Supplementary Table S1

Essentiality evaluations for AOPs 305, 306, 307.

Event	Evidence	Uncertainties, inconsistencies, and contradictory evidence
	Upstream event in AOP 306	
MIE-26 Antagonism, AR **HIGH:** This MIE is usually measured in vitro whereas the downstream events in the AOP are in most cases measured in vivo. Canonical knowledge of normal male reproductive development provides strong support for essentiality along with AR knockout models.	**Biological plausibility provides strong support for the essentiality of this event, as androgens, acting through AR, are essential for normal male perineal development.** **Indirect evidence of impact of AR antagonism (MIE-26) in vitro on AR activity in vitro:** • Several chemical substances, including flutamide and vinclozolin, are known AR antagonists and have been shown to decrease AR activity in vitro (Sonneveld 2005; Pedersen 2022). **Direct evidence of impact of AR antagonism (MIE-26) in vivo on decreased AGD (AO-1688):** • KO of AR in mice leads to reduced AGD and rat in vivo exposure to vinclozolin, procymidone and flutamide, which are known AR antagonists, leads to decreased AGD in male offspring (see KE-1614).	
	Upstream events in AOP 305	
MIE-1617 Inhibition, 5α-reductase **HIGH:** This MIE is usually measured in vitro whereas the downstream events in the AOPN are usually measured in vivo. Canonical knowledge of normal male reproductive development provides strong support for essentiality along with 5α-reductase knockout models.	**Biological plausibility provides strong support for the essentiality of this event, as DHT, produced by 5α-reductase, is essential for normal male perineal development.** **Indirect evidence of impact of inhibition of 5α-reductase (MIE-1617) in vitro on AR activity in vitro:** • Finasteride, a specific inhibitor of 5α-reductase, can decrease proliferation of prostate cancer cells in vitro, a proxy read-out of AR activity (Bologna et al., 1995). **Direct evidence of impact of inhibition of 5α-reductase (MIE-1617) in vivo on decreased DHT levels (KE-1613):** • Lack of 5α-reductase type 2 activity by e.g. inhibitor or KO decrease DHT levels locally in tissues and blood. This is demonstrated in humans, rats, monkeys, and mice (Robitaille & Langlois, 2020). **Indirect evidence of impact of inhibition of 5α-reductase (MIE-1617) in vivo on decreased DHT levels (KE-1613):**	

	• Men with androgenic alopecia treated with finasteride or dutasteride presented with decreased DHT levels in serum (Drake et al., 1999; Clark et al., 2004). **Direct evidence of impact of inhibition of 5α-reductase (MIE-1617) in vivo on decreased AGD (AO-1688):** • Empirical evidence for 5α-reductase inhibitor finasteride decreasing AGD in male pups after in utero exposure was judged to be strong by literature review (Holmer et al., 2024). • Human case studies show that 5α-reductase deficiency leads to feminized genitalia (Robitaille & Langlois, 2020).	
KE-1613 Decrease, DHT levels **HIGH:** DHT insufficiency in e.g. humans (5α-reductase mutations) lead to feminized male offspring despite presence of testosterone during development and rescue studies specifically demonstrate how DHT is essential for normal male perineal development.	**Biological plausibility provides strong support for the essentiality of this event, as DHT is essential for normal male perineal development.** **Indirect evidence of impact of decreased DHT levels (KE-1613) on AR activity in vivo (KE-1614):** • Androgen deprivation is used as treatment for prostate cancer, including 5α-reductase inhibitors, to reduce DHT levels and cancer growth (Aggarwal et al., 2010). **Indirect evidence of impact of decreased DHT levels (KE-1613) on AR activity in vitro:** • Increasing concentrations of DHT lead to increasing AR activation in vitro in AR reporter gene assays (OECD, 2020; Williams et al., 2017). **Indirect evidence of impact of decreased DHT levels (KE-1613) on AR activity in vitro:** • 5α-reductase 2 deficiency is an autosomal recessive condition in which 46,XY subjects with bilateral testes and normal testosterone production have impaired virilization during fetal life due to diminished DHT (Mendonca et al 2016). **Direct evidence of impact of decreased DHT levels (KE-1613) on decreased AGD (AO-1688)** • Treatment with 5α-reductase inhibitor in utero shortened male rat AGD, but simultaneous treatment with DHT restored AGD length (George & Peterson, 1988).	
Downstream events shared by AOP 305-307		
KE-2298 Decrease, intratesticular testosterone (ITT) levels	**Biological plausibility provides strong support for the essentiality of this event, as the testes are the primary testosterone producing organs and essential for normal male perineal development.**	**Inconsistencies with indirect evidence of impact on the AO:** • Empirical evidence supports dose concordance between intratesticular T and AGD, but with

MODERATE: Testis is the primary organ in males for testosterone synthesis and required for serum testosterone. Several studies show concordance with intratesticular T and serum T, as well as AGD.	**Indirect evidence of impact of decreased ITT (KE-2298) on decreased AGD (AO-1688):** • Numerous rat studies have evidenced a relationship between reduced intratesticular testosterone levels caused by chemical exposure and short AGD in male offspring (see empirical evidence table in KER-3449). • Majority of studies report LOAEL concordance between reduced intratesticular testosterone and short AGD. **Indirect evidence of impact of decreased ITT (KE-2298) on decreased circulating T (KE-1690)** • Castrated males have significantly reduced serum T. Although at different life stage, it is highly likely same relationship exists in fetal males, with loss of testosterone from testis resulting in loss of circulating testosterone and finally decreased AGD.	some inconsistencies regarding dose-concordance and LOAELs in minority of studies on anti-androgenic substances.
KE-1690 Decrease, circulating testosterone (CT) levels **MODERATE:** Although DHT may be the main androgen responsible for virilization of external genitalia and perineal region, T is substrate for DHT also locally and numerous studies have shown strong relationships between reduced serum T and decreased AGD.	**Biological plausibility provides strong support for the essentiality of this event. Although DHT may be the main androgen responsible for virilization of external genitalia and perineal region, testosterone is substrate for DHT production also locally.** **Indirect evidence of impact of decreased CT (KE-1690) on decreased AGD (AO-1688):** • DEHP exposure during prenatal development in rats results in reduced fetal testosterone levels and decreased AGD in male offspring after exposure. Literature review on the relationship has judged the link to be strongly evidenced (Holmer et al., 2024). **Indirect evidence of impact of decreased CT (KE-1690) on AR activity in vitro:** • Increasing concentrations of testosterone lead to increasing AR activation in vitro (US EPA, 2023) (see also KER-2131).	**Inconsistencies with indirect evidence of impact on the AO:** • Some inconsistencies were observed in the empirical evidence regarding decreased AGD in male pups after in utero exposure to DEHP. However, all could be explained by exposure doses, statistical power and species differences (Holmer et al., 2024)
Downstream events shared by AOP 305-307		
KE-1614 Decrease, AR activation **HIGH:** There is direct experimental evidence from KO studies that the AR is essential for	**Biological plausibility provides strong support for the essentiality of this event, as androgens, acting through AR, are essential for normal male perineal development.** **Direct evidence of the impact of decreased AR activation (KE-1614) on altered gene transcription by AR (KE-286):**	**Inconsistencies in indirect evidence of impact on the AO** • Some inconsistencies were observed in the empirical evidence showing a decreased AGD in male pups after exposure in utero to procymidone and vinclozolin. All conflicting results could be explained by exposure doses, species differences and

growth of AGD in male offspring. There is also evidence from exposure studies in animals that substances antagonizing AR induce shorter AGD in male pups.	• Male AR KO mice have altered gene expression pattern in a broad range of organs (refer to KER-2124). **Indirect evidence of the impact of decreased AR activation (KE-1614) on altered gene transcription by AR (KE-286):** • Exposure to known anti-androgenic chemicals induce a changed gene expression pattern, e.g. in neonatal pig ovaries (Knapczyk-Stwora et al. 2019). **Direct evidence of impact of decreased AR activation (KE-1614) on reduced AGD (AO-1688):** • Male AR KO mice present with shorter AGD than wildtypes, indistinguishable from wildtype female littermates (Yeh et al., 2002, Sato et al., 2004) (see also KER 2820). **Indirect evidence of impact of decreased AR activation (KE-1614) on decreased AGD (AO-1688):** • AR antagonist flutamide, procymidone and vinclozolin can decrease AGD in male pups after exposure in utero, with overall literature review judging the link to be strongly evidenced (Holmer et al., 2024).	uncertainties in measurements (Holmer et al., 2024).
KE-286 Altered, transcription of genes by AR **MODERATE:** Strongest support for essentiality comes from biological plausibility. However, exact transcriptional effects and causality remain to be fully characterized.	**Biological plausibility provides strong support for the essentiality of this event. AR is a nuclear receptor and transcription factor regulating transcription of genes, and androgens, acting through AR, are essential for normal male perineal development.** **Indirect evidence of impact of altered gene transcription by AR (KE-286) on decreased AGD (AO-1688):** • Gestational exposure of rats to 5α-reductase inhibitor finasteride decreases AGD in male offspring. Gene transcript profiles in perineum were shown sexually dimorphic, with the profile of finasteride-exposed males being intermediary to the male and female control groups (Schwartz et al 2019b). • Gestational exposure of rats to the AR antagonist triticonazole induced decreased AGD in male offspring and a differentially expressed set of genes in the perineum (Draskau et al 2022).	

AGD = anogenital distance, AO = adverse outcome, AR = androgen receptor, DHT = dihydrotestosterone, KE = key event, KER = key event relationship, KO = knockout, LOAEL = lowest observed adverse effect level, MIE = molecular initiating event, T = testosterone

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