AOP: 491 – Annex 1, assessment of the relative level of confidence in the overall AOP based on rank ordered weight of evidence elements.

Defining Question	High (Strong)	Moderate	Low (Weak)
1. Support for Biological Plausibility of KERS			
a) Is there a mechanistic relationship between KE_{up} and KE_{down} consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance.	KER is plausible based on analogy to accepted biological relationships, but scientific understanding is incomplete	Empirical support for association between KEs , but the structural or functional relationship between them is not understood.
Relationship 2731: Decrease GLI1/2 target gene expression (Event 2040) leads to Decrease, SHH second messenger production (Event 2043)		**WEAK** While it is understood that there is extensive crosstalk between SHH and other pathways during development there is an incomplete understanding of these interactions and their feedback and feed forward loops.	
Relationship 2732: Decrease SHH second messenger production (Event 2043) leads to Decrease, cell proliferation (Event 1821)		**STRONG** SHH is a known mitogen and known to regulate cellular proliferation.	
Relationship 2724: Decrease, Cell proliferation (Event 1821) leads to Decrease, outgrowth (Event 2041)		**MODERATE** The SHH pathway is well known to be associated with cellular proliferation and growth of the facial prominences.	
Relationship 2726: Decrease, outgrowth (Event 2041) leads to OFC (Event 2042)		**STRONG** OFCs caused by disruption to SHH are believed to be due to a reduction in epithelial induced proliferation and the subsequent decrease in tissue outgrowth and the failure of the facial processes to meet and fuse (Lipinski, Song et al. 2010, Heyne, Melberg et al. 2015).	
Relationship 2792: Apoptosis (Event 1262) leads to Decrease, outgrowth (Event 2041)		**WEAK** The SHH pathway is known to be associated with cell survival and that disruption of SHH signaling can lead to increased apoptosis. The understanding of this relationship is weak and further work is warranted to increase understanding.	
Relationship 2882: Decrease, GLI1/2 target gene expression (Event 2040) leads to Apoptosis (Event 1262)		**WEAK** The SHH pathway is well known to be associated with cellular proliferation and cell survival. Further investigation into how GLI1/2 gene expression regulates cellular survival is needed	
Defining Question	**High (Strong)**	**Moderate**	**Low (Weak)**
1. Support for Essentiality of KEs			
Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.
Essentiality of the KEs was assessed for the AOP as a whole – rationale for the individual KE calls is provided.	To date, few studies have addressed the essentiality of the proposed sequence of key events. Evidence linking SHH disruption through a decrease in proliferation exists. The hypothesized sequence of events has a high temporal concordance for canonical SHH signaling pathway and orofacial development. • Studies have shown that SHH signaling is required for normal facial development and plays a critical role in the growth of the facial processes that form the upper palate and lip (Bush and Jiang 2012, Kurosaka 2015). • The epithelial derived SHH drives orofacial development through 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).		
Defining Question	**High (Strong)**	**Moderate**	**Low (Weak)**
3. Empirical Support for KERs			
Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.

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

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