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AMH and *AMHR2* mutations: a spectrum of reproductive phenotypes across vertebrate species

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Abstract

Anti-Müllerian hormone (AMH) is a member of the Transforming Growth Factor-β family of secreted signaling proteins. AMH is expressed in Sertoli cells of the fetal and adult testes and granulosa cells of the postnatal ovary. AMH is required for the regression of the Müllerian ducts in mammalian fetuses during male differentiation. AMH signals through its Type II receptor, AMHR2. *AMHR2* is expressed in mesenchyme adjacent to the Müllerian ducts, and in Sertoli, Leydig, and granulosa cells. Although *AMH* and *AMHR2* genes have been identified in numerous vertebrate species, spontaneous or engineered mutations or variants have been found or created in only a few mammals and teleost fishes. *AMH* or *AMHR2* mutations in mammals lead to the development of Persistent Müllerian Duct Syndrome (PMDS), a recessive condition in which affected males are fully virilized but retain Müllerian duct-derived tissues, including a uterus and oviducts, and in human and dog, undescended testes. *Amh* mutant female mice had accelerated ovarian primordial follicle recruitment, suggesting a role for AMH in regulating germ cells, *amh* and *amhr2* mutations have also been experimentally generated in various teleost fishes. Depending on the fish species, loss of AMH signaling results in infertility, germ cell tumors, or male-to-female sex reversal. Here we compare the spectrum of phenotypes caused by *AMH* and *AMHR2* mutations in a variety of vertebrate species. There are both common and unique phenotypes between species, highlighting the range of biological processes regulated by AMH signaling.

Keywords

Müllerian duct regression; testicular descent; gametogenesis; fertility; sex determination

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Introduction

Pioneering fetal transplant surgery experiments by Alfred Jost first established that a secreted protein from the fetal testis was required for the regression of the nascent female reproductive tract (Müllerian ducts) in males (Jost, 1953). Jost called this I'hormone inhibitrice that later became known as anti-Müllerian hormone (AMH) (Josso, 1973). In mammals, AMH, a Transforming Growth Factor-β (TGF-β) family member glycoprotein hormone, is expressed in Sertoli cells of fetal and adult testis and granulosa cells of postnatal ovary (Cate et al., 1986; Josso, 1973, 1974; Takahashi et al., 1986). AMH binds to Type I receptors shared with the bone morphogenetic protein (BMP) pathway (ACVR1 and BMPR1A) and its sole Type II receptor AMHR2 in the Müllerian duct mesenchyme, activating the AMH signaling pathway and initiating regression of the Müllerian ducts (reviewed in (Mullen and Behringer, 2014)). The absence of functional AMH or *AMHR2* results in a rare recessive disorder known as Persistent Müllerian Duct Syndrome (PMDS) that is characterized by the presence of Müllerian duct-derived tissues including oviducts, uterus and vagina in a fully virilized male (Josso et al., 2005).

While the essential role of AMH in Müllerian duct regression led to its discovery, it is not the only function of AMH, nor what is postulated to be AMH's evolutionarily ancient function. Analysis across vertebrate species, including mammals, birds and fishes, suggest that AMH initially evolved to regulate germ ceil proliferation (Adolfi et al., 2018). The phenotypes resulting from mutations in the *AMH* and *AMHR2* genes across species highlight both shared roles for AMH-signaling and species-specific differences. Here we review *AMH* and *AMHR2* mutant phenotypes in vertebrates, revealing various roles for AMH signaling in reproductive organ development.

Identification of *AMH* and *AMHR2* mutations in humans with Persistent Müllerian Duct Syndrome

AMH and *AMHR2* mutations have been identified in human males with PMDS (Josso et al., 2005). The first described mutation in the *AMH* gene in humans was found in three brothers of Moroccan descent. The brothers, aged 2 months, 5 years and 7 years, had been diagnosed with PMDS. No AMH was detected in their testicular tissue however *AMH* mRNA was present. DNA sequencing detected a homozygous mutation in the *AMH* gene that resulted in a premature stop codon in exon 5 (NM_000479.3: c.1144G>T, p. (Glu382*)). This mutation was expected to produce AMH protein lacking the bioactive C-terminus. Although AMH protein was not detected in the patients, a truncated AMH protein was secreted from cells transfected with a gene construct carrying the same mutation in vitro, suggesting rapid degradation of the mutant protein occurs in vivo (Knebelmann et al., 1991).

Since this first case, 64 additional unique mutations in the *AMH* gene have been found that result in PMDS. Together these include 38 missense mutations, 10 stop mutations, 1 non-stop mutation, 9 deletions, 2 insertions and 5 splicing mutations (Altincik et al., 2017; Picard et al., 2017). Mutations have occurred throughout the *AMH* gene with a slightly higher rate of mutation found in the biologically active C-terminal region. *AMH* mutations are homozygous in approximately 65% of patients and more commonly born of consanguineous

parents (second cousins or closer). Nineteen mutations have occurred in 2 or more families. These recurrent mutations may be the result of founder effects in regional populations including Brazil, the Middle East and Northern Europe (Nishi et al., 2012; Picard et al., 2017).

AMHR2 mutations are suspected in PMDS patients that are positive for active AMH and/or have serum AMH levels within the normal range (Josso et al., 2005). The first described human *AMHR2* gene mutation was found following a screen of 21 AMH-positive PMDS patients (Imbeaud et al., 1995). One patient had a homozygous *AMHR2* gene mutation at the invariant GT dinucleotide of the splicing donor site 5' of intron 2. His parents were both heterozygous for this mutation. His serum AMH levels were normal and bioactive AMH was found in his testicular biopsy. Reverse transcription PCR of his testicular biopsy showed two forms of *AMHR2* mRNA: transcripts lacking exon 2 and transcripts generated by use of a cryptic splice site leading to an amino acid change (Gly78Asp) and the addition of 4 residues (EWQR) encoded at the end of exon 2.

To date, a total of 58 additional unique gene mutations in AMHR2 have been described. Together these include 36 missense, 11 stop, 8 deletions and 4 splicing defects (Picard et al., 2017; Ren et al., 2017). Mutations resulting in PMDS occur in all 11 exons of the *AMHR2* gene and are found as both homozygous and compound heterozygous mutations. Ten *AMHR2* gene mutations occur in multiple families (Picard et al., 2017). The most common, a 27-bp deletion in exon 10 (NM_020547.2:c.1332_1358del, p(Gly445_Glu453del) occurring in 30 patients, is found predominantly in Northern Europeans and presumed to be the result of a founder effect (Imbeaud et al., 1996).

Men with mutations in either the *AMH* or *AMHR2* gene are fully virilized with male secondary sex characteristics. The presence of a uterus in these men is usually detected early in childhood because of cryptorchidism (undescended testes) and/or associated hernias (Josso et al., 1993a). In men with PMDS, the uterus is attached to the pelvis via the broad ligament and the testes are closely joined to the uterus. If the uterus is strongly attached to the pelvis, the testes will remain in an abdominal position. In cases where the uterus is loosely attached the testis descends into the scrotum along with the attached uterus (Miller et al., 2004). During testicular descent in unaffected males the gubernacular cord, tethered to the scrotum, thickens and shortens followed by constriction of the inguinal canal. In cases of human *AMH* or *AMHR2* gene mutations, the gubernacular cord remains thin and elongated and the inguinal canal fails to constrict. This, in addition to the presence of the uterus, may also contribute to the predisposition of cryptorchidism in men with *AMH* or *AMHR2* gene mutations (Hutson et al., 1994; Hutson and Lopez-Marambio, 2017).

A little over half of patients with *AMH* or *AMHR2* gene mutations have bilateral cryptorchidism where both testes fail to descend. Inguinal hernias are present when either one testis (hernia uteri inguinalis) or both testes (transverse testicular ectopia) along with the retained uterus are found in one inguinal sac (Fig. 1A). Approximately 20% of patients with *AMH* or *AMHR2* mutations present with hernia uteri inguinalis. Transverse testicular ectopia occurs in about 25% of patients with *AMH* or *AMHR2* mutations but has not been observed in cases of idiopathic PMDS and is considered indicative of *AMH* or *AMHR2*

gene mutations (Picard et al., 2017). The position of the testes is not specific for *AMH* or *AMHR2* mutations and can vary between siblings and families sharing the same mutation (Abduljabbar et al., 2012; Knebelmann et al., 1991; Nalbantoglu et al., 2015).

The majority of patients with *AMH* or *AMHR2* gene mutations are infertile. However review of past literature has found that approximately 19% of patients have naturally fathered at least one child (Picard et al., 2017). In all cases, they had either transverse testicular ectopia or hernia uteri inguinalis. Infertility in PMDS patients can be overcome with testicular sperm extraction followed by intracytoplasmic sperm injection (Picard et al., 2017).

If cryptorchidism is not surgically corrected there is a higher risk of cancer development in the undescended testis. In a recent review, Picard et al. (2017) estimated that testicular cancer arose in 33% of adult PMDS patients, exceeding the risk associated with isolated cryptorchidism. Previous estimates have suggested risk of testicular cancer is similar in PMDS and isolated cryptorchidism (Bucci et al., 2002; Picard et al., 2017; Shamim, 2007). Although very rare, malignant transformation of the Müllerian-derived tissues has also been observed (Farikullah et al., 2012).

Thus far, female relatives carrying homozygous mutations of *AMH* or *AMHR2* have no abnormal phenotypes and are fertile (Picard et al., 2017). However, heterozygous variants of the *AMH* and *AMHR2* gene with reduced AMH signaling activity in vitro have been found in women with premature menopause (Alvaro Mercadal et al., 2015; Li et al., 2016). Thus, it is possible that women with homozygous mutations of *AMH* or *AMHR2* may later undergo premature menopause but at present it is not known.

Generation of *Amh* and *Amhr2* mutant mouse models

The development of gene targeting and embryonic stem (ES) cell technologies led to the generation of *Amh* and *Amhr2* mutant mouse models (Arango et al., 2008; Arango et al., 1999; Behringer et al., 1994; Jamin et al., 2002; Mishina et al., 1996). These *Amh* and *Amhr2* alleles are listed in Table 1. These mouse models recapitulate many aspects of human PMDS but there were differences and new insights into the biological roles of the AMH signaling pathway.

The first targeted mutation in the AMH pathway was achieved in the *Amh* locus (Behringer et al., 1994). This initial *Amh* knockout allele had a drug resistance gene expression cassette introduced into the *Amh* gene that simultaneously deleted ~0.6 kb, including a portion of exon 1, all of intron 1, and all of exon 2, encoding part of the N-terminal domain of AMH (Fig. 2, Table 1). This mutation resulted in a complete loss-of-function allele. Both male and female mice heterozygous for the *Amh* mutation were phenotypically normal and fertile. In addition, females homozygous for the mutation had morphologically normal uteri and ovaries and were fertile. Subsequent studies showed that *Amh* heterozygous and homozygous mutant females, despite morphologically normal ovaries, had alterations in primordial follicle recruitment in the ovary (Durlinger et al., 1999). 25-day old and 4-month old homozygous mutant females had more preantral and small antral follicles in their ovaries

compared to wild-type females. At 13 months of age, the *Amh* homozygous mutant females had very few primordial follicles, i.e. an early depletion of primordial follicles compared to wild type.

Morphological abnormalities were found in males homozygous for the *Amh* mutation. These males had developed Müllerian duct derivatives, including a uterus, oviducts, and vaginal tissue, in addition to fully descended, normal sized testes and a properly differentiated Wolffian duct system (seminal vesicles, vasa deferentia, and epididymides) identical to the defects observed in *Amhr2* mutants shown in Fig. 1B, C. In addition, the homozygous mutant males had fertility defects with only 13% of them able to sire litters. The mutant males were able to produce vaginal plugs in females. Spermatogenesis appeared normal because spermatozoa isolated from the epididymides were able to fertilize wild-type oocytes in vitro.

As predicted from human studies, the loss of *Amh* in male mice led to the formation of Müllerian duct derivatives, including the oviduct, uterus, and vaginal tissue. The normal descent of the testes in the mutant mice was in contrast to human PMDS that present with undescended testes or inguinal hernia (Josso et al., 1993b). This may be due in part to the bicornuate uterus of the mouse compared to the simplex uterus of human. The fertility defects observed in the *Amh* mutant male mice were thought to be caused by the mechanical repercussions of having a female reproductive tract superimposed onto a male reproductive tract.

A series of mutant *Amh* alleles were subsequently generated to investigate the requirement of cis-regulatory sequences located 5’ of the start of transcription (Table 1) (Arango et al., 1999; Shen et al., 1994). In vitro studies had indicated that the nuclear hormone orphan receptor, steroidogenic factor 1 (SF1, also known as NR5A1), bound a 20-bp motif within 100-bp upstream of the TATAA sequence that was essential for *Amh* transcription (Shen et al., 1994). Approximately 50-bp upstream of the SF1 binding site is a conserved high mobility group protein binding site that was shown to bind the SOX9 transcription factor to activate *Amh* transcription (De Santa Barbara et al., 1998). Point mutations were introduced into ES cells in the SF1, SOX9, or both binding sites to abolish binding. The SF1 binding site mutation was identical to the one used in the previous in vitro studies (Shen et al., 1994). These alleles were called *R1* (SF1 binding site mutation), *R2* (SOX9 binding site mutation), and *R3* (SF1 and SOX9 binding site mutations) (Arango et al., 1999). In contrast to the in vitro studies, *Amh R1/R1* males expressed *Amh* transcripts in fetal and post-natal testes but had 3-fold lower levels compared to wild type. Thus, the SF1 binding site mutation reduces but does not block *Amh* transcription and is considered a hypomorphic allele. Surprisingly, Müllerian duct regression occurred normally in *Amh R1/R1* males. *Amh R2/R2* and *Amh R3/R3* mutant males had a complete block in Müllerian duct regression, essentially a phenocopy of the *Amh*-null male phenotype. These results suggest that SF1 contributes to *Amh* transcription and that SOX9 is essential for *Amh* transcription.

The initial gene targeting manipulations in ES cells to generate the *Amh R1* allele required the inclusion of a neomycin drug resistance expression cassette that was placed in intron 1 in reverse orientation relative to the direction of *Amh* transcription (Arango et al., 1999). The

allele called *R1-neo* was effectively null because *Amh R1-neo/R1-neo* males had a block in Müllerian duct regression. The *Amh R1* hypomorphic allele was combined with the *Amh R1-neo* null allele to generate males with even more reduced levels of *Amh* transcripts compared to *Amh R1/R1* males. *Amh R1/R1-neo* males developed some uterine tissue, indicating that Müllerian duct regression was incomplete. Thus, AMH levels must be severely reduced to observe partial Müllerian duct regression. Biologically, it appears that the levels of *Amh* transcription lead to more than sufficient AMH to regress the Müllerian system. Perhaps this is to ensure elimination of the Müllerian duct derivatives during male differentiation to prevent fertility defects.

The second targeted mutation in the AMH pathway was in the *Amhr2* gene (Mishina et al., 1996). The mutation was designed to delete ~4.4 kb of the *Amhr2* locus, including exons 1-6, replacing them with a drug resistance gene expression cassette (Fig. 2, Table 1). *Amhr2* heterozygous mutant ES cells from two clones were injected into blastocysts to generate male chimeras that were bred with controls to produce progeny carrying the targeted allele. All mice that were heterozygous for the mutation were phenotypically normal and fertile. Homozygous mutant females were phenotypically normal and fertile. The homozygous mutant males all had normal sized, descended testes and normally differentiated Wolffian duct derivatives. They all developed Mullerian duct derivatives, including a uterus, oviducts, and partial vagina that were superimposed onto the male reproductive system (Fig. 1B, C). This phenotype was identical to that of *Amh*-null mice. Additionally, *Amh* mRNA was expressed in both *Amhr2* heterozygous and homozygous mutant males at the stage for the onset of Mullerian duct regression. The homozygous mutant males were able to sire offspring but at less than a 50% rate, with decreased litter size relative to controls. Similar to *Amh* homozygous mutant males, *Amhr2* homozygous mutant males initially had normal spermatogenesis. However, by two months of age they displayed focal atrophy of the germinal epithelium in seminiferous tubules, which led to significantly reduced spermatogenesis by 9 months of age (Mishina et al., 1996).

Two additional *Amhr2* alleles have been generated by gene targeting in mouse ES cells (Fig. 2). Both are so-called “knock-in” alleles to express an introduced gene in a pattern similar to *Amhr2* transcription. The bacterial *lacZ* gene was introduced into the *Amhr2* locus to visualize *Amhr2* expression using a simple histochemical stain and also to follow Müllerian duct regression (Arango et al., 2008; Arango et al., 1999). An intraribosomal entry site (IRES)-*lacZ-pA* cassette followed by a drug resistance expression cassette was introduced into exon 5 of the *Amhr2* locus. The resulting mice expressed *lacZ* in an *Amhr2* pattern, including Sertoli cells of the testes, preantral and antral ovarian follicles, Müllerian duct mesenchyme and postnatal uterine myometrium. *Amhr2 lacZ/lacZ* males exhibited PMDS exactly like *Amhr2*-null males, indicating that the *lacZ* insertion caused a loss-of-function mutation.

The bacterial Cre DNA recombinase gene was also introduced into the *Amhr2* locus to genetically modify the target tissue for AMH action, the *Amhr2-expressing* Müllerian duct mesenchyme, Sertoli and granulosa cells (Jamin et al., 2002). Similar to the *lacZ* knock-in strategy, an *IRES-Cre-pA* cassette followed by a drug resistance expression cassette was introduced into exon 5 of the *Amhr2* locus to generate the *Amhr2 Cre* allele. Crosses with

mice carrying Cre reporter alleles demonstrated that Cre was expressed in Müllerian duct mesenchyme and fetal gonads. *Amhr2 Cre/Cre* males develop PMDS just like *Amh*- and *Amhr2*-null males (Mullen and Behringer, unpublished results). Thus, similar to the *Amhr2-lacZ* allele, *Amhr2-Cre* is likely a null allele.

AMH pathway mutations in dogs

Numerous *AMH* and *AMHR2* recessive mutations have been reported in humans with PMDS (Picard et al., 2017). *Amh* and *Amhr2* knockouts generated by gene targeting in ES ceils in mice have also resulted in PMDS (Behringer et al., 1990; Mishina et al., 1996). The only other mammalian species in which an AMH pathway gene mutation has been identified is the dog (Wu et al., 2009).

There are numerous reports of PMDS in dogs (Meyers-Wallen, 2012). However, a causative mutation has only been identified in *AMHR2* (*MISRII*) in the miniature schnauzer breed (Fig. 3A) (Wu et al., 2009). A non-sense mutation was identified in exon 3 of *AMHR2* (C241T) (Fig. 2, Table 1). This is predicted to result in a truncated protein, lacking part of the extracellular region and the transmembrane and serine/threonine kinase domain and therefore no ability to transduce AMH signals. Males homozygous for C241T have uterine horns attached to and located parallel to the vasa deferentia (Fig. 3B, C). 50% of the males C241T homozygotes had unilateral or bilateral cryptorchidism. Mutant males with at least one scrotal testis were fertile (Fig. 3D). The female C241T homozygotes were described as normal (Wu et al., 2009). Two female C241T homozygous produced progeny (Vicki Meyers-Wallen, personal communication). The frequency of this mutant allele was found to be 16% in a cohort of 216 miniature schnauzers (Smit et al., 2018).

A male German shepherd with PMDS was screened for *AMH* or *AMHR2* mutations (De Lorenzi et al., 2018). Single base pair variants were identified in *AMHR2* exons 6 and 7. However, neither variant resulted in a predicted amino acid change. No mutations were found in exons 1-4 of *AMH.* However, technical difficulties precluded obtaining sequence for 21 bp at the 3’ end of exon 4 and all of exon 5. Thus, it is formally possible that the causative mutation resides in these regions. A male Belgian Malinois with PMDS was identified (Smit et al., 2018). However, no coding or splicing mutations were found in *AMH* or *AMHR2.* Likewise, two male Basset Hounds were found to have PMDS but sequencing of their *AMHR2* exons did not identify coding mutations (Pop et al., 2017).

The regulatory sequences that direct *Amhr2* transcription in the Müllerian duct mesenchyme have not been identified. Thus, it is possible that mutations in the *Amh* promoter region or the predicted *Amhr2* Müllerian duct mesenchyme-specific enhancer could lead to PMDS. More studies are required to identify the Müllerian duct mesenchyme transcriptional enhancer. The miniature schnauzer *AMHR2* model and other PMDS dog models provide useful complementary information relative to human PMDS patients and mouse knockout models. Comparisons of these diverse mammalian species may reveal how AMH signaling to conserved target tissues (Müllerian duct mesenchyme, Seroli cells, Leydig cells, granulosa cells) results in a spectrum of reproductive phenotypes influenced by species-specific anatomy.

AMH ligand and receptor gene mutations in teleost fishes

Teleost fish genomes possess *amh* and *amhr2* orthoiogous genes (Pfennig et al., 2015). At first, this seems counterintuitive because teleosts do not possess Müllerian ducts that give rise to reproductive tract organs (Suzuki and Shibata, 2004). In mammals, in addition to its role in Müllerian duct regression, AMH has been shown to regulate germ cell development. As mentioned above, *Amh* regulates primordial follicle recruitment in the mouse ovary (Durlinger et al., 1999). *Amh* and *amhr2*, have been shown to be expressed in at least 20 different teleost fish species (Pfennig et al., 2015). *amh* transcripts are detected in Sertoli cells of the testis and granulosa cells of the ovary. In some species, *amh* expression has also been detected in extragonadal tissues, including pituitary gland, brain and heart (Halm et al., 2007). *amhr2* expression has been reported in four teleost species: tiger pufferfish (*Takifugu rubripes*), black porgy (*Acanthopagrus schlegeli*), medaka (*Oryzias latipes*) and Nile tilapia (*Oreochromis niloticus*) (Pfennig et al., 2015). Interestingly, *amhr2* expression in Nile tilapia is found in the brain and gonads; *amhr2* expression in medaka and fugu is gonad-specific. Thus, *amh* and *amhr2* expression in somatic gonadal cells is conserved between fish and mammals. Does the AMH signaling pathway regulate germ cell development in teleosts? Does it regulate other processes?

The first non-mammalian vertebrate in which the function of an AMH signaling pathway gene was studied was the Japanese rice fish, medaka (*Oryzias latipes*). An *N*-ethyl-*N*-nitrosourea (ENU) recessive mutagenesis screen for gonadal defects identified a mutation in the autosomal *amhr2* gene (Morinaga et al., 2007). Chromosomal mapping and sequencing identified an A to G mutation in exon 9 of *amhr2.* This point mutation results in an amino acid substitution from Tyr to Cys in the receptor kinase domain (Fig. 2, Table 2). Phenotypically, XX female homozygous mutants had enlarged abdomens due to excessive proliferation of germ cells and therefore, enlarged ovaries (Fig. 4A–C). One-half of XY homozygous mutants showed sex reversal, and the remaining half displayed enlarged abdomens due to enlarged testis with overproliferation of germ cells. Analysis of adult fish showed that both male and female mutants where infertile and had a shorter lifespan compared to wild-type controls. These findings suggest that *amhr2* regulates germ cell proliferation and male development in medaka.

The tiger pufferfish (*Takifugu rubripes*) uses an XX-XY sex determination system (Kikuchi et al., 2007). Genetic mapping for the sex-determining locus identified a single nucleotide polymorphism (SNP) in the *amhr2* gene (Kamiya et al., 2012). The SNP (C to G) in exon 9 produces an amino acid substitution at position 384 from His to Asp and is located in the receptor kinase domain (Fig. 2, Table 2). Biochemical studies suggest that the $amhr2^{H384}$ allele has reduced activity compared to $amhr2^{D384}$. All males were heterozygous ($amhr2^{H384/D384}$) and all females were homozygous for $amhr2^{H384}$. This *amhr2* SNP is also present in *Takifugu pardalis* and *Takifugu poecilonotus* and shows a perfect correlation with sex phenotypes. These results suggest that AMH signaling is required for male development in three species of *Takifugu* pufferfish. Interestingly, the *T. rubripes amhr2* SNP is not found in the green spotted freshwater pufferfish, *Tetraodon nigroviridis* (Kamiya et al., 2012).

The Patagonian pejerrey (*Odonthestes hatcheri*) has an XX-XY system of sex determination (Hattori et al., 2010). A male-specific duplication of the *amh* gene was identified in *O. hatched*, designated *amhy* (Y chromosome-specific *amh*) (Hattori et al., 2012). *amhy* encodes the mature AMH ligand, is expressed earlier than autosomal *amh*, and was found to have a 557-bp insertion in intron 3. The authors performed an *amhy* morpholino knockdown and found that XY embryos resulted in male-to-female sex reversal and upregulation of two genes important for ovarian development: *foxl2* and *cyp19a1a.* Sex-reversed XY fish had ovaries with oocytes that were identical to wild-type XX female fish.

The Nile tilapia (*Oreochromis niloticus*) also uses an XX-XY sex determination system (Eshel et al., 2011; Lee et al., 2003). The *amh* gene resides on the X chromosome. Interestingly, *O. niloticus* has two male-specific duplications of *amh* designated *amhy* and *amhΔ-y* (Li et al., 2015). The coding region of *amhy* was found to be identical to *amh* except for a SNP (C to T) in exon 2 that causes an amino acid change from Ser to Leu in the N-terminal domain, *amhy* has also lost a large (5,608-bp) portion of the promoter region compared to the *amh* locus. In contrast, *amhΔ-y* has a 5-bp insertion in exon 6 that produces a frameshift that leads to a premature stop codon. In addition, *amhΔ-y* also has a 233-bp deletion in exon 7 and an insertion and numerous deletions 5’ of the start codon. The authors used CRISPR/Cas9 technologies to knockout *amh, amhy* and *amhΔ-y* genes in XY fish. Guide RNAs were targeted individually to exons 2 and 3 of all three genes (Fig. 2, Table 2). All *amhy* mutants showed male-to-female sex reversal (Fig. 4D–F), whereas *amhΔ-y* had no sex-specific mutant phenotype. No results were reported on *amh* single mutants. In addition, overexpression of *amhy* but not *amh* in XX individuals led to female-to-male sex reversal. Furthermore, CRISPR/Cas9-induced mutations in exons 2 and 3 of *amhr2* led to 100% male-to-female sex reversal (Fig. 2, Table 2). These results suggest that AMH signaling is required for male sex determination in Nile tilapia.

In zebrafish (*Danio rerio), amh* was mutated within the AB line genetic background, using CRISPR/Cas9 technology (Lin et al., 2017). Guide RNAs were designed to target exon 6 that would disrupt the expression of the mature AMH ligand (Fig. 2, Table 2). Two alleles were chosen for analysis: a 5-bp deletion and a 17-bp insertion; both produced premature stop codons. Homozygotes for the *amh* mutant alleles displayed a female-biased (−70%) sex ratio compared to wild-type siblings that were 40% female. Both male and female homozygous mutants showed an enlarged abdomen secondary to hypertrophic gonads. Histological analysis of ovaries and testes revealed increased germ cell proliferation with abnormal differentiation. These findings demonstrate that *amh* regulates germ cell proliferation and sex ratios in zebrafish. Interestingly, zebrafish does not have an *amhr2* gene (Adolfi et al., 2018), suggesting that AMH signals through a different Type 2 receptor in this teleost.

Summary and perspectives

AMH was discovered because of its role in Müllerian duct regression in mammals (Cate et al., 1986; Jost, 1953; Knebelmann et al., 1991; Picard et al., 1986). In humans, PMDS is predominantly caused by recessive mutations in either *AMH* or *AMHR2* (Picard et al., 2017). Similar genetic observations are found in the mouse and dog (Behringer et al., 1994;

Mishina et al., 1996; Wu et al., 2009). Perhaps this is not surprising because human, dog and mouse are eutherian mammals. Mouse knockout studies also indicated a role for AMH signaling in regulating germ cell development, specifically in primordial follicle recruitment (Durlinger et al., 1999). However, there are differences among these mammals that may be related to species-specific anatomy and physiology, including testicular descent and male fertility. PMDS has been observed in other mammals (Meyers-Wallen, 2012; Panasiewicz et al., 2015). However, *Amh* or *Amhr2* mutations have not yet been found in those species.

amh and *amhr2* genes have also been identified in non-mammalian species, including teleost fishes, i.e. animals that do not form a Müllerian duct. A primary role for the AMH pathway in germ cell proliferation has emerged through studies of *amh* and *amhr2* mutants in teleost species. In teleost fishes, the AMH pathway also regulates gonadal sex determination. Thus, it appears that the biological function of AMH in germ cell development precedes the evolution of Müllerian ducts. Interestingly, many sex-determining genes in mammals, reptiles, birds, and frogs, encode transcription factors (Capel, 2017). The above studies demonstrate that the AMH signaling pathway is a non-transcription factor sex-determining system at least in some species of teleost fishes (Hattori et al., 2012; Kamiya et al., 2012; Li et al., 2015). There is a wide variety of teleost and other fish species in which *amh* and *amhr2* are expressed (Pfennig et al., 2015). Further mutant studies in other fishes may provide insights into the evolutionary origins of these genes and their roles distinct from Müllerian duct regression.

A comparison of *AMH* and *AMHR2* mutant phenotypes in mammals and teleost fishes highlights the various biological processes regulated by AMH signaling, including Müllerian duct regression, primordial follicle recruitment, germ cell proliferation, and gonadal sex determination. CRISPR-CAS9 gene editing technologies have opened up new opportunities to create targeted mutations in species that previously were not amenable for this type of mutagenesis (Bier et al., 2018; DeLay et al., 2018; Farboud, 2017; Moravec and Pelegri, 2019). It will be very interesting to explore the spectrum of phenotypes for *AMH* and *AMHR2* mutations in other vertebrate species. Amniotes, including birds, reptiles, and mammals form Müllerian ducts that differentiate into organs of the female reproductive tract (Roly et al., 2018). However, *AMH* and *AMHR2* mutations have only been observed in eutherian mammals among amniote species. It seems reasonable to expect that Müllerian duct regression in males would be blocked in such mutants. However, would there be a germ cell phenotype? Would gonadal sex determination be altered as in teleost fishes? *Amh* and *Amhr2* genes are also found in amphibians (Jansson et al., 2016; Piprek et al., 2013). Müllerian ducts have been reported in *Xenopus laevis* and *Xenopus tropicalis* (Jansson et al., 2016). What Müllerian duct, germ cell, and gonadal sex determination phenotypes might occur in an amphibian if AMH signaling was ablated by mutation (Tandon et al., 2017)? It will be very interesting to determine the spectrum of reproductive phenotypes associated with *Amh* and *Amhr2* mutations that block AMH signaling across vertebrate species.

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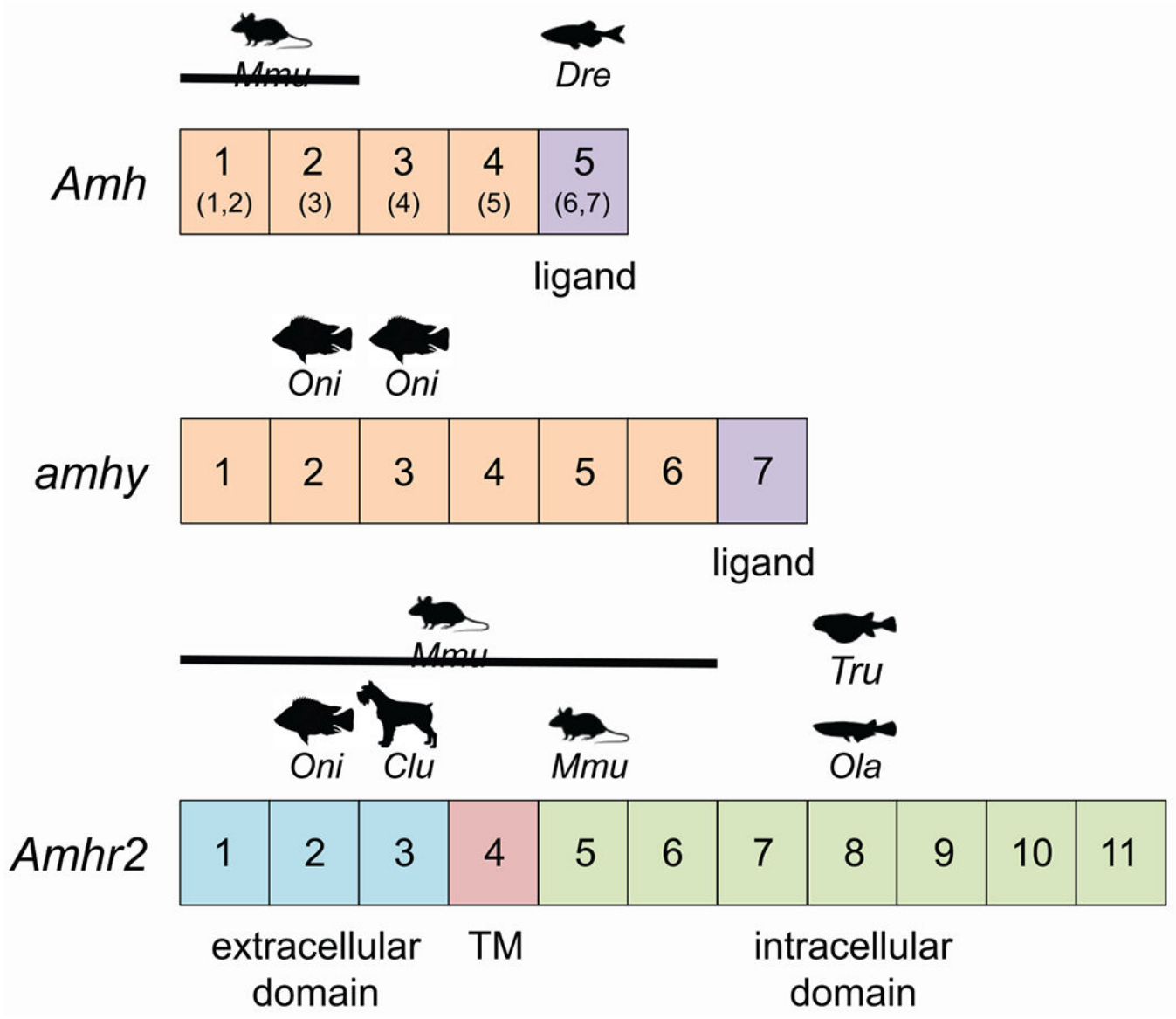

Fig. 1. AMH signaling mutant phenotypes in human and mouse.

A, Human; **B, C,** mouse. A, Diagram showing variation in position of testes and uterus in human Persistent Müllerian Duct Syndrome. **B, C.** Images of male reproductive tract organs from control **(B)** and *Amhr2*-null **(C)** mice. *Amh*-null male mice have the same phenotype as *Amhr2*-null mice. Ep, epididymis; ft, fallopian tube; gb, gubernaculum; scr, scrotum; sv, seminal vesicle; t, testis; ut, uterus; vd, vas deferens. Diagram in A modified from Hutson, Thorup, & Beasley, 2016.

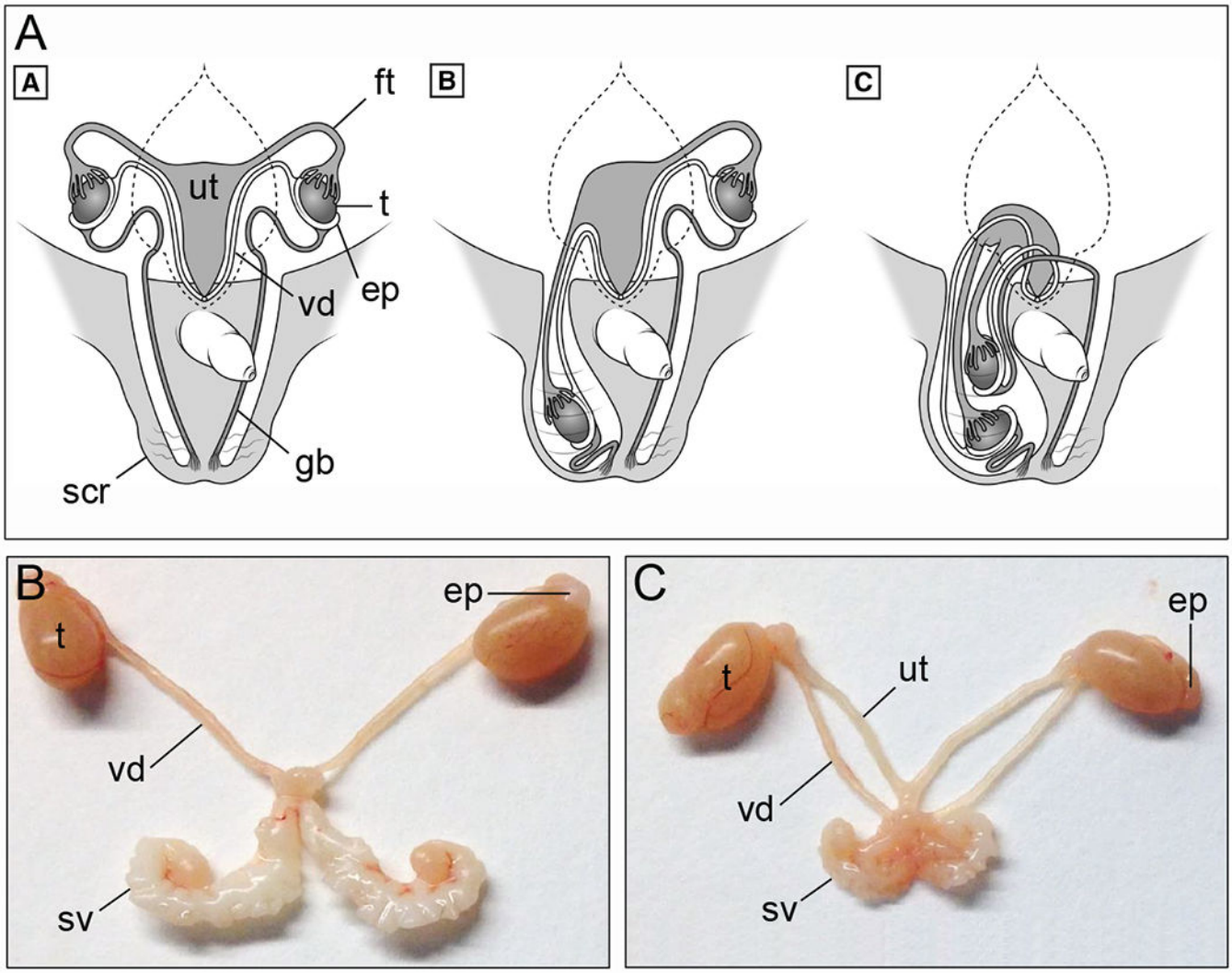

Fig. 2. *Amh, amhy*, and *Amhr2* mutations in vertebrates.

Diagram of *Amh, amhy*, and *Amhr2* gene and protein domains. Exons, numbered boxes not to scale. Numbers in parentheses correspond to *Dre* exons. Exon encoding TGF-beta domain, purple; extracellular domain, blue; transmembrane domain (TM), red; intracellular region containing kinase domain, green. Species symbols are indicated over the exons containing spontaneous or targeted mutations or SNPs. *Clu, Canis lupis*/dog; *Dre, Danio rerio*/zebrafish; *Mmu, Mus musculus*/mouse; *Ola, Oryzias latlpes*/medaka; *Oni, Oreochromis nitoticus*/Patagonian pejerrey; *Tru, Takifugu rubripes*/tiger pufferfish. Lines associated with species symbols indicate multiple exons in a deletion.

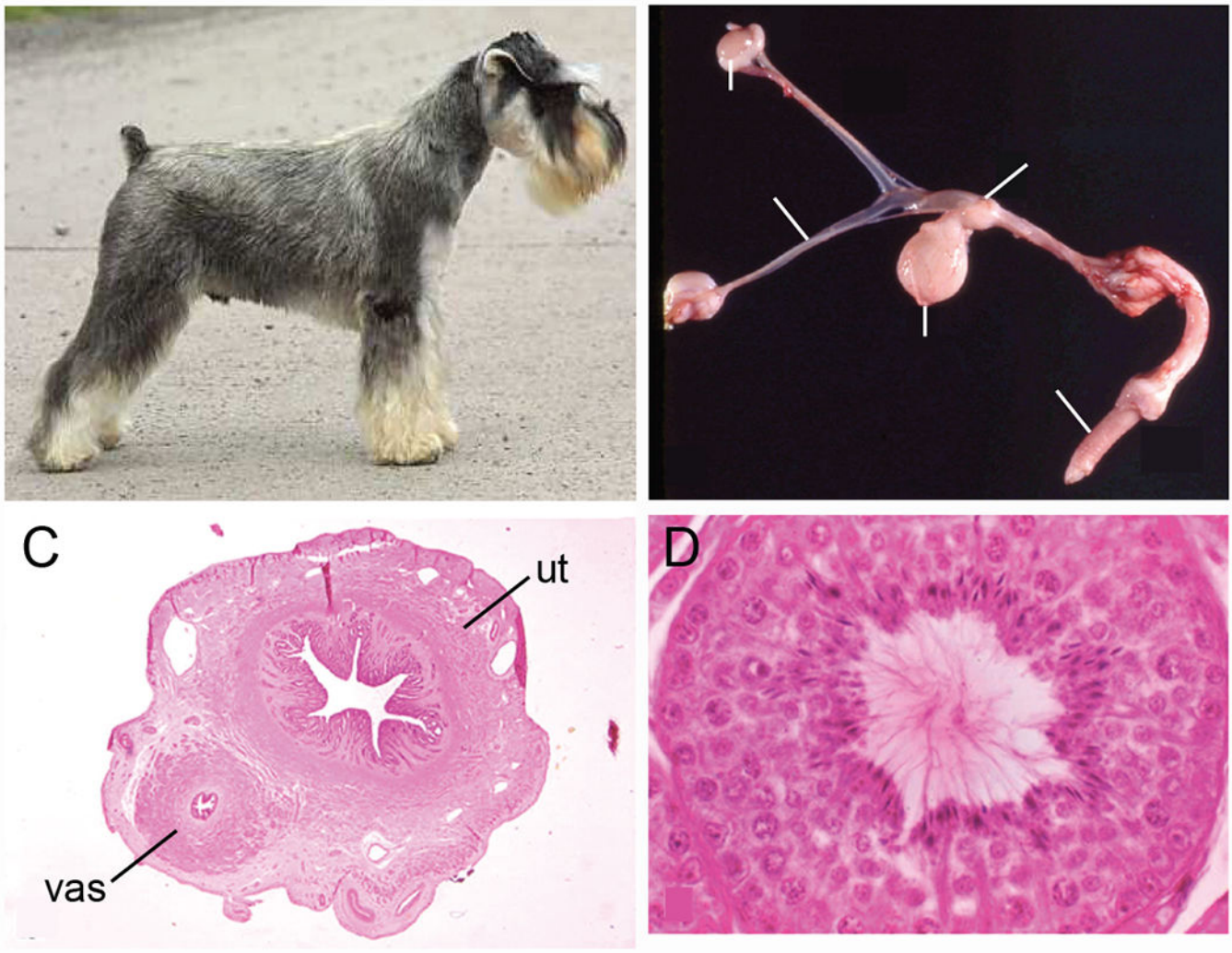

Fig. 3. PMDS in the miniature schnauzer.

A. miniature schnauzer. **B.** reproductive tract from 60-day old male homozygous for *AMHR2* missense mutation. **C.** H&E-stained histological section of vas deferens and connected uterine horn. **D.** H&E-stained histological section of seminiferous tubule from testis of PMDS male, showing productive spermatogenesis. Image in A from Wikipedia. Images in B-D modified from Wu et al., 2009.

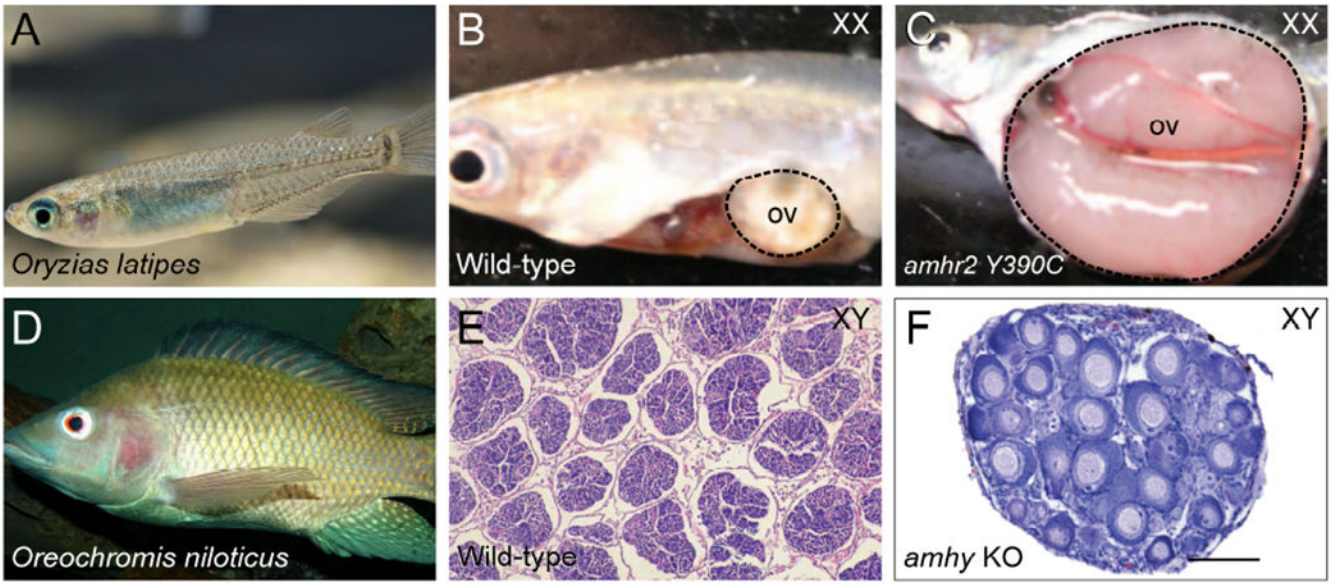

Fig. 4. Germ cell tumors and sex reversal in AMH signaling mutant teleost fish.

A-C, Japanese rice fish, also known as medaka (*Oryzias latipes*). **B,** Wild-type female dissection, showing ovary (ov, dotted line). **C,** *Amhr2 Y390C/Y390C* female, showing hypertrophic ovary (dotted line). **D-F,** Nile tilapia (*Oreochromis niloticus*). **E,** Histological section of testis from wild-type XY. **F,** Histological section of gonad, showing typical ovarian histology in *amhy* knockout XY. Image in A modified from www.seriouslyfish.com. Images B and C modified from Morinaga et al., 2007, copyright 2007 National Academy of Sciences. Image in D from Wikipedia. Image in E from El-Sayed et al., 2014. Image in F modified from Li et al., 2015.

Table 1.

Amh and *Amhr2* mutations in the mouse and dog.

Mouse *Amh*				
Allele	**Variant**	**Dominant/recessive**	**Phenotype**	**References**
Exon 1, intron 1, exon 2 deletion with *neo* cassette (*tm1Bhr*)	induced	recessive	male PMDS, infertility, Leydig cell hyperplasia; female primordial follicle recruitment alterations	Behringer 1994; Durlinger 1999
SF1-binding site mutation in promoter region with *neo* cassette in intron 1 (*tm2Bhr*)	induced	recessive	male PMDS	Arango 1999
SF1-binding site mutation in promoter region (*tm2. 1Bhr*)	induced	recessive	wild-type, decreased *Amh* transcript levels	Arango 1999
SOX9-binding site mutation in promoter region with *neo* cassette in intron 1 (*tm3Bhr*)	induced	recessive	male PMDS	Arango 1999
SOX9-binding site mutation in promoter region (i*tm3. 1Bhr*)	induced	recessive	male PMDS	Arango 1999
SF1- & SOX9-binding site mutation in promoter region with *neo* cassette in intron 1 (*tm4. 1Bhr*)	induced	recessive	male PMDS	Arango 1999
SF1- & SOX9-binding site mutation in promoter region (*tm4.1Bhr*)	induced	recessive	male PMDS	Arango 1999
neo cassette in intron 1 (.*tm5Bhr*)	induced	recessive	male PMDS	Arango 1999
*lox*P in intron 1 (*tm5.1Bhr*)	induced	N/A	wild-type	Arango 1999
Mouse *Amhr2*				
Allele	**Variant**	**Dominant/recessive**	**Phenotype**	**References**
Exons 1-6 deletion with neo cassette (*tm1Bhr*)	induced	recessive	male PMDS	Mishina 1996
Insertion of *IRES-cre* cassette with neo cassette into exon 5 with 50-bp deletion (*tm3(cre)Bhr*)	induced	recessive	male PMDS	Jamin 2002
Insertion of *IRES-lacZ* cassette with neo cassette into exon 5 with 50-bp deletion (*tm2Bhr*)	induced	recessive	male PMDS	Arango 2008
Dog *AMHR2*				
Allele	**Variant**	**Dominant/recessive**	**Phenotype**	**Reference**
Exon 3, C241T	spontaneous	recessive	male PMDS; some males with undescended testes and infertility	Wu 2009

Table 2.

amh, amhy, and *amhr2* mutations in teleost fishes.

Fish *amh*					
Species	**Allele**	**Variant**	**Dominant/recessive**	**Phenotype**	**Reference**
zebrafish (*Danio rerio*)	Exon 6, 5-bp deletion	induced	recessive	enlarged gonads, germ ceil proliferation; female-biased sex ratio	Lin 2017
zebrafish (*Danio rerio*)	Exon 6, 17-bp insertion	induced	recessive	enlarged gonads, germ cell proliferation; female-biased sex ratio	Lin 2017
Fish *amhy*					
Species	**Allele**	**Variant**	**Dominant/recessive**	**Phenotype**	**Reference**
Patagonian pejerrey (*Odonthestes hatchteri*)	Intron 3, 557-bp insertion	spontaneous	Y-linked	Male to female sex reversal (morpholino knockdown)	Hattori 2012
Nile tilapia (*Oreochromis niloticus*)	Exons 2 and 3, indels (*amhΔ-y*)	induced	Y-linked	wild-type	Li 2015
Nile tilapia (*Oreochromis niloticus*)	Exons 2 and 3, indels (*amhy*)	induced	Y-linked	Male to female sex reversal	Li 2015
Fish *amhr2*					
Species	**Allele**	**Variant**	**Dominant/recessive**	**Phenotype**	**Reference**
Medaka (*Oryzias latipes*)	Exon 9, Y390C	induced	recessive	enlarged gonads, germ cell proliferation; male to female sex reversal	Morinaga 2007
Tiger pufferfish (*'Takifugu rubripes*)	Exon 9, H384D	variant	male heterozygotes; female homozygotes	N/A	Kamiya 2012
Nile tilapia (*Oreochromis niloticus*)	Exon 2, indels	induced	recessive	male to female sex reversal	Li 2015
Nile tilapia (*Oreochromis niloticus*)	Exon 3, indels	induced	recessive	male to female sex reversal	Li 2015