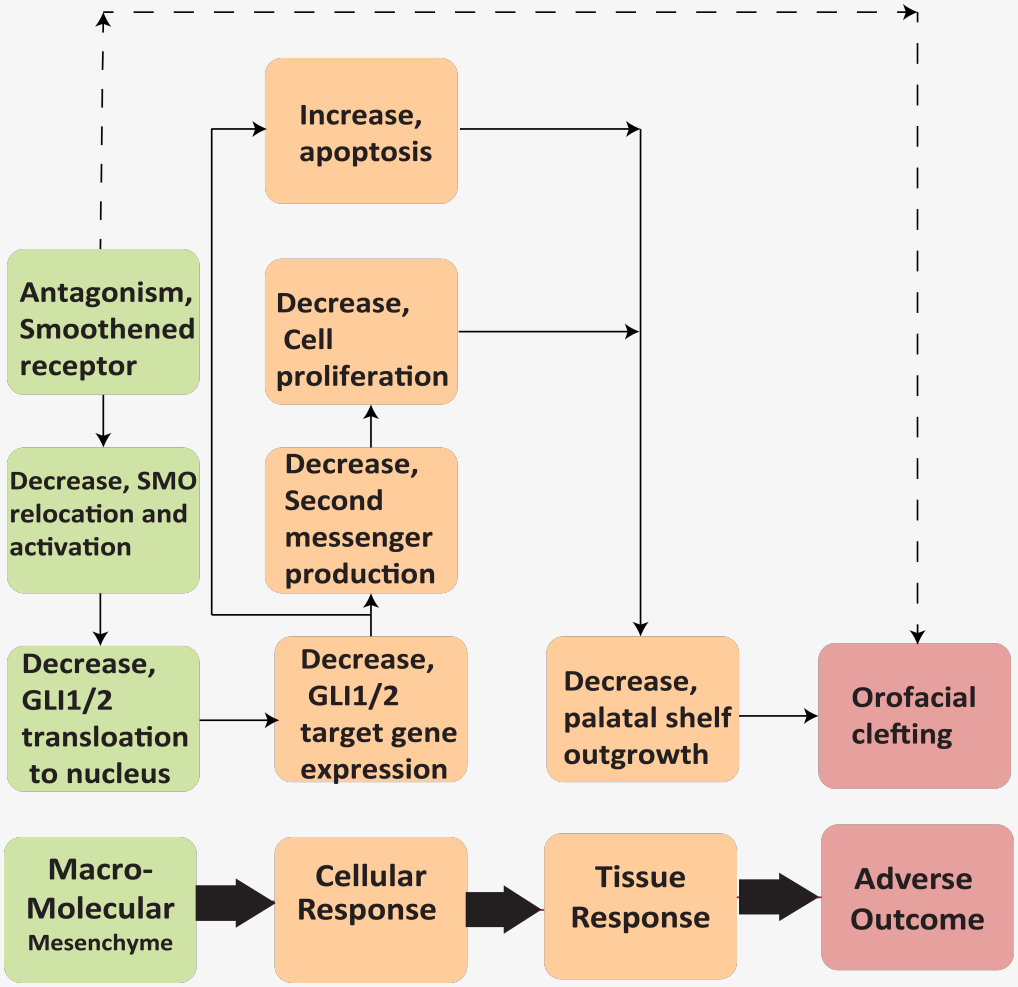
AOP ID and Title:

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 Do not cite	Under Review	1.101	Included in OECD Work Plan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.

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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).

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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).

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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).

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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).

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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
Aop:595 - Nanoplastic effect	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)

- *Apoptosis applicable to fishes, hence be used to study as models (dos Santos, N. M., et al. (2008).*
- *Apoptosis in humans and baboon ovaries (Kugu, K., et al. (1998)*
- *Apoptosis in amphibians during metamorphosis (Ishizuya-Oka, A., et al. (2010).*
- *Apoptosis in Drosophila melanogaster (Steller, H. (2008)*
- *Apoptosis is a highly conserved and essential process across a broad taxonomic range, from unicellular eukaryotes to complex multicellular animals, it is also evident in metazoans (Suraweera, C. D., et al. (2022).*

- *Sex Applicability:*
  Both sexes. Apoptosis occurs in male and female systems (e.g., oocyte and sperm cell turnover).

- *Life Stage Applicability:*
  All stages. Especially critical during embryonic development and in maintaining adult tissue homeostasis.

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.

In mammals, the foetal ovary produces hundreds of thousands of oocytes. But most of them die before birth due to apoptosis (Kaur, S., & Kurokawa, M., 2023). The apoptotic process has a specific pattern at different stages: in foetal ovaries, the majority of apoptotic activity was found in germ cells, whereas in adult quiescent cortical follicles, apoptosis occurred from both granulosa and oocyte cells. The oocyte has been shown to be the one that triggers the apoptotic process and causes follicular atresia (Jin, X., et al. (2011). In humans, the primordial follicles' ovarian endowment is formed throughout foetal development. Apoptotic cell death, which is carried out with the assistance of multiple players and routes conserved from worms to humans, depletes this endowment by at least two-thirds prior to birth. As of right now, apoptosis has been linked to atresia, oocyte loss/selection, folliculogenesis, and oogenesis (Hussein MR, 2005)

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]

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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 ID and Name	Event Type
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

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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: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:333 - Excessive reactive oxygen species leading to growth inhibition via lipid peroxidation and reduced cell proliferation	KeyEvent
Aop:591 - DBDPE-induced DNA damage increase in liver leading to Non-alcoholic fatty liver disease via liver steatosis and inhibition of regeneration	KeyEvent
Aop:326 - Excessive reactive oxygen species leading to growth inhibition via uncoupling of oxidative phosphorylation and reduced cell proliferation	KeyEvent
Aop:598 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and reduced cell proliferation	KeyEvent
Aop:602 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	KeyEvent
Aop:603 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell cycle disruption	KeyEvent
Aop:601 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and reduced cell proliferation	KeyEvent

Stressors

Name

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
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.

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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: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

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 6th and 12th 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).

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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 ID and Name	Event Type
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.

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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

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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.

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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)

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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
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

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.

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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.

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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.

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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.

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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 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 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

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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)
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.

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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
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.

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