberts and P. A. Beachy (2017). "Rapid, direct activity assays for Smoothened reveal Hedgehog pathway regulation by membrane cholesterol and extracellular sodium." Proc Natl Acad Sci U S A **114**(52): E11141-E11150.

Myers, Benjamin R., N. Sever, Yong C. Chong, J. Kim, Jitendra D. Belani, S. Rychnovsky, J. F. Bazan and Philip A. Beachy (2013). "Hedgehog Pathway Modulation by Multiple Lipid Binding Sites on the Smoothened Effector of Signal Response." Developmental Cell **26**(4): 346-357.

Nachtergaele, S., L. K. Mydock, K. Krishnan, J. Rammohan, P. H. Schlesinger, D. F. Covey and R. Rohatgi (2012). "Oxysterols are allosteric activators of the oncoprotein Smoothened." Nat Chem Biol **8**(2): 211-220.

Nachtergaele, S., L. K. Mydock, K. Krishnan, J. Rammohan, P. H. Schlesinger, D. F. Covey and R. Rohatgi (2012). "Oxysterols are allosteric activators of the oncoprotein Smoothened." Nature Chemical Biology **8**(2): 211-220.

Qi, X., H. Liu, B. Thompson, J. McDonald, C. Zhang and X. Li (2019). "Cryo-EM structure of oxysterol-bound human Smoothened coupled to a heterotrimeric Gi." Nature **571**(7764): 279-283.

Rana, R., C. E. Carroll, H.-J. Lee, J. Bao, S. Marada, C. R. R. Grace, C. D. Guibao, S. K. Ogden and J. J. Zheng (2013). "Structural insights into the role of the Smoothened cysteine-rich domain in Hedgehog signalling." Nature Communications **4**(1): 2965.

Rohatgi, R., L. Milenkovic, R. B. Corcoran and M. P. Scott (2009). "Hedgehog signal transduction by Smoothened: pharmacologic evidence for a 2-step activation process." Proc Natl Acad Sci U S A **106**(9): 3196-3201.

Rohatgi, R., L. Milenkovic and M. P. Scott (2007). "Patched1 regulates hedgehog signaling at the primary cilium."Science

317(5836): 372-376.

Rohatgi, R. and M. P. Scott (2007). "Patching the gaps in Hedgehog signalling."Nat Cell Biol **9**(9): 1005-1009.

Rohatgi, R. and W. J. Snell (2010). "The ciliary membrane."Curr Opin Cell Biol **22**(4): 541-546.

Taipale, J., J. K. Chen, M. K. Cooper, B. Wang, R. K. Mann, L. Milenkovic, M. P. Scott and P. A. Beachy (2000). "Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine." Nature **406**(6799): 1005-1009.

Taipale, J., M. K. Cooper, T. Maiti and P. A. Beachy (2002). "Patched acts catalytically to suppress the activity of Smoothened." Nature **418**(6900): 892-896.

Von Ohlen, T. and J. E. Hooper (1997). "Hedgehog signaling regulates transcription through Gli/Ci binding sites in the wingless enhancer." Mech Dev **68**(1-2): 149-156.

Wang, C., H. Wu, V. Katritch, G. W. Han, X. P. Huang, W. Liu, F. Y. Siu, B. L. Roth, V. Cherezov and R. C. Stevens (2013). "Structure of the human smoothened receptor bound to an antitumour agent." Nature **497**(7449): 338-343.

Wang, Y., A. C. Arvanites, L. Davidow, J. Blanchard, K. Lam, J. W. Yoo, S. Coy, L. L. Rubin and A. P. McMahon (2012). "Selective identification of hedgehog pathway antagonists by direct analysis of smoothened ciliary translocation." ACS Chem Biol **7**(6): 1040-1048.

Relationship: 2735: Decrease, SMO relocation leads to Decrease, GLI1/2 translocation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mice	Mus sp.	High	NCBI
human	Homo sapiens	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

The relationship between a decrease in translocation of SMO and a decrease in GLI1/2 translocation to the nucleus has been shown repeatedly in mice models as detailed in the empirical evidence section. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question. The SHH pathway is well understood to be fundamental to proper embryonic development. For this reason, this KER is applicable to the embryonic stage with a high level of confidence.

Key Event Relationship Description

The Smoothened (SMO) receptor is Class F G protein coupled receptor involved in signal transduction of the Sonic Hedgehog (SHH) pathway. It includes distinct functional groups including ligand binding pockets, cysteine rich domain (CRD), transmembrane helix (TM), extracellular loop (ECL), intracellular loop (ICL), and a carboxyl-terminal tail (C-term tail) (Arensdorf, Marada et al. 2016). SMO signaling is dependent upon its relocation to a subcellular location. This relocation occurs in the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005). Relocation of SMO to the PC typically occurs within ~20 minutes of agonist stimulation (Arensdorf, Marada et al. 2016).

The Glioma-associated onocogene (Gli) family of zinc finger transcription factors (Gli1, Gli2, Gli3) are the primarily downstream effectors of the Hedgehog (HH) signaling cascade. When HH ligand binds to Patched (PTCH), its' inhibition on SMO is relieved. SMO this then able to accumulate to the tip of primary cilium in its' active form (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Kim, Kato et al. 2009). SMO causes the GLI family to become dislodged from their complex with the negative regulator of HH signaling, Suppressor of Fused (Sufu) (Kogerman, Grimm et al. 1999,

Pearse, Collier et al. 1999, Stone, Murone et al. 1999, Tukachinsky, Lopez et al. 2010). The GLI-Sufu complex maintains retention of Gli in the cytosol allowing for exposure to phosphorylation via protein kinase A (PKA) which inhibits downstream signal transduction (Tuson, He et al. 2011). When SMO is activated, the GLI2/3-Sufu complex is dismantled allowing for retrograde transport of GLI back into the nucleus (Kim, Kato et al. 2009).).

The GLI family is found in both a long activator form (GliA) or a proteolytically cleaved repressor form (GliR). Current understanding is that Gli3 functions primarily as a repressor while Gli1 and Gli2 function mainly as activators of the pathway and that recruitment of SMO to the cilium leads to an increase in the ratio of GliA:GliR (Hui and Angers 2011, Liu 2016). Downstream transcription is primarily activated by Gli2 and repressed by Gli3(Wang, Fallon et al. 2000, Bai, Auerbach et al. 2002, Persson, Stamataki et al. 2002). Gli1 serves primarily as an activator of transcription and works through amplification of the activated state (Park, Bai et al. 2000).

Evidence Supporting this KER

Biological Plausibility

SMO signaling is dependent upon its relocation to a subcellular location. This relocation occurs in the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005). It has been shown that SMO localization to the tip of the primary cilia is essential for the SHH signaling cascade via the GLI transcription factors(Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Rohatgi, Milenkovic et al. 2009)

Empirical Evidence

- In vitro
  - NIH 3T3 clones with stable HA-Gli2 expression were created and a line with low HA-Gli2 expression was selected for further study. The reporter activity was induced by ShhN and fully inhibited by cyclopamine. When stimulated with ShhN, antibody staining was used to verify that Gli2 accumulates at the tip of the primary cilia. Immunostaining was also used to find that Gli2 accumulated in the nucleus of cells treated with ShhN. Using nuclear extracts of unstimulated cells HA-Gli2R was predominantly localized in the nucleus while in stimulated cells HA-Gli2 increased and HA-Gli2 decreased. Cells treated with Shh agonist SAG also had SMO accumulation in the primary cilia and increased HA-Gli2A in the nucleus (Kim, Kato et al. 2009).
  - NIH 3T3 cells were used to study whether the oxysterols and/or cholesterol are required for SHH signaling. Cells were depleted of sterols via incubation with methyl-β-cyclodextrin (MCD). Fluorinated sterols were added back as soluble components and the cells were stimulated with Shh ligand. Assays were performed for recruitment of endogenous SMO to the primary cilia and for pathway activation using a transcriptional reporter assay. Sterol depletion blocked relocation of SMO to the cilia and SHH activation. Cholesterol and 25-fluorocholesterol both rescued sterol depleted cells and restored SHH pathway activation (Huang, Nedelcu et al. 2016).
  - MMS1 (human myeloma) cells were used to study whether activation of Gli1 is required for its' translocation to the nucleus. Forskolin (FSK) which acts by blocking GLI1 access to PKA was added to culture for 24h at 10µm. The nuclear localization of GLI1 was significantly decreased in the Prescence of FSK (Blotta, Jakubikova et al. 2012).
- In vivo
  - none identified

Uncertainties and Inconsistencies

While we know that entry to the cilia is tightly controlled, the exact mechanism of SMO ciliary trafficking is not fully understood. The PC is separated from the plasma membrane by the ciliary pockets and the transition zone which function together to regulate the movement of lipids and proteins in and out of the organelle (Goetz, Ocbina et al. 2009, Rohatgi and Snell 2010). The SHH receptor PTCH contains a ciliary localization sequence in its' carboxy tail. Localization of PTCH to the PC is essential for inhibition of SMO as deletion of the CLS in PTCH prevents PTCH localization as well as inhibition of SMO (Kim, Hsia et al. 2015) (53). SMO also contains a CLS, but only accumulates in the PC upon ligand binding (Corbit, Aanstad et al. 2005). The entry of SMO into the PC is thought to occur either laterally through the ciliary pockets or internally via recycling endosomes (Milenkovic, Scott et al. 2009). Once inside the PC, SMO can diffuse freely, however it will usually accumulate in specific locations depending upon its' activation state. Inactive SMO will accumulate more at the base of the PC while active SMO will accumulate in the tip of the PC (Milenkovic, Weiss et al. 2015).

Quantitative Understanding of the Linkage

The data presented in support of this KER includes in vitro studies. The in vitro work offers data that SMO relocates to the tip of the primary cilium and that this plays a role in the translocation of the GLI transcription factors to the nucleus. The quantitative understanding of this linkage is low as studies including dose-response and time-course were not found.

Time-scale

Relocation of SMO to the PC typically occurs within ~20 minutes of agonist stimulation(Arensdorf, Marada et al. 2016). No data was found with regards to GLI1/2 translocation.

References

Arensdorf, A. M., S. Marada and S. K. Ogden (2016). "Smoothened Regulation: A Tale of Two Signals." Trends Pharmacol Sci **37**(1): 62-72.

Bai, C. B., W. Auerbach, J. S. Lee, D. Stephen and A. L. Joyner (2002). "Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway." Development **129**(20): 4753-4761.

Blotta, S., J. Jakubikova, T. Calimeri, A. M. Roccaro, N. Amodio, A. K. Azab, U. Foresta, C. S. Mitsiades, M. Rossi, K. Todoerti, S. Molica, F. Morabito, A. Neri, P. Tagliaferri, P. Tassone, K. C. Anderson and N. C. Munshi (2012). "Canonical and noncanonical Hedgehog pathway in the pathogenesis of multiple myeloma." Blood **120**(25): 5002-5013.

Brugmann, S. A., D. R. Cordero and J. A. Helms (2010). "Craniofacial ciliopathies: A new classification for craniofacial disorders." Am J Med Genet A **152A**(12): 2995-3006.

Corbit, K. C., P. Aanstad, V. Singla, A. R. Norman, D. Y. R. Stainier and J. F. Reiter (2005). "Vertebrate Smoothened functions at the primary cilium." Nature **437**(7061): 1018-1021.

Goetz, S. C., P. J. Ocbina and K. V. Anderson (2009). "The primary cilium as a Hedgehog signal transduction machine." Methods Cell Biol **94**: 199-222.

Huang, P., D. Nedelcu, M. Watanabe, C. Jao, Y. Kim, J. Liu and A. Salic (2016). "Cellular Cholesterol Directly Activates Smoothened in Hedgehog Signaling." Cell **166**(5): 1176-1187.e1114.

Huangfu, D. and K. V. Anderson (2005). "Cilia and Hedgehog responsiveness in the mouse." Proc Natl Acad Sci U S A **102**(32): 11325-11330.

Hui, C. C. and S. Angers (2011). "Gli proteins in development and disease." Annu Rev Cell Dev Biol **27**: 513-537.

Kim, J., E. Y. Hsia, A. Brigui, A. Plessis, P. A. Beachy and X. Zheng (2015). "The role of ciliary trafficking in Hedgehog receptor signaling." Sci Signal **8**(379): ra55.

Kim, J., M. Kato and P. A. Beachy (2009). "Gli2 trafficking links Hedgehog-dependent activation of Smoothened in the primary cilium to transcriptional activation in the nucleus." Proc Natl Acad Sci U S A **106**(51): 21666-21671.

Kogerman, P., T. Grimm, L. Kogerman, D. Krause, A. B. Undén, B. Sandstedt, R. Toftgård and P. G. Zaphiropoulos (1999). "Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1." Nat Cell Biol **1**(5): 312-319.

Liu, K. J. (2016). "Craniofacial Ciliopathies and the Interpretation of Hedgehog Signal Transduction." PLoS Genet **12**(12): e1006460.

Milenkovic, L., M. P. Scott and R. Rohatgi (2009). "Lateral transport of Smoothened from the plasma membrane to the membrane of the cilium." J Cell Biol **187**(3): 365-374.

Milenkovic, L., L. E. Weiss, J. Yoon, T. L. Roth, Y. S. Su, S. J. Sahl, M. P. Scott and W. E. Moerner (2015). "Single-molecule imaging of Hedgehog pathway protein Smoothened in primary cilia reveals binding events regulated by Patched1." Proc Natl Acad Sci U S A **112**(27): 8320-8325.

Millington, G., K. H. Elliott, Y. T. Chang, C. F. Chang, A. Dlugosz and S. A. Brugmann (2017). "Cilia-dependent GLI processing in neural crest cells is required for tongue development." Dev Biol **424**(2): 124-137.

Park, H. L., C. Bai, K. A. Platt, M. P. Matise, A. Beeghly, C. C. Hui, M. Nakashima and A. L. Joyner (2000). "Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation." Development **127**(8): 1593-1605.

Pearse, R. V., 2nd, L. S. Collier, M. P. Scott and C. J. Tabin (1999). "Vertebrate homologs of Drosophila suppressor of fused interact with the gli family of transcriptional regulators." Dev Biol **212**(2): 323-336.

Persson, M., D. Stamataki, P. te Welscher, E. Andersson, J. Böse, U. Rüther, J. Ericson and J. Briscoe (2002). "Dorsal-ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity." Genes Dev **16**(22): 2865-2878.

Rohatgi, R., L. Milenkovic, R. B. Corcoran and M. P. Scott (2009). "Hedgehog signal transduction by Smoothened: pharmacologic evidence for a 2-step activation process." Proc Natl Acad Sci U S A **106**(9): 3196-3201.

Rohatgi, R., L. Milenkovic and M. P. Scott (2007). "Patched1 regulates hedgehog signaling at the primary cilium." Science **317**(5836): 372-376.

Rohatgi, R. and W. J. Snell (2010). "The ciliary membrane." Curr Opin Cell Biol **22**(4): 541-546.

Stone, D. M., M. Murone, S. Luoh, W. Ye, M. P. Armanini, A. Gurney, H. Phillips, J. Brush, A. Goddard, F. J. de Sauvage and A. Rosenthal (1999). "Characterization of the human suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli." J Cell Sci **112 (Pt 23)**: 4437-4448.

Tukachinsky, H., L. V. Lopez and A. Salic (2010). "A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes." J Cell Biol **191**(2): 415-428.

Tuson, M., M. He and K. V. Anderson (2011). "Protein kinase A acts at the basal body of the primary cilium to prevent Gli2 activation and ventralization of the mouse neural tube." Development **138**(22): 4921-4930.

Wang, B., J. F. Fallon and P. A. Beachy (2000). "Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb." Cell **100**(4): 423-434.

Relationship: 2721: Decrease, GLI1/2 translocation leads to Decrease, GLI1/2 target gene expression

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

All presented evidence for the relationship is performed in mice. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question.

Key Event Relationship Description

The Glioma-associated onocogene (Gli) family of zinc finger transcription factors (Gli1, Gli2, Gli3) are the primarily downstream effectors of the Hedgehog (HH) signaling cascade. When HH ligand binds to Patched (PTCH), its' inhibition on SMO is relieved. SMO this then able to accumulate to the tip of primary cilium in its' active form (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Kim, Kato et al. 2009). SMO causes the GLI family to become dislodged from their complex with the negative regulator of HH signaling, Suppressor of Fused (Sufu) (Kogerman, Grimm et al. 1999, Pearse, Collier et al. 1999, Stone, Murone et al. 1999, Tukachinsky, Lopez et al. 2010). The GLI-Sufu complex maintains retention of Gli in the cytosol allowing for exposure to phosphorylation via protein kinase A (PKA) which inhibits downstream signal transduction (Tuson, He et al. 2011). When SMO is activated, the GLI2/3-Sufu complex is dismantled allowing for retrograde transport of GLI back into the nucleus (Kim, Kato et al. 2009). This relocation then leads to signaling to effectors resulting in the activation of the GLI transcription factors and the subsequent induction of SHH target gene expression (Alexandre, Jacinto et al. 1996, Von Ohlen and Hooper 1997)

The GLI family is found in both a long activator form (GliA) or a proteolytically cleaved repressor form (GliR). Current understanding is that Gli3 functions primarily as a repressor while Gli1 and Gli2 function mainly as activators of the pathway and that recruitment of SMO to the cilium leads to an increase in the ratio of GliA:GliR (Hui and Angers 2011, Liu 2016). Downstream transcription is primarily activated by Gli2 and repressed by Gli3(Wang, Fallon et al. 2000, Bai, Auerbach et al. 2002, Persson, Stamataki et al. 2002). Gli1 serves primarily as an activator of transcription and works through amplification of the activated state (Park, Bai et al. 2000).

Evidence Supporting this KER

The evidence presented for this KER is low.The relationship between GLI1/2 translocation and a decrease in GLI1/2 target gene expression relocation has been shown indirectly in multiple mouse models through disruption of SHH signaling at the level of SMO. From our understanding of the SHH pathway, we can infer that disruption of the SHH signaling pathway at the level of SMO is causing a decrease in GLI1/2 translocation and it is this that is causing the altered gene expression While clear evidence that disruption of SHH signaling leads to altered gene expression especially those of the Fox family, insufficient evidence exists for the direct relationship between GLI1/2 translocation and SHH target gene expression. The evidence also lacks direct human applicability as all presented work was

performed *in vitro* on murine models or *in vitro* on murine cell lines.

Biological Plausibility

SHH signaling is well established to be essential for proper embryonic development in vertebrates including mice and humans. Activation of the pathway results in a downstream signaling cascade resulting in the relocation of GLI to the nucleus and subsequent gene transcription (Carballo, Honorato et al. 2018).

Empirical Evidence

- In vitro
  - A mouse cNCC line (09-1) with the expression signature (AP-2alpha (Tfap2a, Twist1, Sox9, Cd44) was used to study whether foxf2 is a target of SHH signalling. Addition of SHH ligand (0.4µg/ml) was found to upregulate both GLI1 and Foxf2. This upregulation was completely blocked by the addition of vismodegib (120nm)(Everson, Fink et al. 2017).
  - To determine if SHH pathway inhibition was downstream for GANT 61 and GANT 58, a Sufu-null MEF cell line was used. Treatment of cells with either GANT at 10µm led to a significant reduction of SHH target genes GLI1 and Hip1 as determined by qPCR. As expected, cyclopamine was unable to inhibit signalling in this system as activation occurs downstream of SMO. GANT 61 is believed to act through addition of the modification to GLI1 that compromises its' ability to properly bind DNA (Lauth, Bergström et al. 2007).
  - GLI activators bind to the GACCACCCA motif to promote transcription of GLI1, PTCH1, PTCH2, HHIP1, MYCN, CCND1, CCND2, BCL2, CFLAR, FOXF1, FOXL1, PRDM1 (BLIMP1), JAG2, GREM1, and Follistatin (Katoh and Katoh 2009)
  - Using a 3D microphysiological model loaded with 3T3 SHH lightII and GMSM-K GFP SHH cells a gradient of PTCH1 correlating with the distance from the epithelium secreting SHH ligand (Johnson, Vitek et al. 2021).
- In vivo
  - In situ hybridization was used to determine expression of GLI1 in C57BL/6J mice to better understanding temporal SHH signalling. At GD 9.0 no difference was found between control and embryos exposed to cyclopamine (120mg/kg/day). GLI1 was downregulated in the ventral frontonasal prominence (FNP) of clomipramine exposed embryos by GD 9.25. FNP tissue was micro dissected and cDNA microarray analysis was performed. 210 genes were found to be dysregulated including a significant enrichment to the forkhead box (Fox) family. RT-PCR confirmed significant down regulation of the SHH target genes GLI1 and PTCH1 as well as nine Fox members: Foxa2, Foxb2, Foxc1, Foxc2, Foxd1, Foxe1, Foxf1, Foxf2, Foxl1. Two members of the fox family, Foxm1 and Foxo1 were not found to differentially expressed in either the cDNA microarray or RT-PCR (Everson, Fink et al. 2017).
  - Using mutant Osr2-IresCre;Smo$^{C/C}$ mice Foxf2 and Foxf1were found to be positively regulated by SHH-SMO signalling. Expression of Osr2 was found to be reduced by E13.5 in the mutants. Expression of Osr1, Pax9, Tbx22 were not found to be altered (Lan and Jiang 2009).
  - o To study whether SHH signaling regulates the developmental fate of the ecto-mesenchyme via regulation of gene activity in the facial primordia, Wnt1-Cre;Smon/c, (removal of SHH signaling) and Wnt1-Cre;R26SmoM2 (activation of SHH signaling). Positive regulation from SHH activity was found for Foxc2, Foxd1, Foxd2, Foxf1, and Foxf2. The Fox genes were found to be dissimilar in expression pattern with spatial activation even with uniform activation of the SHH pathway. Foxc2 and Foxd1 were found to be expressed ubiquitously in the MNA except at the midline, while Foxf1 is expressed at the lateral ends. Foxd2 and Foxf2 are both expressed along the mediolateral axis with Foxd2 having an increasing gradient from medial to lateral and Foxf2 having an opposing gradient (Jeong, Mao et al. 2004). These data support that disrupting GLI1/2 translocation via disruption of the SHH signaling pathway disrupts transcription of Foxc2, Foxd1, Foxd2, Foxf1, and Foxf2.

Uncertainties and Inconsistencies

None identified

Quantitative Understanding of the Linkage

The quantitative understanding for this KER is low. Studies to investigate response-response relationship as well as time scale have not been conducted or were not found in the literature review. The empirical evidence presented establishes that disruption of SHH signaling results in the altered gene expression of SHH target genes. There is a need for more studies to address the dose-response and time course relationship of this linkage.

Known Feedforward/Feedback loops influencing this KER

Positive feedback loop of gene expression from GLI1 and negative feedback loop for PTCH1, PTCH2, HHIP1(Katoh and Katoh 2009)

References

Alexandre, C., A. Jacinto and P. W. Ingham (1996). "Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins." Genes Dev **10**(16): 2003-2013.

Bai, C. B., W. Auerbach, J. S. Lee, D. Stephen and A. L. Joyner (2002). "Gli2, but not Gli1, is required for initial Shh signaling and

ectopic activation of the Shh pathway." Development **129**(20): 4753-4761.

Carballo, G. B., J. R. Honorato, G. P. F. de Lopes and T. C. L. d. S. e. Spohr (2018). "A highlight on Sonic hedgehog pathway." Cell Communication and Signaling **16**(1): 11.

Corbit, K. C., P. Aanstad, V. Singla, A. R. Norman, D. Y. R. Stainier and J. F. Reiter (2005). "Vertebrate Smoothened functions at the primary cilium." Nature **437**(7061): 1018-1021.

Everson, J. L., D. M. Fink, J. W. Yoon, E. J. Leslie, H. W. Kietzman, L. J. Ansen-Wilson, H. M. Chung, D. O. Walterhouse, M. L. Marazita and R. J. Lipinski (2017). "Sonic hedgehog regulation of Foxf2 promotes cranial neural crest mesenchyme proliferation and is disrupted in cleft lip morphogenesis." Development **144**(11): 2082-2091.

Hui, C. C. and S. Angers (2011). "Gli proteins in development and disease." Annu Rev Cell Dev Biol **27**: 513-537.

Jeong, J., J. Mao, T. Tenzen, A. H. Kottmann and A. P. McMahon (2004). "Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia." Genes Dev **18**(8): 937-951.

Johnson, B. P., R. A. Vitek, M. M. Morgan, D. M. Fink, T. G. Beames, P. G. Geiger, D. J. Beebe and R. J. Lipinski (2021). "A Microphysiological Approach to Evaluate Effectors of Intercellular Hedgehog Signaling in Development." Front Cell Dev Biol **9**: 621442.

Katoh, Y. and M. Katoh (2009). "Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation." Curr Mol Med **9**(7): 873-886.

Kim, J., M. Kato and P. A. Beachy (2009). "Gli2 trafficking links Hedgehog-dependent activation of Smoothened in the primary cilium to transcriptional activation in the nucleus." Proc Natl Acad Sci U S A **106**(51): 21666-21671.

Kogerman, P., T. Grimm, L. Kogerman, D. Krause, A. B. Undén, B. Sandstedt, R. Toftgård and P. G. Zaphiropoulos (1999). "Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1." Nat Cell Biol **1**(5): 312-319.

Lan, Y. and R. Jiang (2009). "Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth." Development **136**(8): 1387-1396.

Lauth, M., A. Bergström, T. Shimokawa and R. Toftgård (2007). "Inhibition of GLI-mediated transcription and tumor cell growth by small-molecule antagonists." Proc Natl Acad Sci U S A **104**(20): 8455-8460.

Liu, K. J. (2016). "Craniofacial Ciliopathies and the Interpretation of Hedgehog Signal Transduction." PLoS Genet **12**(12): e1006460.

Park, H. L., C. Bai, K. A. Platt, M. P. Matise, A. Beeghly, C. C. Hui, M. Nakashima and A. L. Joyner (2000). "Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation." Development **127**(8): 1593-1605.

Pearse, R. V., 2nd, L. S. Collier, M. P. Scott and C. J. Tabin (1999). "Vertebrate homologs of Drosophila suppressor of fused interact with the gli family of transcriptional regulators." Dev Biol **212**(2): 323-336.

Persson, M., D. Stamataki, P. te Welscher, E. Andersson, J. Böse, U. Rüther, J. Ericson and J. Briscoe (2002). "Dorsal-ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity." Genes Dev **16**(22): 2865-2878.

Rohatgi, R., L. Milenkovic and M. P. Scott (2007). "Patched1 regulates hedgehog signaling at the primary cilium." Science **317**(5836): 372-376.

Stone, D. M., M. Murone, S. Luoh, W. Ye, M. P. Armanini, A. Gurney, H. Phillips, J. Brush, A. Goddard, F. J. de Sauvage and A. Rosenthal (1999). "Characterization of the human suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli." J Cell Sci **112 (Pt 23)**: 4437-4448.

Tukachinsky, H., L. V. Lopez and A. Salic (2010). "A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes." J Cell Biol **191**(2): 415-428.

Tuson, M., M. He and K. V. Anderson (2011). "Protein kinase A acts at the basal body of the primary cilium to prevent Gli2 activation and ventralization of the mouse neural tube." Development **138**(22): 4921-4930.

Von Ohlen, T. and J. E. Hooper (1997). "Hedgehog signaling regulates transcription through Gli/Ci binding sites in the wingless enhancer." Mech Dev **68**(1-2): 149-156.

Wang, B., J. F. Fallon and P. A. Beachy (2000). "Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb." Cell **100**(4): 423-434.

Relationship: 2731: Decrease, GLI1/2 target gene expression leads to Decrease, SHH second messenger production

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Low	Low
Decrease, GLI1/2 target gene expression leads to orofacial clefting	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	High

The relationship between a decrease in GLI1/2 target gene expression and a decrease in secondary messenger production has been shown in mouse models. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question.

Key Event Relationship Description

Activation of the Sonic Hedgehog (SHH) pathway results in a downstream signaling cascade resulting in the relocation of GLI to the nucleus and subsequent gene transcription (Carballo, Honorato et al. 2018). This gene expression drives secondary messenger signaling for the pathway. The following genes are believed to be regulated by GLI as a component of SHH signaling: FGF10, BMP2, BMP4.

Evidence Supporting this KER

Biological Plausibility

SHH signaling is well established to be essential for proper embryonic development in vertebrates including mice and humans. Activation of the pathway results in a downstream signaling cascade resulting in the relocation of GLI to the nucleus and subsequent gene transcription (Carballo, Honorato et al. 2018). SHH cross talks with other developmental pathways including FGF and BMP.

Empirical Evidence

- In Osr2-IresCre;Smo$^{c/c}$ (SHH pathway inactive) mutant mice Fgf10 mRNA was found to be significantly reduced in the anterior palatal mesenchyme. The expression of Fgf10 correlated with a downregulation of PTCH1 (Lan and Jiang 2009).
- To determine if SHH can induce Fgf10, SHH overexpressing cells were implanted in the anterior region of the wing bud of chick embryos. By 27 hours, the expression of Fgf10 had significantly increased and expanded from the anterior mesenchyme to the bifurcating wing bud (Ohuchi, Nakagawa et al. 1997).
- To investigate whether MSX-1 is in the same pathway as Fgf10, MSX-1 expression was examined in Fgf10-/- mice and Fgf10 expression was examined in Msx-1-/- mice. No change in expression was found and it is concluded that MSX-1 is not a downstream target of Fgf10 (Alappat, Zhang et al. 2005).
- SHH expression is reduced in the palatal epithelium of both Fgf10-/- and Fgfr2b -/- mutants. Exogenous Fgf10 induced SHH in WT palatal epithelium (Rice, Spencer-Dene et al. 2004).
- BMP2 and BMP4 is downregulated in the anterior palate of Osr2-IresCre;Smo$^{c/c}$ (SHH pathway inactive) mutant mice (Lan and Jiang 2009).
- Upregulation of mesenchymal BMP4 by SHH via Foxf1 or Foxl1(Katoh and Katoh 2009).

Uncertainties and Inconsistencies

The relationships and feedback/feedforward loops that exist between SHH and its' secondary messengers primary Fgf10 and BMP4 is not well understood. Some evidence exists that expression of both Fgf10 and BMP4 correlates with that of SHH. The state of evidence is lacking and no dose response data was found.

References

Alappat, S. R., Z. Zhang, K. Suzuki, X. Zhang, H. Liu, R. Jiang, G. Yamada and Y. Chen (2005). "The cellular and molecular etiology of the cleft secondary palate in Fgf10 mutant mice." Dev Biol **277**(1): 102-113.

Carballo, G. B., J. R. Honorato, G. P. F. de Lopes and T. C. L. d. S. e. Spohr (2018). "A highlight on Sonic hedgehog pathway." Cell Communication and Signaling **16**(1): 11.

Cobourne, M. T. and J. B. Green (2012). "Hedgehog signalling in development of the secondary palate." Front Oral Biol **16**: 52-59.

Katoh, Y. and M. Katoh (2009). "Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation." Curr Mol Med **9**(7): 873-886.

LaBonne, C. and M. Bronner-Fraser (1999). "Molecular mechanisms of neural crest formation." Annu Rev Cell Dev Biol **15**: 81-112.

Lan, Y. and R. Jiang (2009). "Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth." Development **136**(8): 1387-1396.

Ohuchi, H., T. Nakagawa, A. Yamamoto, A. Araga, T. Ohata, Y. Ishimaru, H. Yoshioka, T. Kuwana, T. Nohno, M. Yamasaki, N. Itoh and S. Noji (1997). "The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor." Development **124**(11): 2235-2244.

Rice, R., B. Spencer-Dene, E. C. Connor, A. Gritli-Linde, A. P. McMahon, C. Dickson, I. Thesleff and D. P. Rice (2004). "Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate." J Clin Invest **113**(12): 1692-1700.

Zhang, Y., X. Zhao, Y. Hu, T. St Amand, M. Zhang, R. Ramamurthy, M. Qiu and Y. Chen (1999). "Msx1 is required for the induction of Patched by Sonic hedgehog in the mammalian tooth germ." Dev Dyn **215**(1): 45-53.

Zhang, Z., Y. Song, X. Zhao, X. Zhang, C. Fermin and Y. Chen (2002). "Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis." Development **129**(17): 4135-4146.

Relationship: 2732: Decrease, SHH second messenger production leads to Decrease, Cell proliferation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Low	Low
Decrease, GLI1/2 target gene expression leads to orofacial clefting	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus		NCBI
chicken	Gallus gallus		NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

The relationship between a decrease in SHH secondary messengers and a decrease in cellular proliferation translocation has been demonstrated in both mouse and chick models. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question.

Key Event Relationship Description

SHH is a mitogen that regulates cell proliferation during development. SHH regulation of proliferation works at least in part through regulation of cyclin D1 (ccnd 1) and cyclin D2 (Ccnd 2) (Kenney and Rowitch 2000, Ishibashi and McMahon 2002, Lobjois, Benazeraf et al. 2004, Mill, Mo et al. 2005, Hu, Mo et al. 2006). The regulation of ccnd 1 and ccnd 2 by SHH is not fully understood but is believed to be in part by regulation via SHH signaling and its signaling to SHH secondary messengers, namely the fibroblast growth factor family and GLI. GLI1 has been shown to directly bind and regulate ccnd1 and ccnd2 (Yoon, Kita et al. 2002). This signaling is largely comprised of a network between bone morphogenic protein (BMP), Fibroblast growth factor (Fgf), and SHH (SHH) (Zhang, Song et al. 2002, Rice, Spencer-Dene et al. 2004). The SHH signaling cascade results in the expression of secondary messengers. Proper Msx1 activity in the mesenchyme is required for the expression of SHH in the overlying epithelium (Zhang, Song et al. 2002). Maintenance of SHH expression in the epithelium is believed to be dependent on Fgf10 expression in the mesenchyme and its' signaling through Fgfr2b in the epithelium (Rice, Spencer-Dene et al. 2004).

Evidence Supporting this KER

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Biological Plausibility

The SHH pathway is well known to be associated with cellular proliferation. There is a high biological probability that this proliferation results through regulation of SHH secondary messengers.

Empirical Evidence

- In vitro
  - Mouse cerebellar granule cells exposed to cycloheximide and SHH did not promote upregulation of ccnd 1, ccnd 2, or ccn3 mRNA. This supports that there is a protein intermediate between the SHH pathway and regulation of the G1 cyclins(Kenney and Rowitch 2000).
  -
- In vivo

  o In mouse palate explants application of SHH was found to induce proliferation in the palatal mesenchyme as measured by BrdU (Rice, Spencer-Dene et al. 2004).
  o In CD-1 WT and MSX-1-/-, SHH soaked beads were able to induce proliferation in palatal mesenchyme explants at 24hr but not after 8hr suggesting the induction of proliferation is through an indirect mechanism (Zhang, Song et al. 2002).
  o IHC staining for Ccnd-1 and Ccnd-2 in Osr2-IresCre Smoc/c (SHH inactive) and control embryos was used to determine if expression patterns differed between the mesenchyme and epithelium in mutants. Expression for both Ccnd-1 and Ccnd-2 was found to be reduced in the mesenchyme for mutants. mRNA was found to be reduced for both Ccnd-1 and Ccnd-2 in the palatal mesenchyme (Lan and Jiang 2009).
  o In Osr2-IresCre;Smoc/c (SHH pathway inactive) mutant mice Fgf10 mRNA was found to be significantly reduced in the anterior palatal mesenchyme. The expression of Fgf10 correlated with a downregulation of PTCH1 (Lan and Jiang 2009).
  o SHH expression is reduced in the palatal epithelium of both Fgf10-/- and Fgfr2b -/- mutants. Exogenous Fgf10 induced SHH in WT palatal epithelium (Rice, Spencer-Dene et al. 2004).
  o Decreased proliferation correlating with downregulation of GLI1 and PTCH1 was found in E10.25 mouse embryos treated with cyclopamine (Everson, Fink et al. 2017).

Uncertainties and Inconsistencies

The relationship between a decrease is SHH secondary messenger production and a decrease in cellular proliferation is plausible and data is shown that supports a decrease in ccnd 1 and 2 in correlation with the Fgf and SHH pathways. Further studies are needed to further out understanding of the regulation of proliferation by SHH.

References

Alappat, S. R., Z. Zhang, K. Suzuki, X. Zhang, H. Liu, R. Jiang, G. Yamada and Y. Chen (2005). "The cellular and molecular etiology of the cleft secondary palate in Fgf10 mutant mice." Dev Biol **277**(1): 102-113.

Carballo, G. B., J. R. Honorato, G. P. F. de Lopes and T. C. L. d. S. e. Spohr (2018). "A highlight on Sonic hedgehog pathway." Cell Communication and Signaling **16**(1): 11.

Everson, J. L., D. M. Fink, J. W. Yoon, E. J. Leslie, H. W. Kietzman, L. J. Ansen-Wilson, H. M. Chung, D. O. Walterhouse, M. L. Marazita and R. J. Lipinski (2017). "Sonic hedgehog regulation of Foxf2 promotes cranial neural crest mesenchyme proliferation and is disrupted in cleft lip morphogenesis." Development **144**(11): 2082-2091.

Hu, M. C., R. Mo, S. Bhella, C. W. Wilson, P. T. Chuang, C. C. Hui and N. D. Rosenblum (2006). "GLI3-dependent transcriptional repression of Gli1, Gli2 and kidney patterning genes disrupts renal morphogenesis." Development **133**(3): 569-578.

Ishibashi, M. and A. P. McMahon (2002). "A sonic hedgehog-dependent signaling relay regulates growth of diencephalic and mesencephalic primordia in the early mouse embryo." Development **129**(20): 4807-4819.

Kenney, A. M. and D. H. Rowitch (2000). "Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors." Mol Cell Biol **20**(23): 9055-9067.

Lan, Y. and R. Jiang (2009). "Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth." Development **136**(8): 1387-1396.

Lobjois, V., B. Benazeraf, N. Bertrand, F. Medevielle and F. Pituello (2004). "Specific regulation of cyclins D1 and D2 by FGF and Shh signaling coordinates cell cycle progression, patterning, and differentiation during early steps of spinal cord development." Dev Biol **273**(2): 195-209.

Mill, P., R. Mo, M. C. Hu, L. Dagnino, N. D. Rosenblum and C. C. Hui (2005). "Shh controls epithelial proliferation via independent pathways that converge on N-Myc." Dev Cell **9**(2): 293-303.

Ohuchi, H., T. Nakagawa, A. Yamamoto, A. Araga, T. Ohata, Y. Ishimaru, H. Yoshioka, T. Kuwana, T. Nohno, M. Yamasaki, N. Itoh and S. Noji (1997). "The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor." Development **124**(11): 2235-2244.

Rice, R., B. Spencer-Dene, E. C. Connor, A. Gritli-Linde, A. P. McMahon, C. Dickson, I. Thesleff and D. P. Rice (2004). "Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate." J Clin Invest **113**(12): 1692-1700.

Yoon, J. W., Y. Kita, D. J. Frank, R. R. Majewski, B. A. Konicek, M. A. Nobrega, H. Jacob, D. Walterhouse and P. Iannaccone (2002). "Gene expression profiling leads to identification of GLI1-binding elements in target genes and a role for multiple downstream pathways in GLI1-induced cell transformation." J Biol Chem **277**(7): 5548-5555.

Zhang, Z., Y. Song, X. Zhao, X. Zhang, C. Fermin and Y. Chen (2002). "Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis." Development **129**(17): 4135-4146.

Relationship: 2724: Decrease, Cell proliferation leads to Decrease, facial prominence outgrowth

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Low	Low
Decrease, GLI1/2 target gene expression leads to orofacial clefting	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

The relationship between a decrease in cellular proliferation and a decrease in outgrowth has been demonstrated in both mouse and chick models. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question.

Key Event Relationship Description

SHH is a mitogen that regulates cell proliferation during development. SHH regulation of proliferation works at least in part through regulation of cyclin D1 (ccnd 1) and cyclin D2 (Ccnd 2) (Kenney and Rowitch 2000, Ishibashi and McMahon 2002, Lobjois, Benazeraf et al. 2004, Mill, Mo et al. 2005, Hu, Mo et al. 2006). The regulation of ccnd 1 and ccnd 2 by SHH is not fully understood but is believed to be in part by regulation via SHH signaling and its signaling to SHH secondary messengers, namely the fibroblast growth factor family. A network of reciprocal growth factor signaling between the epithelium and mesenchyme is required for proper growth and patterning of the early palatal shelves.

The development of the face occurs early in embryogenesis and involves precise coordination of multiple tissues. The oropharyngeal membrane appears early in the 4th week of gestation and gives rise to the frontonasal process and the 1st pharyngeal arch. The frontonasal process is derived from the neural crest and in turn gives rise to two medial nasal process and two lateral nasal processes that later fuse and form the intermaxillary process. The pharyngeal arch is derived from mesoderm and the neural crest. It gives rise to two mandibular process and two maxillary processes (Som and Naidich 2013). These processes are comprised of mesenchymal cells from neural crest migration and the craniopharyngeal ectoderm and are coated in an epithelium (Ferguson 1988). The upper lip is formed during weeks 5-7 when the maxillary processes grow towards the midline and fuse intermaxillary process that have formed the philtrum and columella (Warbrick 1960, Kim, Park et al. 2004). The palate develops between week 6-12 from a median palatine process and a pair of lateral palatine processes. The primary palate is formed from the posterior extension of the intermaxillary process. The lateral palatine processes arise as medial mesenchymal processes from both maxillary processes. These processes initially grow inferiorly until the tongue is pulled downwards by the elongation of the maxilla and mandible. Once above the tongue, the lateral processes grow medially until they make contact and fuse (Som and Naidich 2014). For normal facial development and growth coordinated multivariate signaling is required. For example, retinoic acid, BMP, FGF, and SHH signal together to control facial growth (Liu, Rooker et al. 2010). SHH is an important modulator of epithelial-mesenchyme interaction (EMi) during development. SHH has been shown to regulate growth and formation of the palatal shelves prior to elevation and fusion (Rice, Connor et al. 2006). During development, SHH ligand is secreted by the epithelium into the underlying mesenchyme. This causes a gradient of signaling where mesenchyme proximal to the epithelium is exposed to higher concentrations of SHH than more distal cells (Cohen, Kicheva et al. 2015). Disruption of SHH during critical windows of development is believed to work in an EMi dependent, but epithelial-mesenchyme transition (Emt) independent manner. OFCs caused by disruption to SHH are believed to be due to a reduction in epithelial induced proliferation and the subsequent decrease in tissue outgrowth and the failure of the facial processes to meet and fuse (Lipinski, Song et al. 2010, Heyne, Melberg et al. 2015).

Evidence Supporting this KER

Biological Plausibility

The SHH pathway is well known to be associated with cellular proliferation and growth of the facial prominences. There is a high biological probability that disruption to proliferation of the facial prominences disrupts outgrowth.

Empirical Evidence

- In vitro
  - None identified
- In vivo
  - To investigate how SHH might regulate early pharyngeal arch (PA1) development SHH-/- embryos were generated. At E9.5, the mutant embryos were thinner with hypoplasia on PA1. Morphometrics of PA1 of mutant vs. control showed a significant decrease in size in the mutant ($P<0.05$) for both the dorsal-ventral and the anteroposterior axis. Hypoplasia was quantified using a Pax3-Cre/R26R transgenic mouse line marked with LacZ and stained with X-gal (Yamagishi, Yamagishi et al. 2006).
  - SHH expressed in thickened palatal epithelium prior to palatal shelf outgrowth (E13.0-14.5) (Rice, Connor et al. 2006)
  - Using Wnt1-Cre;Smon/c embryos, a significant decrease in the growth of the mandibular arch in both the proximodistal and dorsoventral (D-V) axes. This supports that observation that the wild type, but not the mutants undergo rapid growth in the D-V axis around E11.5 (Jeong, Mao et al. 2004).
  - SHH is expressed in oral epithelium and shown as a key signal for palatal shelf outgrowth in explant culture (Lan and Jiang 2009)

Uncertainties and Inconsistencies

The regulation of proliferation by SHH has been shown but questions to the exact mechanism of regulation remain. Evidence exists that there is likely an intermediate between SHH and regulation of ccnd 1 and ccnd 2. Some evidence exists that the intermediate could be a member(s) of the Fgf family. The relationship between a decrease in cellular proliferation and a decrease in outgrowth is plausible and data is shown that supports that disruption of the SHH pathway leads to decrease in palatal outgrowth. Further studies are needed to further out understanding of the regulation of proliferation by SHH and its subsequent impact on outgrowth of the facial prominences.

References

en, M., A. Kicheva, A. Ribeiro, R. Blassberg, K. M. Page, C. P. Barnes and J. Briscoe (2015). "Ptch1 and Gli regulate Shh signalling dynamics via multiple mechanisms." Nature Communications **6**(1): 6709.

Ferguson, M. W. (1988). "Palate development." Development **103 Suppl**: 41-60.

Heyne, G. W., C. G. Melberg, P. Doroodchi, K. F. Parins, H. W. Kietzman, J. L. Everson, L. J. Ansen-Wilson and R. J. Lipinski (2015). "Definition of critical periods for Hedgehog pathway antagonist-induced holoprosencephaly, cleft lip, and cleft palate." PLoS One **10**(3): e0120517.

Hu, M. C., R. Mo, S. Bhella, C. W. Wilson, P. T. Chuang, C. C. Hui and N. D. Rosenblum (2006). "GLI3-dependent transcriptional repression of Gli1, Gli2 and kidney patterning genes disrupts renal morphogenesis." Development **133**(3): 569-578.

Ishibashi, M. and A. P. McMahon (2002). "A sonic hedgehog-dependent signaling relay regulates growth of diencephalic and mesencephalic primordia in the early mouse embryo." Development **129**(20): 4807-4819.

Jeong, J., J. Mao, T. Tenzen, A. H. Kottmann and A. P. McMahon (2004). "Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia." Genes Dev **18**(8): 937-951.

Kenney, A. M. and D. H. Rowitch (2000). "Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors." Mol Cell Biol **20**(23): 9055-9067.

Kim, C. H., H. W. Park, K. Kim and J. H. Yoon (2004). "Early development of the nose in human embryos: a stereomicroscopic and histologic analysis." Laryngoscope **114**(10): 1791-1800.

Lan, Y. and R. Jiang (2009). "Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth." Development **136**(8): 1387-1396.

Lipinski, R. J., C. Song, K. K. Sulik, J. L. Everson, J. J. Gipp, D. Yan, W. Bushman and I. J. Rowland (2010). "Cleft lip and palate results from Hedgehog signaling antagonism in the mouse: Phenotypic characterization and clinical implications." Birth Defects Res A Clin Mol Teratol **88**(4): 232-240.

Liu, B., S. M. Rooker and J. A. Helms (2010). "Molecular control of facial morphology."Semin Cell Dev Biol **21**(3): 309-313.

Lobjois, V., B. Benazeraf, N. Bertrand, F. Medevielle and F. Pituello (2004). "Specific regulation of cyclins D1 and D2 by FGF and Shh signaling coordinates cell cycle progression, patterning, and differentiation during early steps of spinal cord development." Dev Biol **273**(2): 195-209.

Mill, P., R. Mo, M. C. Hu, L. Dagnino, N. D. Rosenblum and C. C. Hui (2005). "Shh controls epithelial proliferation via independent pathways that converge on N-Myc." Dev Cell **9**(2): 293-303.

Rice, R., E. Connor and D. P. C. Rice (2006). "Expression patterns of Hedgehog signalling pathway members during mouse palate development." Gene Expression Patterns **6**(2): 206-212.

Som, P. M. and T. P. Naidich (2013). "Illustrated review of the embryology and development of the facial region, part 1: Early face and lateral nasal cavities." AJNR Am J Neuroradiol **34**(12): 2233-2240.

Som, P. M. and T. P. Naidich (2014). "Illustrated review of the embryology and development of the facial region, part 2: Late development of the fetal face and changes in the face from the newborn to adulthood." AJNR Am J Neuroradiol **35**(1): 10-18.

Warbrick, J. G. (1960). "The early development of the nasal cavity and upper lip in the human embryo."J Anat **94**(Pt 3): 351-362.

Yamagishi, C., H. Yamagishi, J. Maeda, T. Tsuchihashi, K. Ivey, T. Hu and D. Srivastava (2006). "Sonic Hedgehog Is Essential for First Pharyngeal Arch Development." Pediatric Research **59**(3): 349-354.

Relationship: 2726: Decrease, facial prominence outgrowth leads to orofacial cleft

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Moderate	Low
Decrease, GLI1/2 target gene expression leads to orofacial clefting	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

The relationship is biologically plausible in human, but to date no specific experiments have addressed this question.

The SHH pathway is well understood to be fundamental to proper embryonic development and that aberrant SHH signaling during embryonic development can cause birth defects including orofacial clefts (OFCs). For this reason, this KER is applicable to the embryonic stage with a high level of confidence.

Key Event Relationship Description

Orofacial clefts (OFCs) are one of the most common human birth defects and occur in approximately 1 in 700 live births (Mossey, Little et al. 2009, Dixon, Marazita et al. 2011) Formation of the upper lip and palate requires the orchestrated proliferation and fusion of embryonic facial growth centers and is dependent on paracrine intercellular signaling through multiple pathways. Genetic and chemical disruption of the Sonic Hedgehog (SHH), Transforming growth factor-beta (Tgf-β), bone morphogenic protein (BMP), epidermal growth factor (EGF) etc. pathways have been shown to cause OFCs (Jiang, Bush et al. 2006, Bush and Jiang 2012, Lan, Xu et al. 2015). Early orofacial development involves epithelial ectoderm derived SHH ligand driving tissue outgrowth through an induced gradient of SHH dependent transcription in the underlying mesenchyme, which is thought to drive mesenchymal proliferation (Lan and Jiang 2009, Kurosaka 2015).

The development of the face occurs early in embryogenesis and involves precise coordination of multiple tissues. The oropharyngeal membrane appears early in the 4^{th} week of gestation and gives rise to the frontonasal process and the 1^{st} pharyngeal arch. The frontonasal process is derived from the neural crest and in turn gives rise to two medial nasal process and two lateral nasal processes that later fuse and form the intermaxillary process. The pharyngeal arch is derived from mesoderm and the neural crest. It gives rise to two mandibular process and two maxillary processes (Som and Naidich 2013). These processes are comprised of mesenchymal cells from neural crest migration and the craniopharyngeal ectoderm and are coated in an epithelium (Ferguson 1988). The upper lip is formed during weeks 5-7 when the maxillary processes grow towards the midline and fuse intermaxillary process that have formed the philtrum and columella (Warbrick 1960, Kim, Park et al. 2004). The palate develops between week 6-12 from a median palatine process and a pair of lateral palatine processes. The primary palate is formed from the posterior extension of the intermaxillary process. The lateral palatine processes arise as medial mesenchymal processes from both maxillary processes. These processes initially grow inferiorly until the tongue is pulled downwards by the elongation of the maxilla and mandible. Once above the tongue, the lateral processes grow medially until they make contact and fuse (Som and Naidich 2014). For normal facial development and growth coordinated multivariate signaling is required. For example, retinoic acid, BMP, FGF, and SHH signal together to control facial growth (Liu, Rooker et al. 2010). SHH is an important modulator of epithelial-mesenchyme interaction (EMi) during development. SHH has been shown to regulate growth and formation of the palatal shelves prior to elevation and fusion (Rice, Connor et al. 2006). During development, SHH ligand is secreted by the epithelium into the underlying mesenchyme. This causes a gradient of signaling where mesenchyme proximal to the epithelium is exposed to higher concentrations of SHH than more distal cells (Cohen, Kicheva et al. 2015). Disruption of SHH during critical windows of development is believed to work in an EMi dependent, but epithelial-mesenchyme transition (Emt) independent manner. OFCs caused by disruption to SHH are believed to be due to a reduction in epithelial induced proliferation and the subsequent decrease in tissue outgrowth and the failure of the facial processes to meet and fuse (Lipinski, Song et al. 2010, Heyne, Melberg et al. 2015).

Evidence Supporting this KER

Biological Plausibility

The SHH pathway is well known to be associated with development of the face including the lip and palatal. Disruption of SHH at critical periods of development has been shown to cause OFCs.

Empirical Evidence

- In vitro
  - None identified
- In vivo
  - ~85% of K14-Cre;$Shh^{c/n}$ mice had cleft palate with rudimentary palatal shelves spaced apart without contact suggesting that the cleft is due to insufficient outgrowth of the shelves (Rice, Spencer-Dene et al. 2004).
  - 100% (n=22) Osr2-IresCre;$Smo^{c/c}$ had a cleft palate. At E14.5 the palatal shelves were underdeveloped and had not grown out to make contact compared to control littermates that had met and initiated fusion. This supports that disruption of SHH signalling impairs palatal shelf outgrowth and can lead to cleft palate (Lan and Jiang 2009)

Uncertainties and Inconsistencies

The quantitative understanding of this relationship is low. No studies were found to exist to address dose response or time-scale data. Further work is needed to address these questions and create a better understanding of this relationship.

References

Bush, J. O. and R. Jiang (2012). "Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development." Development **139**(2): 231-243.

Cohen, M., A. Kicheva, A. Ribeiro, R. Blassberg, K. M. Page, C. P. Barnes and J. Briscoe (2015). "Ptch1 and Gli regulate Shh signalling dynamics via multiple mechanisms." Nature Communications **6**(1): 6709.

Dixon, M. J., M. L. Marazita, T. H. Beaty and J. C. Murray (2011). "Cleft lip and palate: understanding genetic and environmental influences." Nature Reviews Genetics **12**(3): 167-178.

Ferguson, M. W. (1988). "Palate development." Development **103 Suppl**: 41-60.

Heyne, G. W., C. G. Melberg, P. Doroodchi, K. F. Parins, H. W. Kietzman, J. L. Everson, L. J. Ansen-Wilson and R. J. Lipinski (2015). "Definition of critical periods for Hedgehog pathway antagonist-induced holoprosencephaly, cleft lip, and cleft palate." PLoS One **10**(3): e0120517.

Jiang, R., J. O. Bush and A. C. Lidral (2006). "Development of the upper lip: morphogenetic and molecular mechanisms."Dev Dyn **235**(5): 1152-1166.

Kim, C. H., H. W. Park, K. Kim and J. H. Yoon (2004). "Early development of the nose in human embryos: a stereomicroscopic and histologic analysis." Laryngoscope **114**(10): 1791-1800.

Kurosaka, H. (2015). "The Roles of Hedgehog Signaling in Upper Lip Formation."Biomed Res Int **2015**: 901041.

Lan, Y. and R. Jiang (2009). "Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth." Development **136**(8): 1387-1396.

Lan, Y., J. Xu and R. Jiang (2015). "Cellular and Molecular Mechanisms of Palatogenesis."Curr Top Dev Biol **115**: 59-84.

Lipinski, R. J., C. Song, K. K. Sulik, J. L. Everson, J. J. Gipp, D. Yan, W. Bushman and I. J. Rowland (2010). "Cleft lip and palate results from Hedgehog signaling antagonism in the mouse: Phenotypic characterization and clinical implications." Birth Defects Res A Clin Mol Teratol **88**(4): 232-240.

Liu, B., S. M. Rooker and J. A. Helms (2010). "Molecular control of facial morphology."Semin Cell Dev Biol **21**(3): 309-313.

Mossey, P. A., J. Little, R. G. Munger, M. J. Dixon and W. C. Shaw (2009). "Cleft lip and palate."The Lancet **374**(9703): 1773-1785.

Rice, R., E. Connor and D. P. C. Rice (2006). "Expression patterns of Hedgehog signalling pathway members during mouse palate development." Gene Expression Patterns **6**(2): 206-212.

Rice, R., B. Spencer-Dene, E. C. Connor, A. Gritli-Linde, A. P. McMahon, C. Dickson, I. Thesleff and D. P. Rice (2004). "Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate." J Clin Invest **113**(12): 1692-1700.

Som, P. M. and T. P. Naidich (2013). "Illustrated review of the embryology and development of the facial region, part 1: Early face and lateral nasal cavities." AJNR Am J Neuroradiol **34**(12): 2233-2240.

Som, P. M. and T. P. Naidich (2014). "Illustrated review of the embryology and development of the facial region, part 2: Late development of the fetal face and changes in the face from the newborn to adulthood." AJNR Am J Neuroradiol **35**(1): 10-18.

Warbrick, J. G. (1960). "The early development of the nasal cavity and upper lip in the human embryo."J Anat **94**(Pt 3): 351-362.

Relationship: 2792: Apoptosis leads to Decrease, facial prominence outgrowth

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Low	Low
Decrease, GLI1/2 target gene expression leads to orofacial clefting	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

The relationship between an increase in apoptosis and a decrease in palatal shelf outgrowth has been shown in mice models as detailed in the empirical evidence section. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question. The SHH pathway is well understood to be fundamental to proper embryonic development and that aberrant SHH signaling during embryonic development can cause birth defects including orofacial clefts (OFCs). For this reason, this KER is applicable to the embryonic stage with a high level of confidence.

Key Event Relationship Description

The development of the face occurs early in embryogenesis and involves precise coordination of multiple tissues. The oropharyngeal membrane appears early in the 4^{th} week of gestation and gives rise to the frontonasal process and the 1^{st} pharyngeal arch. The frontonasal process is derived from the neural crest and in turn gives rise to two medial nasal process and two lateral nasal processes that later fuse and form the intermaxillary process. The pharyngeal arch is derived from mesoderm and the neural crest. It gives rise to two mandibular process and two maxillary processes (Som and Naidich 2013). These processes are comprised of mesenchymal cells from neural crest migration and the craniopharyngeal ectoderm and are coated in an epithelium (Ferguson 1988). The upper lip is formed during weeks 5-7 when the maxillary processes grow towards the midline and fuse intermaxillary process that have formed the philtrum and columella (Warbrick 1960, Kim, Park et al. 2004). The palate develops between week 6-12 from a median palatine process and a pair of lateral palatine processes. The primary palate is formed from the posterior extension of the intermaxillary process. The lateral palatine processes arise as medial mesenchymal processes from both maxillary processes. These processes initially grow inferiorly until the tongue is pulled downwards by the elongation of the maxilla and mandible. Once above the tongue, the lateral processes grow medially until they make contact and fuse (Som and Naidich 2014). For normal facial development and growth coordinated multivariate signaling is required. For example, retinoic acid, BMP, FGF, and SHH signal together to control facial growth (Liu, Rooker et al. 2010). SHH is an important modulator of epithelial-mesenchyme interaction (EMi) during development. SHH has been shown to regulate growth and formation of the palatal shelves prior to elevation and fusion (Rice, Connor et al. 2006). During development, SHH ligand is secreted by the epithelium into the underlying mesenchyme. This causes a gradient of signaling where mesenchyme proximal to the epithelium is exposed to higher concentrations of SHH than more distal cells (Cohen, Kicheva et al. 2015). Disruption of SHH during critical windows of development is believed to work in an EMi dependent, but epithelial-mesenchyme transition (Emt) independent manner. OFCs caused by disruption to SHH are believed to be due to a decrease in cellular proliferation and an increase in apoptosis leading to a decrease in tissue outgrowth and the failure of the facial processes to meet and fuse (Lipinski, Song et al. 2010, Heyne, Melberg et al. 2015). In mice, zones of apoptosis within the fusing epithelium of the medial nasal process and the lateral nasal process have been identified (Gaare and Langman 1977). These regions have been shown to be nonproliferative and are actively undergoing apoptosis (Jiang, Bush et al. 2006, Song, Li et al. 2009, Ferretti, Li et al. 2011). These studies demonstrate the importance of apoptosis in orofacial development and indicate that dysregulation of this process could result in OFC formation.

Evidence Supporting this KER

Biological Plausibility

There is a high biological plausibility that increased apoptosis would lead to decreased facial prominence outgrowth.

Empirical Evidence

- In vitro
  - None found in search
- In vivo
  - Wnt1-Cre;$Smo^{n/c}$ have increased apoptosis in the mandibular arch compared to wild type at E9.5, E 10.5. This is combination with a decrease in proliferation at E11.5 leads to a decrease in outgrowth of the process (Jeong, Mao et al. 2004).
  - Chick embryos exposed to 200ul of 10% ethanol with an additional 20ul of 1% ethanol at stage 9-10 display a reduction in the growth of the frontonasal prominence, hypoplastic branchial arches, and increased apoptosis in cranial neural crest cells. Treatment with antibodies that block SHH signalling had the same impact as ethanol exposure supporting that ethanol exposure reduces shh signalling (Ahlgren, Thakur et al. 2002).

Uncertainties and Inconsistencies

Further studies are needed to expand our understanding of the role that apoptosis plays in orofacial development and cleft formation.

Quantitative Understanding of the Linkage

The quantitative understanding of this relationship is low. No studies were found to exist to address dose response or

time-scale data. Further work is needed to address these questions and create a better understanding of this relationship.

Response-response relationship

Insufficient evidence

Time-scale

Insufficient evidence

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Insufficient evidence			

Known Feedforward/Feedback loops influencing this KER

Insufficient evidence

References

Ahlgren, S. C., V. Thakur and M. Bronner-Fraser (2002). "Sonic hedgehog rescues cranial neural crest from cell death induced by ethanol exposure." Proc Natl Acad Sci U S A **99**(16): 10476-10481.

Cohen, M., A. Kicheva, A. Ribeiro, R. Blassberg, K. M. Page, C. P. Barnes and J. Briscoe (2015). "Ptch1 and Gli regulate Shh signalling dynamics via multiple mechanisms." Nature Communications **6**(1): 6709.

Ferguson, M. W. (1988). "Palate development." Development **103 Suppl**: 41-60.

Heyne, G. W., C. G. Melberg, P. Doroodchi, K. F. Parins, H. W. Kietzman, J. L. Everson, L. J. Ansen-Wilson and R. J. Lipinski (2015). "Definition of critical periods for Hedgehog pathway antagonist-induced holoprosencephaly, cleft lip, and cleft palate." PLoS One **10**(3): e0120517.

Jeong, J., J. Mao, T. Tenzen, A. H. Kottmann and A. P. McMahon (2004). "Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia." Genes Dev **18**(8): 937-951.

Kim, C. H., H. W. Park, K. Kim and J. H. Yoon (2004). "Early development of the nose in human embryos: a stereomicroscopic and histologic analysis." Laryngoscope **114**(10): 1791-1800.

Lan, Y. and R. Jiang (2009). "Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth." Development **136**(8): 1387-1396.

Lipinski, R. J., C. Song, K. K. Sulik, J. L. Everson, J. J. Gipp, D. Yan, W. Bushman and I. J. Rowland (2010). "Cleft lip and palate results from Hedgehog signaling antagonism in the mouse: Phenotypic characterization and clinical implications." Birth Defects Res A Clin Mol Teratol **88**(4): 232-240.

Liu, B., S. M. Rooker and J. A. Helms (2010). "Molecular control of facial morphology."Semin Cell Dev Biol **21**(3): 309-313.

Rice, R., E. Connor and D. P. C. Rice (2006). "Expression patterns of Hedgehog signalling pathway members during mouse palate development." Gene Expression Patterns **6**(2): 206-212.

Som, P. M. and T. P. Naidich (2013). "Illustrated review of the embryology and development of the facial region, part 1: Early face and lateral nasal cavities." AJNR Am J Neuroradiol **34**(12): 2233-2240.

Som, P. M. and T. P. Naidich (2014). "Illustrated review of the embryology and development of the facial region, part 2: Late development of the fetal face and changes in the face from the newborn to adulthood." AJNR Am J Neuroradiol **35**(1): 10-18.

Warbrick, J. G. (1960). "The early development of the nasal cavity and upper lip in the human embryo."J Anat **94**(Pt 3): 351-362.

Relationship: 2882: Decrease, GLI1/2 target gene expression leads to Apoptosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

The relationship between a decrease in cellular proliferation and a decrease in outgrowth has been demonstrated in both mouse and chick models. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question.

Key Event Relationship Description

The GLI transcription factors are the main transcription factors of the Sonic Hedgehog (SHH) pathway. Sonic Hedgehog is a major developmental pathway involved in embryonic development. Disruption of SHH during critical windows of development can cause birth defects (ex. Orofacial clefting (OFCs)). OFCs caused by disruption to SHH are believed to be due to a decrease in cellular proliferation and an increase in apoptosis leading to a decrease in tissue outgrowth and the failure of the facial processes to meet and fuse (Lipinski, Song et al. 2010, Heyne, Melberg et al. 2015). This increase is apoptosis is believed to be due to a decrease in GLI1/2 target gene expression.

Evidence Supporting this KER

Biological Plausibility

There is a high biological probability that disruption of GLI1/2 target gene expression leads to an increase in apoptosis.

Empirical Evidence

- In vitro
  - None found
- In vivo
  - Decreased GLI1/2 expression found using in situ hybridization was found on E9.5 embryos of all-trans RA (E 8.5 25mg/kg oral gavage) exposed pregnant dams. An increase in apoptosis of CNCC was also found in the E9.5 embryos. A rescue experiment with SAG (SMO agonist) dosed in combination with RA reduced the incidence of CP and CNCC apoptosis (Wang, Kurosaka et al. 2019).
  - Chick embryos exposed to 200µl of 10% ethanol with an additional 20µl of 1% ethanol at stage 9-10 display saw decreased GLI and SHH expression in the head. These embryos also display a reduction in the growth of the frontonasal prominence, hypoplastic branchial arches, and increased apoptosis in cranial neural crest cells. Treatment with antibodies that block SHH signalling had the same impact (Ahlgren, Thakur et al. 2002).

Uncertainties and Inconsistencies

The relationship between GLI1/2 target gene expression and increased apoptosis has a high biological plausibility although there is currently lack of studies that address this relationship.

Quantitative Understanding of the Linkage

The quantitative understanding of this relationship is low. No studies were found to exist to address dose response or time-scale data. Further work is needed to address these questions and create a better understanding of this relationship.

Response-response relationship

Further work is needed to increase the understanding of this relationship and its' response-response relationship.

Time-scale

Further work is needed to increase the understanding of this relationship and its' time scale.

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Further work is needed to increase the understanding of this relationship and its' modulating factors.			

Known Feedforward/Feedback loops influencing this KER

Further work is needed to increase the understanding of this relationship and shed light on what other feedback/forward loops are at play.

References

Ahlgren, S. C., V. Thakur and M. Bronner-Fraser (2002). "Sonic hedgehog rescues cranial neural crest from cell death induced by ethanol exposure." Proc Natl Acad Sci U S A **99**(16): 10476-10481.

Cohen, M., A. Kicheva, A. Ribeiro, R. Blassberg, K. M. Page, C. P. Barnes and J. Briscoe (2015). "Ptch1 and Gli regulate Shh signalling dynamics via multiple mechanisms." Nature Communications **6**(1): 6709.

Ferguson, M. W. (1988). "Palate development." Development **103 Suppl**: 41-60.

Heyne, G. W., C. G. Melberg, P. Doroodchi, K. F. Parins, H. W. Kietzman, J. L. Everson, L. J. Ansen-Wilson and R. J. Lipinski (2015). "Definition of critical periods for Hedgehog pathway antagonist-induced holoprosencephaly, cleft lip, and cleft palate." PLoS One **10**(3): e0120517.

Kim, C. H., H. W. Park, K. Kim and J. H. Yoon (2004). "Early development of the nose in human embryos: a stereomicroscopic and histologic analysis." Laryngoscope **114**(10): 1791-1800.

Lipinski, R. J., C. Song, K. K. Sulik, J. L. Everson, J. J. Gipp, D. Yan, W. Bushman and I. J. Rowland (2010). "Cleft lip and palate results from Hedgehog signaling antagonism in the mouse: Phenotypic characterization and clinical implications." Birth Defects Res A Clin Mol Teratol **88**(4): 232-240.

Liu, B., S. M. Rooker and J. A. Helms (2010). "Molecular control of facial morphology."Semin Cell Dev Biol **21**(3): 309-313.

Rice, R., E. Connor and D. P. C. Rice (2006). "Expression patterns of Hedgehog signalling pathway members during mouse palate development." Gene Expression Patterns **6**(2): 206-212.

Som, P. M. and T. P. Naidich (2013). "Illustrated review of the embryology and development of the facial region, part 1: Early face and lateral nasal cavities." AJNR Am J Neuroradiol **34**(12): 2233-2240.

Som, P. M. and T. P. Naidich (2014). "Illustrated review of the embryology and development of the facial region, part 2: Late development of the fetal face and changes in the face from the newborn to adulthood." AJNR Am J Neuroradiol **35**(1): 10-18.

Wang, Q., H. Kurosaka, M. Kikuchi, A. Nakaya, P. A. Trainor and T. Yamashiro (2019). "Perturbed development of cranial neural crest cells in association with reduced sonic hedgehog signaling underlies the pathogenesis of retinoic-acid-induced cleft palate." Dis Model Mech **12**(10).

Warbrick, J. G. (1960). "The early development of the nasal cavity and upper lip in the human embryo."J Anat **94**(Pt 3): 351-362.

List of Non Adjacent Key Event Relationships

Relationship: 2894: Antagonism Smoothened leads to orofacial cleft

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	non-adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

The nonadjacent relationship between antagonism of SMO and orofacial clefting (OFCs) has been shown repeatedly in mice models as detailed in the empirical evidence section. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question. The SHH pathway is well understood to be fundamental to proper embryonic development and that aberrant SHH signaling during embryonic development can cause birth defects including orofacial clefts (OFCs). For this reason, this KER is applicable to the embryonic stage with a high level of confidence.

Key Event Relationship Description

The Smoothened (SMO) receptor is Class F G protein coupled receptor involved in signal transduction of the Sonic Hedgehog (SHH) pathway. It includes distinct functional groups including ligand binding pockets, cysteine rich domain (CRD), transmembrane helix (TM), extracellular loop (ECL), intracellular loop (ICL), and a carboxyl-terminal tail (C-term tail) (Arensdorf, Marada et al. 2016). SMO signaling is dependent upon its relocation to a subcellular location. This relocation occurs in the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005). Relocation of SMO to the PC typically occurs within ~20 minutes of agonist stimulation (Arensdorf, Marada et al. 2016).

In the absence of SHH ligand, the Patched (PTCH) receptor suppresses the activation of SMO. When HH ligand binds to PTCH, suppression on SMO is released and SMO can relocate, accumulate, and signal to intracellular effectors (Denef, Neubüser et al. 2000, Rohatgi and Scott 2007). It has been shown that SMO localization to the tip of the primary cilia is essential for the SHH signaling cascade in vertebrates (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Rohatgi, Milenkovic et al. 2009). This relocation then leads to signaling to effectors resulting in the activation of the GLI transcription factors and the subsequent induction of HH target gene expression (Alexandre, Jacinto et al. 1996, Von Ohlen and Hooper 1997). Antagonism of SMO disrupts the downstream signaling cascade of SHH and if disrupted during critical periods of development can lead birth defects including OFCs.

Evidence Supporting this KER

Biological Plausibility

There is high biological plausibility of this relationship.The SHH pathway is well understood to be fundamental to proper embryonic development and that aberrant SHH signaling during embryonic development can cause birth defects including orofacial clefts (OFCs).

Empirical Evidence

- *in vitro*- It should be noted that OFC cannot be evaluated *in vitro*. The evidence presented below is intended to further support the *in vivo* evidence and offers support of which stressors might cause an OFC and their possible mechanism.
  - A small molecule screen of 10,000 compounds identified six inhibitors of SHH signaling, four of which bind directly to SMO (SANT1-4). Screening was conducted using NIH 3T3 SHH LightII cells cultured in media conditioned from HEK 293 transfected to stably express Shh-N. Cells were dosed with the compound library at 0.714ug/ml and SHH activity was quantified at 30h using Renilla luciferase activity. A fluorescent binding assay using BODIPY-cyclopamine was used to verify binding to SMO for the SANT compounds. Dose response reported as IC50 for the inhibition of SHH signaling was conducting in NIH 3T3 SHH light2, NIH 3T3 SmoA1-Light2, P2 Ptch1-/- (mouse embryonic fibroblasts) (Chen, Taipale et al. 2002).

Compound/Cell	SHH-Light2 (nM)	SmoA1-Light2 (nM)	Ptch1-/- (nM)
SANT-1	20	30	20
SANT-2	30	70	50
SANT-3	100	80	80
SANT-4	200	300	300

  - Direct binding of cyclopamine to SMO was verified using a photoaffinity form of cyclopamine (PA-cyclopamine). PA-cyclopamine had previously been shown to inhibit SHH signaling in NIH 3T3 Shh-LightII cells with similar IC50 values to cyclopamine (300nm and 150nm respectively) (Taipale, Chen et al. 2000). Binding to SMO was verified using a COS-1 (fibroblast, monkey) line transfected to over express SMO. The location of cyclopamine binding was further investigated using BODIPY- cyclopamine and COS-1 cells modified to lack either a N-terminal, extracellular cysteine-rich domain, or the cytoplasmic C terminal of SMO. The findings support that cyclopamine does not require these domains and instead binds directly to

the heptahelical domain (Chen, Taipale et al. 2002).

- In vivo
  - The presence of critical periods for disruption of SHH was investigated using C57BL/6J mice. Vismodegib was suspended at 3mg/ml in 0.5% methyl cellulose and 0.2% tween. Pregnant dams were administered 40mg/kg vismodegib at GD7.0, 7.25, 7.5, 7.75, 8.0, 8.25,8.5, 8.625, 8.75, 8.875, 9.0, 9.25, 9.5, 9.75, and 10.0. Cyclopamine was dosed at 120mg/kg/d via subcutaneous infusion between GD8.25-9.375. Pregnant dams were euthanized at GD17 and fetal specimens were collected and fixed for imaging. The control group consisted of fetuses exposed to 0.5% methyl cellulose and 0.2% tween at GD7.75, 8.875, or 9.5. Acute exposure to vismodegib resulted in a peak incidence of lateral cleft lip and palate at GD8.875 (13%). Exposure at GD9.0 and 10.0 resulted in clefts of the secondary palate only (34%). A higher penetrance (81%) was found for cyclopamine exposure (Heyne, Melberg et al. 2015).
  - Timed pregnant C57B1/6J mice were treated with cyclopamine from GD 8.25-9.5 by subcutaneous infusion (160mg/kg/d) or at GD 8.5 with AZ75 (potent cyclopamine analog) via oral gavage (40 or 80mg/kg). Exposure to cyclopamine resulted in lateral cleft lip and cleft palate defects attributed to a deficiency of midline and lower medial nasal prominence tissue. Both drugs infrequently resulted in an intermediate phenotype of median CLP. Cyclopamine caused gross facial malformations in 5/14 litters with an intra-litter penetrance of clefting of 50%. AZ75 dosed at 80mg/kg caused all embryos to resorb. At 40mg/kg AZ75 caused gross facial malformations in 6/7 litters (Lipinski, Song et al. 2010).
  - Timed pregnant C57B1/6J mice were administered cyclopamine via micro osmotic pumps (120mg/kg/d) surgically implanted at GD 8.25. Dams were euthanized on GD 17. 25/45 of the cyclopamine exposed fetuses presented with a cleft compared to 0/39 for the control group (Lipinski, Holloway et al. 2014).
  - Pregnant Sprague Dawley rats were dosed with 240mg/kg of cyclopamine (oral gavage once daily) from GD 6.0-9.0. Craniofacial malformations were noted including cebocephaly, microphthalmia, hydrocephaly, exencephaly, and anencephaly. Parallel experimentation in golden hamsters found that 170mg/kg of cyclopamine was sufficient to cause malformations including cleft lip and palate (Keeler 1975).
  - C57BL/6J and A/J mice were dosed with single doses of jervine (70, 150,300mg/kg gavage) on either GD 8, 9, 10. A dose response pattern of CLP was seen for both strains with dosing on GD 8. A dose response pattern for CP was found for C57BL/6J for treatment on GD 9 or 10 but not at GD 8(Omnell, Sim et al. 1990).

Quantitative Understanding of the Linkage

Response-response relationship

Further work is needed to address these questions and create a better understanding of this relationship.

Time-scale

Relocation of SMO to the PC typically occurs within ~20 minutes of agonist stimulation(Arensdorf, Marada et al. 2016). No data was found on how fast antagonism of SMO will stop its' relocation to the primary cilia. Further work is needed to increase the understanding of this relationship and its' time scale

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Further work is needed to increase the understanding of this relationship and its' modulating factors.			

Known Feedforward/Feedback loops influencing this KER

Further work is needed to increase the understanding of this relationship and shed light on what other feedback/forward loops are at play.

References

Alexandre, C., A. Jacinto and P. W. Ingham (1996). "Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins." Genes Dev **10**(16): 2003-2013.

Arensdorf, A. M., S. Marada and S. K. Ogden (2016). "Smoothened Regulation: A Tale of Two Signals."Trends Pharmacol Sci **37**(1): 62-72.

Byrne, E. F. X., G. Luchetti, R. Rohatgi and C. Siebold (2018). "Multiple ligand binding sites regulate the Hedgehog signal transducer Smoothened in vertebrates." Current Opinion in Cell Biology **51**: 81-88.

Byrne, E. F. X., R. Sircar, P. S. Miller, G. Hedger, G. Luchetti, S. Nachtergaele, M. D. Tully, L. Mydock-McGrane, D. F. Covey, R. P. Rambo, M. S. P. Sansom, S. Newstead, R. Rohatgi and C. Siebold (2016). "Structural basis of Smoothened regulation by its extracellular domains." Nature **535**(7613): 517-522.

Chen, J. K., J. Taipale, M. K. Cooper and P. A. Beachy (2002). "Inhibition of Hedgehog signaling by direct binding of cyclopamine

to Smoothened." Genes Dev **16**(21): 2743-2748.

Chen, J. K., J. Taipale, K. E. Young, T. Maiti and P. A. Beachy (2002). "Small molecule modulation of Smoothened activity." Proc Natl Acad Sci U S A **99**(22): 14071-14076.

Corbit, K. C., P. Aanstad, V. Singla, A. R. Norman, D. Y. R. Stainier and J. F. Reiter (2005). "Vertebrate Smoothened functions at the primary cilium." Nature **437**(7061): 1018-1021.

Denef, N., D. Neubüser, L. Perez and S. M. Cohen (2000). "Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened." Cell **102**(4): 521-531.

Dwyer, J. R., N. Sever, M. Carlson, S. F. Nelson, P. A. Beachy and F. Parhami (2007). "Oxysterols are novel activators of the hedgehog signaling pathway in pluripotent mesenchymal cells." J Biol Chem **282**(12): 8959-8968.

Heyne, G. W., C. G. Melberg, P. Doroodchi, K. F. Parins, H. W. Kietzman, J. L. Everson, L. J. Ansen-Wilson and R. J. Lipinski (2015). "Definition of critical periods for Hedgehog pathway antagonist-induced holoprosencephaly, cleft lip, and cleft palate." PLoS One **10**(3): e0120517.

Huang, P., D. Nedelcu, M. Watanabe, C. Jao, Y. Kim, J. Liu and A. Salic (2016). "Cellular Cholesterol Directly Activates Smoothened in Hedgehog Signaling." Cell **166**(5): 1176-1187.e1114.

Huang, P., S. Zheng, B. M. Wierbowski, Y. Kim, D. Nedelcu, L. Aravena, J. Liu, A. C. Kruse and A. Salic (2018). "Structural Basis of Smoothened Activation in Hedgehog Signaling." Cell **174**(2): 312-324.e316.

Huangfu, D. and K. V. Anderson (2005). "Cilia and Hedgehog responsiveness in the mouse." Proc Natl Acad Sci U S A **102**(32): 11325-11330.

Keeler, R. F. (1975). "Teratogenic effects of cyclopamine and jervine in rats, mice and hamsters." Proc Soc Exp Biol Med **149**(1): 302-306.

Kobilka, B. K. (2007). "G protein coupled receptor structure and activation." Biochimica et Biophysica Acta (BBA) - Biomembranes **1768**(4): 794-807.

Lipinski, R. J., H. T. Holloway, S. K. O'Leary-Moore, J. J. Ament, S. J. Pecevich, G. P. Cofer, F. Budin, J. L. Everson, G. A. Johnson and K. K. Sulik (2014). "Characterization of subtle brain abnormalities in a mouse model of Hedgehog pathway antagonist-induced cleft lip and palate." PLoS One **9**(7): e102603.

Lipinski, R. J., C. Song, K. K. Sulik, J. L. Everson, J. J. Gipp, D. Yan, W. Bushman and I. J. Rowland (2010). "Cleft lip and palate results from Hedgehog signaling antagonism in the mouse: Phenotypic characterization and clinical implications." Birth Defects Res A Clin Mol Teratol **88**(4): 232-240.

Millington, G., K. H. Elliott, Y. T. Chang, C. F. Chang, A. Dlugosz and S. A. Brugmann (2017). "Cilia-dependent GLI processing in neural crest cells is required for tongue development." Dev Biol **424**(2): 124-137.

Myers, Benjamin R., N. Sever, Yong C. Chong, J. Kim, Jitendra D. Belani, S. Rychnovsky, J. F. Bazan and Philip A. Beachy (2013). "Hedgehog Pathway Modulation by Multiple Lipid Binding Sites on the Smoothened Effector of Signal Response." Developmental Cell **26**(4): 346-357.

Nachtergaele, S., L. K. Mydock, K. Krishnan, J. Rammohan, P. H. Schlesinger, D. F. Covey and R. Rohatgi (2012). "Oxysterols are allosteric activators of the oncoprotein Smoothened." Nat Chem Biol **8**(2): 211-220.

Nachtergaele, S., L. K. Mydock, K. Krishnan, J. Rammohan, P. H. Schlesinger, D. F. Covey and R. Rohatgi (2012). "Oxysterols are allosteric activators of the oncoprotein Smoothened." Nature Chemical Biology **8**(2): 211-220.

Omnell, M. L., F. R. Sim, R. F. Keeler, L. C. Harne and K. S. Brown (1990). "Expression of Veratrum alkaloid teratogenicity in the mouse." Teratology **42**(2): 105-119.

Qi, X., H. Liu, B. Thompson, J. McDonald, C. Zhang and X. Li (2019). "Cryo-EM structure of oxysterol-bound human Smoothened coupled to a heterotrimeric Gi." Nature **571**(7764): 279-283.

Rana, R., C. E. Carroll, H.-J. Lee, J. Bao, S. Marada, C. R. R. Grace, C. D. Guibao, S. K. Ogden and J. J. Zheng (2013). "Structural insights into the role of the Smoothened cysteine-rich domain in Hedgehog signalling." Nature Communications **4**(1): 2965.

Rohatgi, R., L. Milenkovic, R. B. Corcoran and M. P. Scott (2009). "Hedgehog signal transduction by Smoothened: pharmacologic evidence for a 2-step activation process." Proc Natl Acad Sci U S A **106**(9): 3196-3201.

Rohatgi, R., L. Milenkovic and M. P. Scott (2007). "Patched1 regulates hedgehog signaling at the primary cilium." Science **317**(5836): 372-376.

Rohatgi, R. and M. P. Scott (2007). "Patching the gaps in Hedgehog signalling." Nat Cell Biol **9**(9): 1005-1009.

Taipale, J., J. K. Chen, M. K. Cooper, B. Wang, R. K. Mann, L. Milenkovic, M. P. Scott and P. A. Beachy (2000). "Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine." Nature **406**(6799): 1005-1009.

Von Ohlen, T. and J. E. Hooper (1997). "Hedgehog signaling regulates transcription through Gli/Ci binding sites in the wingless enhancer." Mech Dev **68**(1-2): 149-156.

Wang, C., H. Wu, V. Katritch, G. W. Han, X. P. Huang, W. Liu, F. Y. Siu, B. L. Roth, V. Cherezov and R. C. Stevens (2013). "Structure of the human smoothened receptor bound to an antitumour agent." Nature **497**(7449): 338-343.