Molecular Mechanisms of AMH Signaling

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Anti-Müllerian Hormone (AMH) is a secreted glycoprotein hormone with critical roles in reproductive development and regulation. Its chemical and mechanistic similarities to members of the Transforming Growth Factor β (TGF-β) family have led to its placement within this signaling family. As a member of the TGF-β family, AMH exists as a noncovalent complex of a large N-terminal prodomain and smaller C-terminal mature signaling domain. To produce a signal, the mature domain will bind to the extracellular domains of two type I and two type II receptors which results in an intracellular SMAD signal. Interestingly, as will be discussed in this review, AMH possesses several unique characteristics which set it apart from other ligands within the TGF-β family. In particular, AMH has a dedicated type II receptor, Anti-Müllerian Hormone Receptor Type II (AMHR2), making this interaction intriguing mechanistically as well as therapeutically. Further, the prodomain of AMH has remained largely uncharacterized, despite being the largest prodomain within the family. Recent advancements in the field have provided valuable insight into the molecular mechanisms of AMH signaling, however there are still many areas of AMH signaling not understood. Herein, we will discuss what is known about the biochemistry of AMH and AMHR2, focusing on recent advances in understanding the unique characteristics of AMH signaling and the molecular mechanisms of receptor engagement.

Keywords: anti-müllerian hormone (AMH), anti-müllerian hormone receptor type II (AMHR2), transforming growth factor-β (TGF-β), bone morphogenetic protein (BMP), activin, persistent müllerian duct syndrome (PMDS), cell signaling, prodomain

INTRODUCTION

Anti-Müllerian Hormone (AMH), also known as Müllerian Inhibiting Substance (MIS), was first described by Alfred Jost in 1946 as a secreted testicular factor which drove the regression of the Müllerian ducts in the male fetus (1). Importantly, dysregulation of this mechanism presents clinically as Persistent Müllerian Duct Syndrome (PMDS), in which loss of function mutations in AMH or its signaling receptor, Anti-Müllerian Hormone Receptor Type II (AMHR2), lead to persistence of Müllerian duct derivatives – uterus, fallopian tubes, cervix, and upper vagina – in males (2, 3). In women, AMH is a negative regulator of folliculogenesis and dysregulation of the signaling pathway has been implicated in two leading causes of female infertility: Polycystic Ovary Syndrome (PCOS) and Primary Ovarian Insufficiency (POI) (4). Since its initial description, more recent characterization of this hormone has provided foundational insights into our current understanding of the structure and function of AMH and its signaling pathway.

AMH is a glycoprotein hormone (5) which shares structural and mechanistic homology with signaling proteins of the Transforming Growth Factor β (TGF-β) family (6). This family consists of over 30 secreted signaling ligands that have essential functions for many processes regulating cell homeostasis and human development, including reproductive development (7). These ligands are synthesized from a precursor consisting of a large N-terminal prodomain and smaller C-terminal mature signaling domain (**Figure 1A**). Folding, dimerization, and secretion are regulated by prodomains, which are cleaved from the smaller signaling domain and, in most cases, remain noncovalently associated (**Figure 1B**) (8). Ligands signal by binding to the extracellular domain (ECD) of two type I and two type II serine/threonine receptor kinases. This complex brings the intracellular kinase domain (ICD) of the constitutively active type II receptor in close enough proximity to phosphorylate the GS domain of the of the type I receptor ICD, relieving inhibition and activating Smad transcription factors (9) (**Figure 1C**). Signaling within the TGF-β family is limited to specific combinations of the seven type I receptors, Activin-like kinases 1-7 (ALK1-7), and five type II receptors, ActRIIA, ActRIIB, BMPR2, TβR2, and AMHR2 (7). It has been shown that AMH will mainly signal using ALK2 (10, 11) or ALK3 (12–14), type I receptors used by the bone morphogenetic protein (BMP) branch of TGF-B ligands, and activation of BMP R-Smads 1, 5, and 9 as well as activation of BMP reporter genes (15, 16). The other BMP type I receptor, ALK6, has a stimulatory or inhibitory effect depending on the tissue type (17, 18). AMHR2 is unique within the TGF-β family as it is the only receptor specific for a single ligand (19). In this review, we will summarize the current biochemical understanding of AMH as a TGF-β ligand from secretion to signal, with a focus on recent efforts to characterize the binding of AMH to AMHR2 and the looming gaps the field must overcome in order to better understand this important biological pathway.
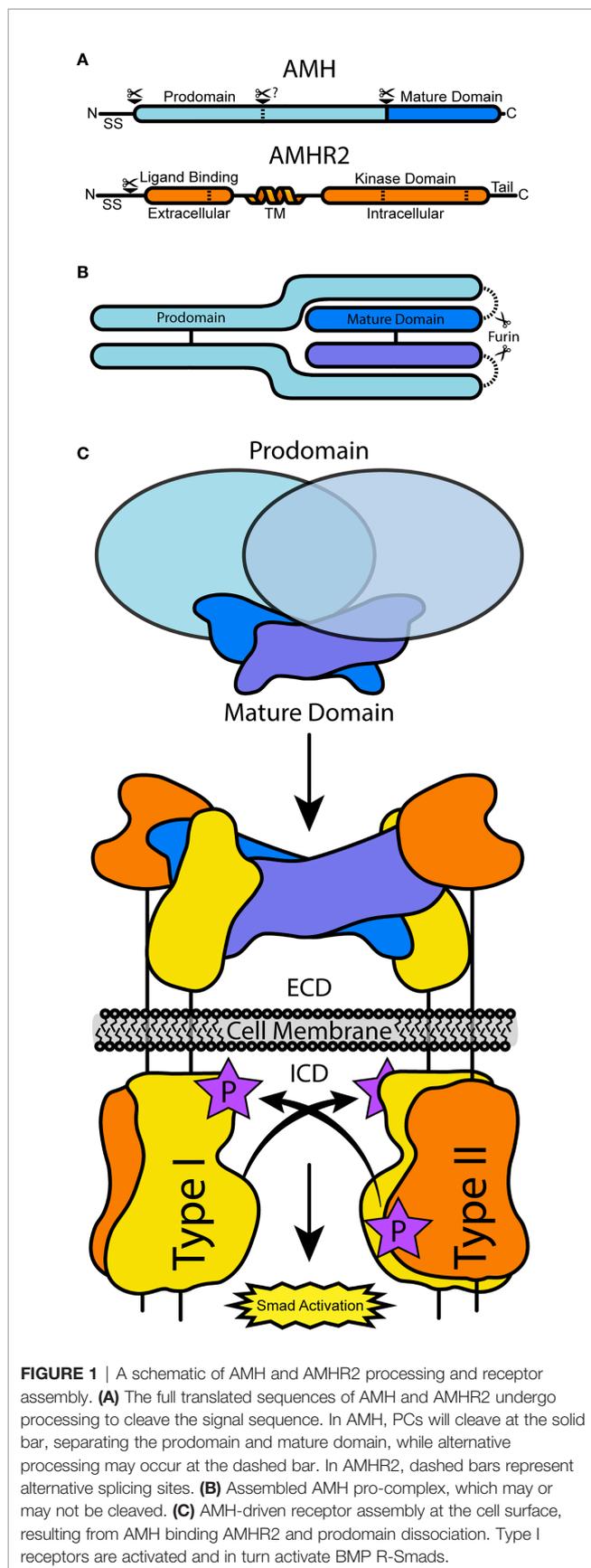
PROCESSING AND REGULATION OF AMH & AMHR2

AMH was first identified as a TGF-β ligand by sequence similarity of its C-terminal mature signaling domain with Activins and TGF-βs (20) and the proteolytic processing of this domain (6). The full open reading frame of human AMH (UniProtKB P03971-1) consists of a signal sequence (SS) (residues 1-24), prodomain (residues 25-451), and mature domain (residues 452-560) (**Figure 1A**) (6). Human AMH is processed canonically; mammalian proprotein convertases (PCs), such as furin, will cleave the proprotein downstream of an a R-X-X-R motif at amino acid position 448-451 to generate the 109 amino acid mature domain (21–24). Similar to other family members, PC cleavage separates the N-terminal prodomain from the C-terminal mature domain, which allows for assembly into a noncovalent complex (**Figure 1B**) (6, 25–27). Only the cleaved, processed, dimer form can properly bind its receptors and induce downstream signaling (28), but evidence of

mixed circulating species of AMH suggests a regulatory role of this processing (29). Both the processed and unprocessed species are found in the serum and follicular fluid (25, 30) in varying ratios depending on age (27), sex (27), and disease state (31, 32). Interestingly, alternative cleavage products resulting from serine proteinase activity within the prodomain (**Figure 1A**) have been described during purification (6, 25, 33–35), however their biological relevance remains unknown.

The processing of AMHR2 (UniProtKB Q16671-1) is less characterized than its ligand. While there has been robust study of type II receptor regulation within the TGF-β family *via* mechanisms of internalization (36–40), homo- and heteromeric complex formation (41–43), and glycosylation (44, 45), for AMHR2, the understanding of regulation is currently limited to biosynthetic processing and surface presentation alone (46, 47). Unlike other type II receptors, it has been suggested that functional presentation of AMHR2 at the membrane is negatively regulated by cleavage or by disulfide-linked oligomerization of the extracellular domain, leading to increased retention within the ER (46). Additionally, those functional receptors that are presented appear to organize in clusters of homo-oligomers, resulting in a lack of lateral mobility (46). In mammals, receptor splice variants have been identified that result in the deletion of either amino acids 377-471 (Amhr2Δ9/10) within the kinase domain, or 17-77 (Amhr2Δ2) within the extracellular domain (47–49) (**Figure 1A**). Although their mRNA expression level in the testes and brain is 5% or less of the normal receptor, the existence of these variants raises interesting questions about their regulatory function in the AMH signaling pathway (47). Thus, continued investigation of the functional consequences of these or other splice variants is necessary to understand their potential impact on signaling. Lastly, Unlike other TGF-β ligands, investigations into mechanisms of extracellular antagonism of AMH have not been definitive (50–52). Nevertheless, regulation of ligands by protein antagonists represents a significant feature of TGF-β ligands, and the lack of known AMH-binding proteins is either a missing piece of the known mechanism or an interesting aberration from other family ligands.

THE ROLE OF THE AMH PRODOMAIN

It is widely accepted that the prodomains of TGF-β ligands are required for proper folding and dimerization of the mature signaling ligand (53–55). While most prodomains are similar in size, an indication of secondary structure elements, there are exceptions. For example, GDF15 maintains the smallest prodomain of 18.5 kDa whereas AMH has evolved the largest of the prodomains at 45 kDa. Furthermore, unlike the ligands which typically have a conserved patter of cystines, the prodomains exhibit significant differences in the number and placement of cysteines, indicating structural divergence (7). For TGF-β1-3 the prodomains from two different chains are joined by a disulfide bond (56). The intermolecular disulfide bond increases the affinity of the prodomains for the mature ligand thorough avidity effects. Similarly, AMH also exhibits an

FIGURE 1 | A schematic of AMH and AMHR2 processing and receptor assembly. **(A)** The full translated sequences of AMH and AMHR2 undergo processing to cleave the signal sequence. In AMH, PCs will cleave at the solid bar, separating the prodomain and mature domain, while alternative processing may occur at the dashed bar. In AMHR2, dashed bars represent alternative splicing sites. **(B)** Assembled AMH pro-complex, which may or may not be cleaved. **(C)** AMH-driven receptor assembly at the cell surface, resulting from AMH binding AMHR2 and prodomain dissociation. Type I receptors are activated and in turn activate BMP R-Smads.

interchain disulfide bond which likely increases its affinity for the mature ligand (6).

For some ligands, such as the TGF-βs, GDF8, and GDF11 the prodomain maintains the ligand in a latent state, and activation occurs thorough proteolysis (57–59) of the prodomain or an integrin-mediated stretching mechanism (56), both of which liberate the bound ligand from the prodomain. For AMH, BMPs, and other activin ligands, the prodomain does not render the ligand latent and the ligand is either thought to signal in the presence of the prodomain or that the prodomain is readily displaced by binding the signaling receptors. For AMH, the prodomain has been shown to allosterically regulate AMH binding to AMHR2 without inhibiting signal (28, 60). This mechanism is similar to the non-latent BMP7 pro-complex, however unlike AMH, the BMP7 prodomain has a weakly competitive interaction with the BMP type II receptors and unchanged type I receptor interactions (61). Further parallels might be drawn from the crystal structure of the BMP9 pro-complex bound by ALK1, which shows that the type I receptor can associate without displacing the prodomain (62), but this remains untested for AMH. Unlike most BMPs, the AMH prodomain has a 10-100 fold higher affinity for the mature ligand (K_d = 0.4 pM) (60). Despite this high-affinity interaction, bivalent binding to AMHR2 presented on a surface is able to disrupt interactions and attenuate binding 1000-fold (60). Displacement appears to be dependent on the avidity as neither monovalent binding nor soluble receptor are able to induce prodomain displacement. Thus, while certain ligands have high affinity for their prodomain and confer latency, many BMP ligands have lower affinity for their prodomains and are more readily displaced by receptor binding (8, 58). AMH appears somewhat unique in that it maintains a very high affinity for the ligand, but the prodomain can also be displaced by cell surface receptors. The high affinity of the prodomain of AMH suggests that the prodomain is likely to play an important role in either protecting AMH or facilitating signaling.

As mentioned, the prodomain seems to be an additional and principal factor of regulation within the signaling pathway. The prodomain is required for proper folding, homo- or heterodimerization, and secretion (7, 63–65), and the presence of PMDS mutations within the prodomain support this mechanism for AMH (2). In the serum, there is no unbound mature AMH ligand (25), suggesting a role for the prodomain in shuttling the mature domain to nearby and distant targets. The endocrine character of AMH is a robust research area, as we have yet to fully comprehend the breadth of extragonadal signaling targets (4, 18, 66, 67). For other ligands, the pro-complex also functions as a shield from extracellular antagonists (23, 33, 64, 68, 69). The interface between BMP antagonist Crossveinless 2 and BMP2 is analogous to the interface between mature BMP9 and the BMP9 prodomain (7), so the large AMH prodomain might function to protect AMH from interactions with a milieu of extracellular matrix (ECM) components. On the other hand, prodomains seem to be important for targeting the mature ligand to the cell surface through interactions with heparin (8), fibrillin (70), and other components of the ECM (7). However, unlike many BMPs, AMH does not have

large positively-charged patches of amino acids which would limit its interactions with heparin; it instead has a significant hydrophobic character (71). We do know, however, that the prodomain is necessary for activity in tissue-based assays (72) but dispensable for cell-based assays (47, 52, 73). This suggests that the prodomain likely does not play a major role in the signaling mechanism but might play a larger role in the availability of the ligand by mediating ECM interactions or conferring protection from degradation or antagonism.

The prodomain itself may also be subject to regulatory mechanisms common to the TGF-β family. In this vein, the previously mentioned alternate cleavage sites of AMH (6, 74) (**Figure 1A**) might have some bearing on the activity of the noncovalent complex. Conformational changes (7), alternative cleavage (57, 75, 76), or other uncharacterized modifications (77) have been shown to prime the noncovalent complex for receptor interactions. Furthermore, there are 2 N-linked glycosylation and several possible O-linked glycosylation sites predicted within the AMH prodomain (78) comprising 13.5% of the complex mass (71). Differential modification of glycosylation may impact protein-protein interactions or cleavage, as observed in drosophila with the ortholog of BMP7, Gbb (76). Largely, we lack understanding of the regulatory role of the AMH prodomain beyond its absolute necessity for secretion and activity in the body. Whether the AMH prodomain, which is the family's largest and most divergent, has additional function beyond increasing the availability of the AMH signaling ligand is not known. While recent advances in modeling using AlphaFold can help to visualize structure and domain architecture of the AMH prodomain (79), the lack of structural definition of this region and low homology cause difficulty in effectively modeling the AMH prodomain (62, 63, 80–82). As such, structural and biochemical characterization of the prodomain structure and its interfacing interactions with the mature ligand and receptors will help ascertain its function.

STRUCTURAL DEFINITION OF AMH AND AMHR2

The TGF-β family is part of the cystine knot growth factor (CKGF) superfamily (7) which have a conserved fold and sequence. The overall shape can be described as an opposite-facing left and right hands in a Vulcan salute joined at the palm (**Figure 2A**). This creates a concave pocket between the wrist helix of one chain and the fingertips of the opposing chain, to which the type I receptor is recruited, and a convex surface on a single chain at the "knuckle" region, to which the type II receptor binds for Activins and BMPs (84). The extracellular ligand-binding domain of the type II receptors adopt a three-finger toxin fold, which has also classically been described with a hand-like morphology, consisting of three anti-parallel beta strand fingers and a central palm region (**Figure 2B**). These features are also conserved for AMH and AMHR2 as shown in the recently solved structure of the extracellular complex (85).

In general, ligands have evolved two central binding modes to interact specifically with their type II receptors (**Figure 2C**).

Ligands of the Activin and BMP class bind at the convex, knuckle surface of the ligand fingers, while the TGF-β class ligands bind the fingertips (84). For AMH, the binding mode was unknown until the recently-solved crystal structure of AMH bound to AMHR2 (PDB:7L0J) (85). This structure provided a critical piece for understanding ligand-receptor interactions and disease-causing mutations (**Figures 2D–E**), revealing that while similar to the general binding mode of BMP and Activins, AMH utilizes a modified mode of type II receptor binding (85).

The receptor binding interaction of AMH and AMHR2 is unique within the family. While TGF-β class ligands bind TβR2 using finger 1 of the receptor and the fingertips of the ligand, Activin and BMP class ligands bind ActRIIA and ActRIIB at the palm of the receptor and the knuckles of the ligand (86–91) (**Figure 2C**). Like Activins and BMPs, AMH binds AMHR2 using the palm of the receptor and the knuckles of the ligand, however this interface is shifted towards the fingertips by about 7.5 Å. Additionally, fingers 1 and 3 of AMHR2 wrap around the ligand making unique contacts with sections of AMH not observed with Activins and BMPs, especially within fingers 3 and 4 of AMH and the connecting loop (85). While the mature ligand is similar to each of the other three classes (the root mean square deviation of the Cα positions is below 2 Å for BMP2, GDF11, and TGF-β1) the AMH ligand adopts a flat character of the fingers akin to TGF-βs.

The structure of AMH bound to AMHR2 highlighted structural differences in each that are likely responsible for specificity. Of note, AMH has a truncated finger 1/2 loop relative to other ligands that facilitates the wrap-around mechanism of AMHR2 (85). More significant variance is observed on the receptor side with differences between AMHR2 and other type II receptors undoubtedly contributing to specificity. Most notably is the conformation of finger 1 of AMHR2 which is extended compared to other receptors and forms a favorable interaction with AMH. While the number of disulfide bonds are similar, a shift of one cysteine (Cys60) results in unique structural character of AMHR2. The altered location of the disulfide bond brings together the finger 2/3 loop and finger 3 to create unique surface for AMH binding (85). These distinct conformational features of AMHR2 promote the selectivity of AMH binding and signaling.

Where we still lack critical information is in our understanding of the nature of interactions between AMH and its type I receptors. The affinity of AMH for its type II receptor has been shown to resemble TGF-βs or Activins, while affinity for the type I receptor, though not yet directly tested, is assumed to follow the same low affinity archetype as the above ligand classes (28, 73, 85). What is known is that AMH lacks two conserved tryptophan residues present in the type I receptor binding site of BMPs. Importantly, these residues have been shown to be necessary for proper signaling in members of the BMP class (92). In fact, the entire type I binding interface is dissimilar to that of BMPs (86, 93, 94) and contains more polar and charged residues, yet AMH will signal using the same type I receptors – ALK2, ALK3, ALK6 – as BMPs. It will be interesting to determine how AMH

accommodates for its binding and specificity of type I receptors with these differences. It is possible that type I receptor binding is shifted relative to BMPs and might even potentially interact with the type II receptor in a cooperative mechanism similar to TGF-β, however, this has yet to be explored.

DISCUSSION

Recent studies have revealed a wealth of information about the molecular mechanisms of AMH signaling, but the field has a long way to go towards a full understanding of the intricacies of this
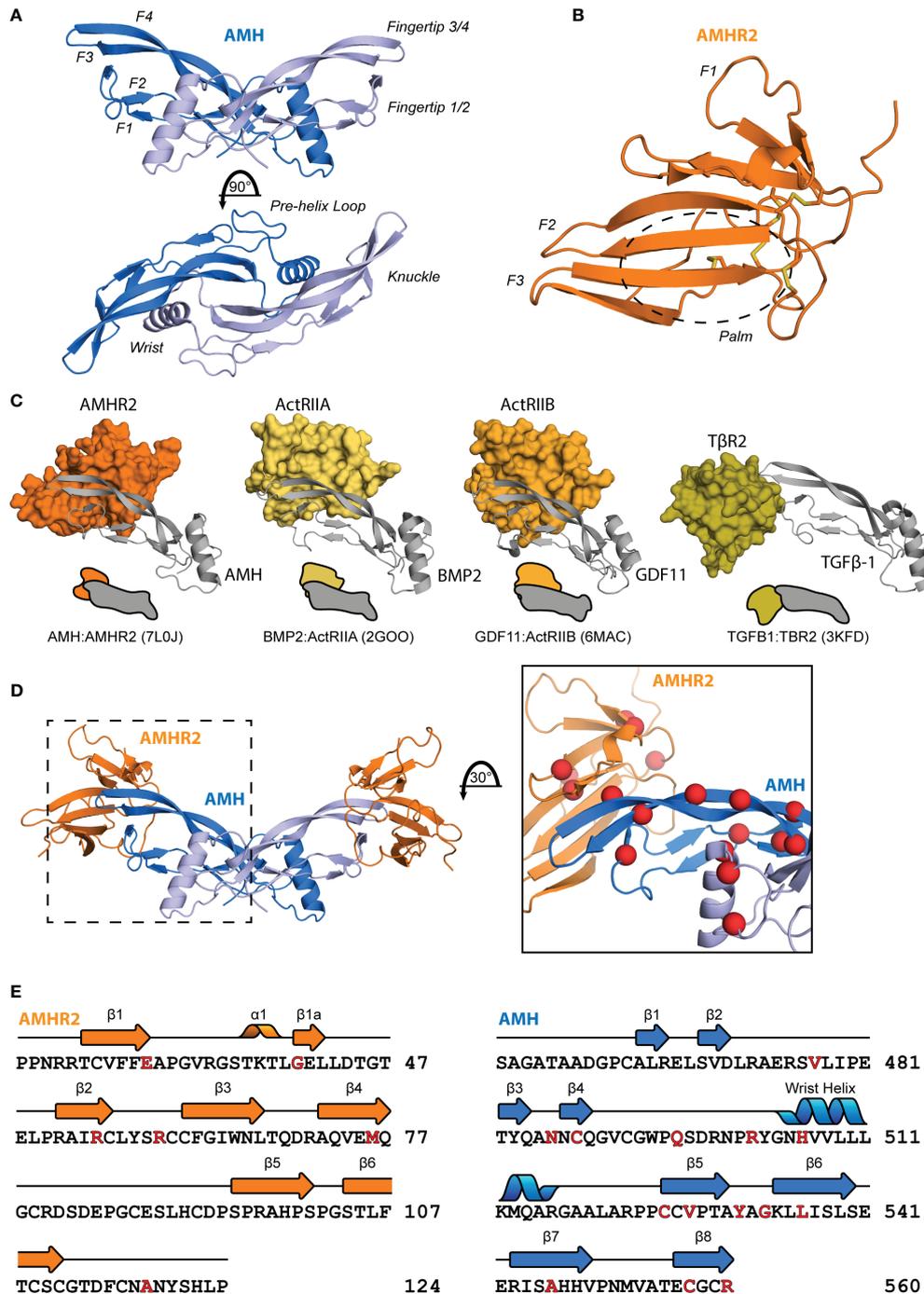

FIGURE 2 | Structural features of AMH and AMHR2. Diagram of the structure of mature AMH (A) and AMHR2 ECD (B). (C) Comparison of the binding modes of each ligand class to a type II receptor. (D) Mapping of PMDS mutations (83), indicated by red spheres, to the binary complex. (E) Sequence of AMH and AMHR2 annotated with secondary structure features and highlighted in red with the above PMDS mutations.

unique pathway. The biochemistry of AMH is certainly less characterized than its TGF-β family counterparts. Knowledge of these structures and their interactions can help explain the expanding genetic information linked to human diseases, such as PMDS and PCOS. For example, several mutations have been identified in the prodomain, however, we lack the structural information needed to better understand how these mutations impact AMH function.

The interaction between mature AMH and the extracellular domain of AMHR2 is perhaps the most actionable piece of recent data characterizing AMH. The crystal structure demonstrates unique features that set AMH apart from other ligand classes, as well as an atomic-level explanation for PMDS mutants which map to the interface (**Figures 2D–E**). This story is, of course, incomplete without a structure of mature AMH bound by a type I receptor. While the mutual exclusivity of the AMH and AMHR2 interaction is an interesting feature within the TGF-β family, equally interesting is how the intracellular kinase domain of AMHR2 can employ type I receptors shared with BMPs yet propagate an AMH-specific signal. Intracellular interactions remain something of a black box for the fields of both TGF-β and AMH biology.

Looking ahead, further structural studies of the AMH ligand and receptor are warranted; these studies must be supported by stronger assay development. Most importantly, the field should address discrepancies between *in vitro* and *in vivo* studies, especially concerning the prodomain. It has been known for some time that the AMH prodomain is required for biological function. This feature is reflected in tissue-based assays but not in cell-based assays where the mature ligand will suffice. Better care

should be taken to include the prodomain, when possible, to better replicate the biological context of AMH and allay concerns about differences between these two assay systems. Additionally, the ability to distinguish between the transcriptional outcome of AMH and BMP signaling would be a great and powerful tool for probing the mechanisms of the signaling pathway at every level. Research into this area might also help to answer a major question of AMH signal in general: is there a signaling cascade unique to AMH, or does AMH modulate a pre-existing BMP signal to generate unique outcomes? Although the research areas in need of attention are difficult, fresh data and new techniques have done wonders to answer critical questions and spark novel hypotheses about how this pathway truly functions.

AUTHOR CONTRIBUTIONS

JH and KH developed the concept. JH wrote the manuscript. KH and TT edited and revised the manuscript for important intellectual content. All authors read and approved the submitted version.

FUNDING

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.