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Non-canonical WNT5A-ROR signaling: new perspectives on an ancient developmental pathway

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Abstract

Deciphering non-canonical WNT signaling is proving to be both fascinating and challenging. Discovered almost 30 years ago, non-canonical WNT ligands signal independently of the transcriptional co-activator β-catenin to regulate a wide range of morphogenetic processes during development. The molecular and cellular mechanisms that underlie non-canonical WNT function however, remain nebulous. Recent results from various model systems have converged to define a core non-canonical WNT pathway consisting of the prototypic non-canonical WNT ligand, WNT5A, the receptor tyrosine kinase ROR, the seven transmembrane receptor Frizzled and the cytoplasmic scaffold protein Dishevelled. Importantly, mutations in each of these signaling components cause Robinow syndrome, a congenital disorder characterized by profound tissue morphogenetic abnormalities. Moreover, dysregulation of the pathway has also been linked to cancer metastasis. As new knowledge concerning the WNT5A-ROR pathway continues to grow, modeling these mutations will likely provide crucial insights into both the physiological regulation of the pathway and the etiology of WNT5A-ROR-driven diseases.

Keywords

WNT5A; ROR; Frizzled; FZD; Dishevelled; DVL; Non-canonical WNT signaling; Robinow syndrome; Omodysplasia; Tissue morphogenesis; Cancer metastasis

1. A brief history of canonical and non-canonical WNT pathways

WNTs make up a highly conserved family of glycoproteins that mediatecell-cell communication in diverse contexts within multicellular organisms. First discovered as drivers of mammary tumors in mice (Nusse and Varmus, 1982) and then as segment polarity genes in *Drosophila* (Baker, 1987; Nusslein-Volhard and Wieschaus, 1980), WNT ligands have garnered several decades of intense study from researchers worldwide. Today, it is recognized that WNT ligands play a pivotal role in most aspects of metazoan biology, from

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the earliest stages of embryonic development to the most severe states of various diseases and many other physiological and pathological activities in-between.

The WNTs identified in these early studies are known as canonical WNTs. Ectopic expression of canonical WNT ligands in *Xenopus* embryos caused alterations in tissue patterning (McMahon and Moon, 1989), most notably an axis duplication phenotype resulting from the dorsalizing effect of WNT misexpression (Figure 1A). In cultured cells, expression of canonical WNTs induced cell transformation (Nusse and Varmus, 1982; Ramakrishna and Brown, 1993; Rijsewijk et al., 1987; Wong et al., 1994). Together, these robust and tractable assays provided the first crucial tools for functional characterization of the pathway. Progress in the field was further propelled by epistasis experiments in *Drosophila* (Noordermeer et al., 1994; Peifer et al., 1994; Siegfried et al., 1992) which helped provide a molecular framework for the canonical WNT signaling cascade.

A defining signature of the canonical WNT pathway is its use of the transcriptional co-activator β-catenin, known as Armadillo in *Drosophila*, to control target gene transcription. In the absence of WNT signaling, β-catenin undergoes constitutive proteolytic turnover mediated by the ubiquitin-proteasome pathway. Upon engagement of the canonical WNT ligands (e.g. WNT1, WNT3A and WNT8B) with the cell surface receptors Frizzled (FZD) and low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6), β-catenin becomes stabilized and translocates into the nucleus to activate target gene transcription (Cadigan and Waterman, 2012; Clevers and Nusse, 2012) (Figure 1C). Through this core mechanism, canonical WNT signaling regulates many important developmental decisions, including cell proliferation and tissue fate determination, as well as tissue homeostasis, such as stem cell maintenance and tissue regeneration, in adult organisms. As a result, defects in canonical WNT signaling can give rise to devastating diseases, including congenital birth defects and cancer (Clevers and Nusse, 2012; Nusse and Varmus, 2012).

The vertebrate genome encodes 19 WNTs. Intriguingly, some early studies observed that a subset of WNT ligands had no effect on tissue patterning or cell transformation, but rather appeared to influence cell rearrangements and tissue shaping (Du et al., 1995; Moon et al., 1993). WNT5A was the first WNT implicated in such changes. In their seminal study, Moon and colleagues found that ectopic expression of WNT5A in *Xenopus* embryos did not produce the canonical WNT phenotype of axis duplication, but instead significantly altered *Xenopus* embryo morphology, producing tadpoles with a truncated head and tail (Figure 1B) (Moon et al., 1993). It was subsequently found that WNT4 and WNT11 also exhibit similar morphogenetic properties when injected into *Xenoups* embryos (Du et al., 1995). These observations indicated that different WNT ligands possessed unique functions and implied the existence of multiple WNT signaling pathways that drive different phenotypic outcomes (Figure 1C, D). This review will focus mainly on WNT5A, as it is thus far the most extenstively characterized among this latter group.

The morphogenetic function of WNT5A is highly conserved throughout evolution (Loh et al., 2016). Other model organisms displayed similar gross morphological defects in response to either genetic ablation or overexpression of WNT5A. For instance, *Wnt5a* knockout mice exhibit truncated body axes, limbs, and tails, as well as widened, flattened

faces and underdeveloped external genitalia (Yamaguchi et al., 1999) (Figure 2A). In addition, overexpressing WNT5A in mice produced similar types of morphogenetic phenotypes (van Amerongen et al., 2012), indicating that the dose and spatiotemporal expression of WNT5A must be tightly controlled within a narrow physiological range during development. Importantly, the morphogenetic signaling activity of WNT5A did not appear to involve β-catenin-mediated functions (Shimizu et al., 1997) (Figure 1D). Thus began the distinction between canonical WNT signaling, dependent on β-catenin, and other β-catenin-independent, or non-canonical, WNT pathways.

Since these early discoveries, the number of biological processes regulated by non-canonical WNT pathways has continued to grow. Collectively, non-canonical WNT pathways appear to culminate in the regulation of cytoskeletal rearrangements (Veeman et al., 2003), thus leading to the cell and tissue morphological changes described above. The largely non-transcriptional responses elicited by non-canonical WNTs, however, also presented challenges for deciphering the underlying mechanisms. For example, the development of a versatile β-catenin-dependent, luciferase-based transcriptional reporter, commonly known as TOPflash (Korinek et al., 1997; Molenaar et al., 1996), greatly accelerated molecular interrogation of the canonical WNT pathway. In contrast, the lack of an equivalent reporter assay for the non-canonical WNT pathway forced investigators to resort to less quantitative readouts such as overexpression phenotypes in embryos. Moreover, as mentioned earlier, genetic studies in *Drosophila* were instrumental in deciphering the canonical WNT pathway (as well as other major developmental signaling pathways, such as Hedgehog, Delta-Notch and Hippo); however, they did not identify a pathway directly equivalent to the vertebrate non-canonical WNT pathway. Together, these limitations severely hindered progress in the non-canonical WNT field.

2. Emergence of WNT5A-ROR signaling as a major non-canonical WNT pathway

It took another decade for the ROR family of receptor tyrosine kinases to enter the spotlight as a pivotal mediator of non-canonical WNT5A signaling. The ROR family has two members in vertebrates, ROR1 and ROR2, which were initially cloned as orphan receptors based on their sequence homologies to other receptor tyrosine kinases (Masiakowski and Carroll, 1992). Even though no ligands or functions were initially known for ROR receptors, sequence analyses suggested that ROR proteins are highly conserved during evolution, with clear homologs found in animals as ancient as sponges and sea slugs (McKay et al., 2001; Sossin, 2006). This implied that these molecules control biological processes fundamental to all metazoan species. Indeed, early genetic studies in *C. elegans* showed that the nematode ROR homolog *Cam-1* plays crucial roles in a wide range of cell and tissue morphogenetic processes, including neuronal migration, axon pathfinding, asymmetric cell division and tissue polarity (Forrester et al., 1999; Koga et al., 1999).

In 2000, two groups independently knocked out the *Ror2* gene in mice (DeChiara et al., 2000; Takeuchi et al., 2000). These mutant mice displayed severe axis, limb and craniofacial truncation phenotypes, which at the time were largely attributed to defects

in skeletal development (See Figure 2B for an example of the phenotypes (Ho et al., 2012)). Subsequently, *Ror1* and *Ror2* double knockout mice were generated and showed exacerbation of the *Ror2* single mutant phenotypes, indicating that the two family members are at least partially redundant during mouse embryogenesis (Ho et al., 2012; Nomi et al., 2001).

Importantly, Xu and Nusse (Xu and Nusse, 1998) noticed that ROR receptors possess a cysteine-rich domain (CRD), a domain originally identified in the FZD family of WNT receptors to mediate WNT binding. In parallel, the striking resemblance of the *Wnt5a* and *Ror* tissue truncation phenotypes prompted Oishi and colleagues to speculate that these molecules might actually function in a common pathway (Oishi et al., 2003) (Figure 2B). Together, these observations raised the hypothesis that RORs might interact with non-canonical WNTs and function as their cognate receptors. Indeed, these same groups subsequently demonstrated a binding interaction between the ROR2 CRD and WNT5A, albeit with a significantly lower affinity compared to FZD (Mikels and Nusse, 2006; Oishi et al., 2003). Furthermore, genetic interactions between *Cam-1* and non-canonical WNT signaling were established in a series of *C. elegans* studies on neural and vulval development (Chien et al., 2015; Green et al., 2007, 2008; Mentink et al., 2014; Rella et al., 2016). The availability of various *Wnt5a* and *Ror* mutant mice also allowed investigators working in mammalian systems to significantly expand our understanding of the developmental role of WNT5A-ROR signaling in myriad tissues and organs, including the nervous, reproductive, pulmonary and renal systems (Cha et al., 2014; Huang et al., 2014; Laird et al., 2011; Ryu et al., 2013; Zhang et al., 2020).

To further establish the molecular connection between WNT5A and ROR beyond phenotypic resemblance and *in vitro* binding interactions, Ho and colleagues (Ho et al., 2012) theorized that any signaling responses further downstream in the pathway should be similarly disrupted in *Wnt5a* or *Ror* mutant mice. Although several non-canonical WNT responses had been suggested in the literature, such as Dishvelled (DVL) phosphorylation and JNK phosphorylation, few had been thoroughly investigated under true genetic loss-of-function conditions. By comparing these signaling responses in primary mouse embryonic fibroblasts (MEFs) isolated from *Wnt5a* KO mice and a novel *Ror1/Ror2* double conditional mouse strain, the group found that DVL2 phosphorylation is strongly diminished in both mutant cell lines (Figure 3A, B) (Ho et al., 2012). Moreover, the same defect was also observed *in vivo* in *Wnt5a* and *Ror1/2* double KO embryos (Ho et al., 2012). These results not only provided strong evidence that WNT5A and RORs function in the same molecular cascade, but also pointed to DVL as a key regulatory target of WNT5A-ROR signaling. In contrast, other WNT5A-induced responses, such JNK phosphorylation, were not affected in *Ror1/2* double KO MEFs (Ho et al., 2012).

The same group next combined the use of the mutant MEF paradigm with whole-proteome mass spectrometry to identify two additional downstream components of the pathway – the atypical kinesin Kif26b and the ubiquitin E3 ligase Pdzrn3 (Konopelski, 2021; Susman et al., 2017). Importantly, in response to WNT5A-ROR pathway activation, both proteins undergo ubiquitin/proteasome-mediated degradation, an observation that inspired the design of new quantitative WNT5A-ROR signaling assays. By expressing a GFP-Kif26b or a

GFP-Pdzrn3 fusion construct in live cells, the strength of WNT5A-ROR signaling could be quantitatively and reliably measured using flow cytometry (Figure 3C, D). This assay together with DVL2 and ROR phosphorylation, which are also specifically induced by WNT5A signaling, represent a panel of novel readouts crucial for molecular examination of the WNT5A-ROR PATHWAY (Figure 3A, C, D) (Karuna et al., 2018; Konopelski Snavely et al., 2021; Susman et al., 2017).

Functionally, both Kif26b and Pdzrn3 mediate the effect of WNT5A-ROR signaling on cell migration in cultured cells (Guillabert-Gourgues et al., 2016; Konopelski, 2021; Sewduth et al., 2014; Susman et al., 2017). Disruption of Kif26b function *in vivo* also compromised axis elongation in zebrafish and primordial germ cell migration in mice, both WNT5A-dependent processes (Susman et al., 2017). The functions of Kif26b and Pdzrn3 in non-canonical WNT signaling and cell migration appear to be highly conserved, as these proteins were independently identified by the Duplaa group as DVL interacting partners that mediate non-canonical WNT function during endothelial cell migration (Guillabert-Gourgues et al., 2016; Sewduth et al., 2014). Likewise, in a *C. elegans* forward genetic screen conducted by the Garriga group more than two decades ago, Cam-1 and the homolog of Kif26b (Vab-8) were both identified as factors crucial for the directional migration of neurons (Forrester et al., 1998).

We note that even though non-canonical WNT signaling is frequently referred to as WNT/PCP (planar cell polarity) in the literature, whether non-canonical WNT signaling and classic PCP signaling work in series or in parallel remains a long-standing, unresolved question (Veeman et al., 2003). PCP is a highly conserved signaling system originally discovered in *Drosophila* that controls the uniform polarization of a field of cells with respect to the plane of a tissue, typically epithelium. Interestingly, WNT signaling components FZD and DVL were originally identified in *Drosophila* as key determinants of PCP (Adler, 2002). However, whether WNT ligands are required in the establishment of *Drosophila* PCP remains controversial (Ewen-Campen et al., 2020; Wu et al., 2013; Yu et al., 2020), with the most recent evidence arguing against a direct role of WNTs. In vertebrates, there is growing evidence that non-canonical WNT signaling and PCP do intersect extensively in certain biological contexts (Dreyer et al., 2022; Gao et al., 2011; Minegishi et al., 2017), and there is no doubt that both pathways play related morphogenetic roles during development and cancer progression (Hatakeyama et al., 2014; VanderVorst et al., 2019; Wallingford and Mitchell, 2011; Yang and Mlodzik, 2015). However, it remains to be determined whether these interactions reflect cross-talks between two independent core pathways that share common components, or whether they represent different parts of a linear cascade. The phenotypic differences between *Ror1/Ror2* double KO mice (which have no WNT5A-ROR signaling) and *Vangl1/Vangl2* double KO mice (which have no PCP signaling) suggest that at least in the case of WNT5A-ROR signaling in the context of embryogenesis, the former scenario is more likely (Ho et al., 2012). We therefore prefer to avoid the term “WNT/PCP” when describing the non-canonical WNT5A-ROR pathway.

3. Robinow syndrome as a disorder of WNT5A-ROR signaling

Another missing piece in the WNT5A-ROR puzzle came from clinical genetics. 10 years prior to the discovery of WNT, Dr. Meinhard Robinow, an American pediatrician, first described Robinow Syndrome (RS) in 1969, noting that patients with the dwarfing syndrome also exhibited unique combination of phenotypes that include mesomelic limb shortening, hyperbrachydactyly, cleft palate, craniofacial dysmorphisms, hypertelorism and genitourinary defects (Figure 2C). Although no candidate genes were known at the time, Dr. Robinow determined that the newly described syndrome was inherited in an autosomal dominant (AD) manner (Robinow et al., 1969) (we refer to this form of the syndrome as ADRS).

Several decades later, the first RS mutations were finally identified in patients with autosomal recessive Robinow syndrome (ARRS), and they were mapped to the human *ROR2* gene (Afzal et al., 2000). In recent years, mutations in additional RS patient cohorts have been mapped to other known components of the WNT5A-ROR pathway, including *WNT5A* itself, the scaffold proteins *DVL1*, *DVL2* and *DVL3*, and the Frizzled receptor *FZD2* (Bunn et al., 2015; Danyel et al., 2018; Nagasaki et al., 2018; Person et al., 2010; Roifman et al., 2015; White et al., 2015; White et al., 2018; White et al., 2016). Importantly, in contrast to the homozygous *ROR2* loss-of-function mutations associated with ARRS, the mutations reported in *WNT5A*, *DVL1*, *DVL2*, *DVL3*, and *FZD2* are all heterozygous and autosomal dominant, suggesting that disrupting the pathway in multiple, unique ways can result in the manifestation of RS.

Remarkably, a frameshift mutation in the canine *DVL2* gene was recently identified in screw-tail dog breeds (colloquially known as bulldogs, French bulldogs and Boston terriers, hereafter referred to as bulldogs; a French bulldog is shown in Figure 2D) (Mansour et al., 2018). Bulldogs are characterized by their shortened and kinked tails (“screw tails”) that arise from vertebral malformations such as fused and/or absent vertebrae (Mansour et al., 2018). In addition to a variety of skeletal malformations, bulldogs also exhibit many other morphological similarities to RS patients, such as short limbs and stature as well as several craniofacial features like flat, short and wide faces, and increased susceptibility to cleft palate. In fact, a recent study further suggested that the *DVL2* mutation contributes to the craniofacial malformations of these dogs (Niskanen et al., 2021). Together, these striking morphological and genetic similarities suggest that bulldogs have a canine version of RS.

4. Molecular insights from Robinow syndrome and related disease mutations

The RS patient and animal reports underscore the increasingly recognized importance of WNT5A-ROR signaling to proper tissue morphogenesis. The growing number of patient mutations also emphasizes a need to deepen our mechanistic understanding of WNT5A-ROR signaling, which will provide insight into the molecular underpinnings that drive RS and facilitate modeling of RS patient mutations. This section will delve into each protein associated with RS or an RS-related congenital disorder, reviewing known molecular

mechanisms and proposing other potential mechanisms by which reported mutations might alter protein function and downstream signaling.

WNT5A

While the first ADRS patients described by Dr. Robinow lacked a molecular diagnosis for several decades (Robinow et al., 1969), reevaluation of the same family identified a missense mutation in the *WNT5A* gene (Person et al., 2010). Since then, many additional *WNT5A* mutations have been identified (Figure 4A), and two of these, $WNT5A^{C83S}$ and $WNT5A^{C182R}$, were analyzed in more detail (Gignac et al., 2019; Hosseini-Farahabadi et al., 2017; Person et al., 2010; Roifman et al., 2015; White et al., 2018). Through overexpression experiments in zebrafish and *Xenopus*, the $WNT5A^{C83S}$ and $WNT5A^{C182R}$ variants were initially described as hypomorphs (Person et al., 2010). However, other work using similar approaches in the chick jaw and limbs suggested that these variants function in a dominant negative manner (Gignac et al., 2019; Hosseini-Farahabadi et al., 2017). Since WNT5A signaling is known to operate within a narrow dose range and under tight spatiotemporal control, the discrepancy between these studies could be due to the use of different model organisms or to differences in experimental conditions.

Based on homology to the published three-dimensional structure of WNT8 (Janda et al., 2012), WNT5A is predicted to have 10 disulfide bonds formed from 20 of the 24 highly conserved cysteine residues (Figure 4A) Notably, nearly all of the reported ADRS-*WNT5A* variants, including the two discussed above, result in the conversion of cysteine residues to other amino acids or vice versa, and the few remaining mutations that do not directly convert amino acids to or from cysteine residues occur in close proximity to existing cysteines. It seems plausible that structural changes caused by disruption of these conserved bonds could lead to the sequestration of WNT5A ligands in the endoplasmic reticulum (ER). How does this mechanism potentially explain the dominant interfering effects then? One possibility is that since WNTs require several modifying enzymes and chaperons for maturation and secretion (Clevers and Nusse, 2012), retention of the improperly folded WNTs in the ER may jam up these enzymes and chaperones, thereby disrupting biogenesis of the wild-type WNT5A pool. Another possibility is that even if some of the mutant WNT5A ligands are secreted, they might bind the receptors but not signal, thereby preventing the receptors from engaging the wild-type WNT5A proteins. Yet another scenario is that the mutant WNT5A might exhibit increased affinity for its receptors, resulting in hyperactivation of the pathway. Evaluation of these possibilities through more direct signaling assays that do not rely on overexpression should help resolve the mechanisms of these pathogenic WNT5A variants.

ROR2

ROR2 is the component of WNT5A-ROR signaling most frequently mutated in RS. The inherence pattern of ROR2-driven RS is recessive, and the clinical features also closely mirror those of *Ror2* null mice (Ali et al., 2007; Chen et al., 2005; Schwabe et al., 2004). This strongly suggests that a loss-of-function mechanism is at play. Interestingly, domesticated pigeons with extra short beaks were recently shown to carry *ROR2* variants analogous to human RS mutations (Boer et al., 2021), underscoring how highly conserved the WNT5A-ROR signaling pathway is within the animal kingdom.

There is tremendous diversity in *ROR2* ARRS mutations, both in terms of location within the protein and type of mutation. The structure of ROR2 includes an N-terminal extracellular region consisting of an immunoglobulin-like domain (Ig) followed by a FZD-like cysteine rich domain (CRD) and a Kringle domain (Kr), connected via one transmembrane (TM) domain to the intracellular region that includes a tyrosine kinase domain (TK), serine/threonine-rich domains (S/TRD) and a proline-rich domain (PRD) (Figure 4B) (Masiakowski and Carroll, 1992; Minami et al., 2010; Stricker et al., 2017). ARRS mutations in *ROR2* are scattered throughout the entire gene and can include missense, nonsense, deletion, and frameshift mutations. The simplest interpretation is that all these mutations cause functional inactivation of the ROR2 protein. A predominant mechanism for this inactivation involves trapping of the misfolded ROR2 in the ER, leading to a loss-of-function phenotype. Indeed, three mechanistic studies have demonstrated that several ARRS-*ROR2* variants ($ROR2^{C182Y,\ R184C,\ R189W,\ Y192D,\ R244W,\ R366W,\ N620K}$) co-localize with ER markers and fail to localize to the plasma membrane (Ali et al., 2007; Chen et al., 2005; Griffiths SC, 2021). These results therefore suggest that ARRS mutations can cause misfolding of ROR2, rendering it incapable of passing through the secretory pathway to reach the cell surface.

It is important to note that these molecular studies have analyzed only a small subset of all the ARRS-*ROR2* mutations reported, and other types of mutations (including nonsense, frameshift and deletions) located in other domains remain unexamined (Figure 4B). These additional mutations could potentially prevent or reduce direct interactions with WNT5A, FZD or downstream signaling components such as DVL, and all of these scenarios would ultimately lead to a reduction in signaling and thus a loss-of-function phenotype. In fact, there is evidence of such a mechanism in the developing short-beaked pigeon. Expression analyses indicated no changes in *ROR2* expression between domesticated pigeons with short beaks versus those with medium or long beaks, suggesting that the mutation itself, rather than changes in expression levels, contributes to this morphological feature (Boer et al., 2021). Additional systematic evaluation of other ARRS-ROR2 variants, particularly those with longer deletions or frameshifts, and their interactions with binding partners, should be undertaken to further test this model.

Interestingly, RS is not the only congenital disorder caused by *ROR2* mutations. In 2000, a different cohort of *ROR2* mutations were identified in patients with brachydactyly type B1 (BDB1) (Schwabe et al., 2000). Brachydactyly is often a clinical symptom of RS, and by itself constitutes a separate congenital disorder characterized by incomplete development or absence of the outermost bones in the fingers and toes (Temtamy and McKusick, 1978). Brachydactyly can be classified into several types, and brachydactyly type B1 (BDB) is the most severe type and includes absence of fingernails, symphalangism (fusion of the phalanges), hypoplasia of the distal and middle phalanges (digits 2–5), and flattening, splitting, or duplication of the distal phalanges of the thumb (Schwabe et al., 2000; Temtamy and Aglan, 2008).

An important genetic distinction is that unlike *ROR2*-driven ARRS, *ROR2* mutations that cause BDB1 are autosomal dominant in nature. In addition, the types and locations of BDB1 and ARRS *ROR2* mutations also differ. As mentioned previously, ARRS *ROR2* mutations

are scattered throughout the protein and represent many diverse mutation types. In contrast, BDB1 mutations are typically nonsense or frameshift mutations that cluster immediately before or after the intracellular TK domain, truncating the C-terminus to varying degrees (Figure 4B).

The most thorough comparison of BDB1-*ROR2* and ARRS-*ROR2* variants was done by Schwarzer and colleagues (Schwarzer et al., 2009), who proposed that the amount of ROR2 present at the cell membrane ultimately determines the phenotypic outcome. The authors demonstrated that ARRS-ROR2 variants (ROR2$^{Q520X,\ N620K,}$ and ^{W720X}) act in a loss-of-function manner due to protein folding issues that lead to their retention in the ER, findings which are consistent with previous reports (Ali et al., 2007; Chen et al., 2005). In contrast, BDB1-ROR2 variants (ROR2$^{W749X,\ Q467fsX57,}$ and ^{R441fsX15}) reach the cell membrane, where they exert a dominant, gain-of-function effect on downstream signaling. In cases where ARRS or BDB1 patients display intermediate phenotypes, variants such as ROR2^{R441X} and ROR2^{R441fsX15} can be observed both at the cell membrane and in the ER. Collectively, these observations suggest that too much ROR2 being trapped in the ER results in ARRS, essentially acting as a loss-of-function and tipping the scale towards ARRS, while sufficient amounts of mutated ROR2 reaching the membrane results in dominant, deleterious effects on downstream signaling resulting in BDB1 (Schwarzer et al., 2009).

DISHEVELLEDS

While early studies largely attributed RS to mutations in *WNT5A* and *ROR2*, several recent studies have identified mutations in all members of the DVL family of scaffold proteins, which drive signal transduction in multiple WNT signaling pathways. Each of the three family members – DVL1, DVL2, and DVL3 – possesses three key modular domains: DIX, PDZ, and DEP (Figure 4C). The DIX and DEP domains are considered essential for interactions with effector proteins specific to canonical and non-canonical WNT signaling, respectively, whereas the PDZ domain is used in multiple modes of signaling (Axelrod et al., 1998; Boutros et al., 1998; Gentzel and Schambony, 2017; Sokol, 1996; Tauriello et al., 2012). While the regions connecting these modular domains tend to be more variable (Gentzel and Schambony, 2017), the C-terminal 30 amino acids, termed “extreme C-terminus”, are highly conserved across each paralog as well as across species, and they comprise a PDZ domain-binding motif (Lee et al., 2015; Qi et al., 2017).

Mutations in all three DVL family members have been found in RS patients. Remarkably, in almost all cases, these mutations are heterozygous −1 frameshifts located in the penultimate or ultimate exon of the corresponding *DVL* genes that result in the generation of a novel sequence in this extreme C-terminus, while leaving the more N-terminal DIX, DEP and PDZ domains intact (Figure 4C) (Bunn et al., 2015; White et al., 2015; White et al., 2018; Zhang et al., 2022; Zhang et al., 2021). Even the bulldog *DVL2* mutation shares this same molecular signature, with a −1 frameshift in the penultimate exon resulting in a novel C-terminus (Mansour et al., 2018).

Although the mechanisms governing DVL-driven RS are still only partially understood, existing data suggest that neither mRNA nor protein instability contribute to the ADRS phenotype (Bunn et al., 2015; Mansour et al., 2018; White et al., 2015; White et al., 2016).

These findings, together with the dominant inheritance pattern of the mutations, suggest that a dominant interfering effect may be affecting other wild-type DVL family members or other pathway components, rather than a simple loss-of-function mechanism. This concept is further supported by the observations that *Dvl1* and *Dvl3* single null mutant mice lack RS-like phenotypes (Etheridge et al., 2008; Hamblet et al., 2002; Lijam et al., 1997), suggesting a more complex pathogenic mechanism.

It also remains possible that ADRS and bulldog *DVL* mutations alter multiple WNT signaling pathways. Unlike WNT5A and ROR receptors, DVL scaffold proteins have well established roles in both canonical and non-canonical WNT pathways, as well as in PCP. When overexpressed with wild-type DVL1 in a heterologous system, the RS DVL1 variant was shown to increase canonical WNT signaling (Bunn et al., 2015). This finding is consistent with data showing that overexpressed bulldog DVL2 exhibits reduced phosphorylation in response to treatment with recombinant WNT3A or WNT5A, suggesting that potentially both WNT pathways may be disrupted (Mansour et al., 2018). However, beyond these studies, data indicating potential signaling defects in non-canonical WNT5A-ROR signaling *per se* have not been reported. More precise genetic manipulations, coupled with more quantitative signaling readouts, will be needed to fully characterize the deleterious effects of *DVL* mutations.

FRIZZLED 2

Although *FZD2* was one of the most recent genes to be mapped in RS, it was previously shown to cause autosomal dominant omodysplasia (ADO), another rare skeletal syndrome with associated craniofacial and genitourinary features (Nagasaki et al., 2018; Saal et al., 2015; Turkmen et al., 2017). In fact, RS and ADO patients share several physical traits, including short stature and limbs in addition to facial characteristics such as hypertelorism, midface hypoplasia, and anteverted nares. These highly similar physical traits, coupled with incomplete penetrance, frequently make it difficult to distinguish ADO from ADRS (Maroteaux et al., 1989; Venditti et al., 2002). Given these highly similar phenotypes and their shared genetics, Zhang and colleagues recently suggested that ADO should be considered a form of ADRS (Zhang et al., 2022). Therefore, we will refer to all ADO *FZD2* mutations as ADRS mutations.

ADRS *FZD2* mutations are primarily missense and frameshift mutations resulting in C-terminal truncations. FZD2, like all FZD receptors, is a seven-pass transmembrane receptor that consists of an extracellular N-terminal domain followed by the seven TM helices with the intervening alternating intracellular and extracellular loops, the last of which culminates in an intracellular C-terminal domain (Figure 4D). Based on this protein topology, FZD2 mutations are predicted to alter binding interactions with downstream cytoplasmic partners, such as DVL (Figure 4D) (Djiane et al., 2005; Saal et al., 2015; Tauriello et al., 2012; Umbhauer et al., 2000). While the most plausible hypothesis is that altered protein-protein interactions would specifically disrupt non-canonical WNT5A-ROR signaling, given that both FZD and DVL also mediate canonical WNT signaling it remains possible that FZD2-driven ADRS arises from some combination of canonical and non-canonical WNT dysfunction.

Of the 10 mammalian *FZD* genes, *FZD2* is in a subfamily together with *FZD1* and *FZD7* (Yu et al., 2010; Yu et al., 2012). This raises the the possibility that additional ADRS mutations might be identified in *FZD1* and/or *FZD7* in the future.

5. Growing connections to cancer metastasis

Like many developmental signaling pathways, WNT5A-ROR signaling is hijacked by cancer cells to drive their oncogenic phenotypes. However, unlike other well studied oncogenic pathways, which often promote tumor initiation and growth through transcription-dependent mechanisms, the WNT5A-ROR pathway appears to specifically drive later stages of oncogenic progress, namely metastasis (Anastas et al., 2012; Asad et al., 2014; Fukuda et al., 2008; Kipps, 2022; Pukrop et al., 2006; Weeraratna et al., 2002). To date, many cancer types have been shown to exhibit elevated expression of WNT5A-ROR signaling components (Asem et al., 2016). For instance, the WNT5A ligand itself is overexpressed in several malignant tumors, most notably gastric cancer, melanoma, pancreatic carcinoma and glioblastoma (Binda et al., 2017; Da Forno et al., 2008; Kurayoshi et al., 2006; Ripka et al., 2007). WNT5A overexpression is also strongly correlated with increased malignant characteristics and poorer prognoses (Da Forno et al., 2008; Weeraratna et al., 2002). Likewise, overexpression of ROR1 or ROR2 has also been described in several cancer types (Baskar et al., 2008; Edris et al., 2012; Morioka et al., 2009; O'Connell et al., 2010; O'Connell et al., 2013; Wright et al., 2009). Significantly, a firm causal relationship between WNT5A-ROR signaling and metastasis *in vivo* was recently established (Obradovic et al., 2019). Specifically, the study showed that *ROR1* is among the genes that are highly expressed during glucocorticoid-induced breast cancer metastasis, and that experimental inhibition of *ROR1* expression reversed this metastatic phenotype.

Interestingly, it was observed early on that normal adult tissues typically lack, or have very low, ROR1 expression (Baskar et al., 2008; Fukuda et al., 2008). However, a number of cancers, including chronic lymphocytic leukemia (CLL), express high levels of ROR1 (Daneshmanesh et al., 2008; Fukuda et al., 2008; Hojjat-Farsangi et al., 2014; Janovska and Bryja, 2017). Moreover, infusion of autologous CLL cells in human patients was found to stimulate the production of anti-ROR1 antibodies (Fukuda et al., 2008). ROR1 is therefore commonly referred to as an oncofetal antigen. This unique expression pattern also suggested that ROR1 can be exploited not only to mark cancer cells but also to kill them, while sparing normal, healthy cells (Kipps, 2022).

Indeed, ROR1 has shown immense promise as a therapeutic target. Zilovertamab (formerly known as cirmtuzumab), a ROR1 neutralizing monoclonal antibody, has successfully completed stage I clinical trials as a treatment option for CLL (Choi et al., 2018). Zilovertamab has also been shown to reduce the growth of breast patient-derived xenograph (PDX) tumors in mice, as well as the formation of secondary tumors upon re-engraftment of treated cells (Zhang et al., 2019). Further, the combined use of zilovertamab with paclitaxel, a classic chemotherapy drug, or other small molecule inhibitors, can generate an additive anti-cancer effect compared to each agent alone in multiple tumor types (Zhang et al., 2019). Lastly, a role for ROR1 in immunotherapy as a target for chimeric antigen receptor (CAR) T cell therapy (Gohil et al., 2017; Wallstabe et al., 2019) was described recently,

and conjugated ROR1 antibodies have been developed into tumor diagnostic tools (Milani et al., 2018). Collectively, these studies demonstrate that selective targeting of ROR1 is a promising treatment option for aggressive cancers with limited cytotoxic side effects (Kipps, 2022).

6. Cell biological functions of WNT5A-ROR signaling

In order to understand how WNT5A-ROR signaling orchestrates development and contributes to diseases, one must also understand its cell biological functions, which are still not characterized in depth. A large body of literature has connected non-canonical WNT signaling to the regulation of convergent extension (CE) during tissue morphogenesis (Heisenberg et al., 2000; Matsui et al., 2005; Tada et al., 2002; Wallingford et al., 2001). CE refers to a process by which a tissue narrows along one axis while lengthening along a perpendicular axis. As one of the best described mechanisms for tissue lengthening, CE plays a crucial role during gastrulation, neurulation, axis formation and likely many other tissue elongation processes (Keller et al., 1985; Wallingford et al., 2002). At the cell biological level, CE involves two related cell behaviors: 1) directed cell migration, a process by which a group of cells polarize and move together in the same direction with little or no exchange of neighbors, and 2) cell intercalation, a process by which mediolateral movements of individual cells within a tissue drive exchange of neighbors, resulting in elongation of the tissue in the anterior-posterior direction (Tada and Heisenberg, 2012; Wallingford et al., 2002). Several early studies implicated WNT5A or WNT5A-ROR signaling in aspects of CE during *Xenopus* and zebrafish embryogenesis (Hikasa et al., 2002; Schambony and Wedlich, 2007; Wallingford and Harland, 2001; Wallingford et al., 2001). In mice, both directed cell migration and cell intercalation have also been observed during morphogenesis of the limb buds, the branchial arches and the palatal shelves, and perturbation of WNT5A function disrupts these characteristic cell behaviors (Gros et al., 2010; He et al., 2008; Tao et al., 2019).

Both directed cell migration and cell intercalation require the precise coordination of multiple cytoskeleton-dependent subcellular processes. During directed cell migration, cells need to uniformly polarize their actin-rich leading edge while migrating as a group. Cell motility itself requires cycles of cell adhesion/deadhesion as well as contractile activity to physically advance the cell body forward. Likewise, during intercalation, cells not only have to polarize and move, but also need to contract their junctions with neighboring cells. Collectively, these cytoskeletal processes provide the mechanical forces necessary to sculpt tissue shape. The key question that remains, however, concerns which of these cytoskeleton-dependent processes – namely motility, adhesion, polarization and contractility – are subject to direct regulation by WNT5A-ROR signaling, and how. The interdependent nature of these processes makes this question all the more challenging to address.

Several existing lines of evidence point to the actomyosin-based contractility machinery as a plausible target of WNT5A-ROR2 signaling. RhoA, a well established regulator of the actomyosin system, has long been implicated in non-canonical WNT function. In zebrafish, experimental suppression of RhoA expression causes the same shortened anterior-posterior axis and tail malformations seen previously in the *WNT5 pipetail* mutants, and

overexpression of RhoA was sufficient to rescue the CE defects of *WNT5* and *WNT11* mutants (Zhu et al., 2006). Experiments conducted in cancer cell lines and other *in vitro* cell culture systems have also shown that WNT5A can activate RhoA and influence aspects of cell migration (Karvonen et al., 2019; Wu et al., 2019; Zhang et al., 2017; Zhu et al., 2012). A subset of these studies further demonstrated that WNT5A can increase the phosphorylation of myosin light chain (pMLC) (Alcantara et al., 2021; Tao et al., 2019), a key target of RhoA, leading to increased focal adhesion stability, actin stress fiber formation and cell contractility. Taken together, these data raised the intriguing hypothesis that WNT5A-ROR signaling might directly impinge on the RhoA-myosin-actin axis to modulate contractility and other biomechanical properties of the cell.

Indeed, direct *in vivo* evidence supporting this model came from a recent study by Tao and colleagues on the role of WNT5A in mandible morphogenesis (Tao et al., 2019). Using an elegant combination of physical measurements, light sheet microscopy and mouse genetics, the investigators showed that 3D mesenchymal cell intercalations are required to shape the mandibular arch. Importantly, WNT5A is required to coordinate actomyosin polarity and oscillatory cortical forces, which in turn decrease tissue rigidity and the energy barrier to cell intercalation. Interestingly, this study along with an earlier study reported that the mechanosensor proteins YAP/TAZ are activated in response to WNT5A signaling (Park et al., 2015; Tao et al., 2019), again supporting the idea that regulation of mechanobiology, possibly through the RhoA-myosin-actin axis, may be a primary cell biological function of the WNT5A-ROR pathway.

Besides tissue elongation, WNT5A-ROR signaling functions in many other developmental processes, ranging from primordial germ cell migration to embryo implantation to neuronal wiring (Cha et al., 2014; Chawengsaksophak et al., 2012; Laird et al., 2011; Ryu et al., 2013). It is presently unclear to what extent the underlying cell biological mechanisms are shared among these processes. Future investigations into the primary cell biological functions of WNT5A-ROR signaling in different developmental and pathological contexts should provide the much needed clarity. Moreover, as the influence of cell contractility and adhesion on higher-order cell behaviors such as migration and intercalation can vary greatly depending on the cell types and experimental contexts, this line of investigation should also help explain why WNT5A-ROR signaling has been reported to promote cell motility in some experiments but inhibit it in others (Bakker et al., 2013; Prasad et al., 2016; Wang et al., 2019; Wang et al., 2018). Such investigations may similarly shed light on the paradoxical observations that WNT5A can exert tumor suppressive as well as tumor promoting activities, even within the same cancer type (Fernandez-Cobo et al., 2007; Jonsson et al., 2002).

7. Concluding remarks

While the past two decades have witnessed tremendous progress in defining WNT5A-ROR signaling as a core non-canonical WNT pathway, many fundamental questions remain. In this final section, we outline what we believe represent some of the most significant outstanding questions in the field.

1. *What is the biochemical nature of the WNT5A receptor complex and the mechanism of signal transduction?* Despite the fact that ROR proteins' requirement in WNT5A signaling has now been well established, we still do not understand how they function at the mechanistic level to transmit the WNT5A signal. Recent structure-functions studies have revealed that the mechanism of WNT5A-ROR interaction is distict from that of the previously described WNT-FZD interaction (Griffiths SC, 2021; Janda et al., 2012; Shi et al., 2021), and that ROR and FZD proteins may function together as part of a lager receptor super-complex to carry the WNT5A signal across the plasma membrane (Griffiths SC, 2021). The precise mechanisms underlying these ligand-receptor interactions, however, remain to be elucidated. Moreover, as the kinase domain of ROR proteins is believed to be catalytically inactivate (Mendrola et al., 2013; Sheetz et al., 2020), it will also be interesting to characterize the functional roles of this and other ROR cytoplasmic domains during WNT5A signal transduction.

2. *How is the WNT5A-ROR signal propagated in the cytoplasm?* Once inside the cytoplasm, little is known about how the WNT5A signal is processed, integrated and relayed. Though DVL is believed to play a central role in this process, more mechanistic information is needed. For example, DVL has been shown to interact with both the cytoplasmic tails of FZD and ROR2, but the functional significance of these interactions is not yet fully characterized. Though several molecules, including Daam1, RhoA, Kif26b and Pdzrn3, have been implicated in WNT5A regulation of the cytoskeleton (Guillabert-Gourgues et al., 2016; Habas et al., 2001; Konopelski Snavely et al., 2021; Sewduth et al., 2014; Susman et al., 2017; Zhu et al., 2012), the signaling cascade that links the upstream WNT5A signal to the downstream cytoskeletal machineries is far from being mapped out. Progress in this area will benefit greatly from identifying and characterizing additional molecules that participate in the pathway.

3. *What are the cytoskeletal effectors of the WNT5A-ROR pathway?* In the previous section, we discussed recent evidence supporting an emerging role of the RhoA-myosin-actin axis in WNT5A-ROR regulation of cell contractility. However, many other cell biological effects of WNT5A-ROR signaling, such as cell migration, adhesion and polarization, have been described. We do not yet know whether these represent fundamentally distinct processes controlled by different effectors, or whether they are overlapping processes controlled by the same or a few shared cytoskeletal regulators downstream of WNT5A-ROR signaling. Ultimately, we also need to understand how the WNT5A-ROR signaling activity is coordinated in time and space in the developing tissue, and through cross-talk with other signaling systems to orchestrate proper morphogenesis.

4. *What can disease mutations teach us about the normal function and regulation of the WNT5-ROR pathway?* It is interesting that even though *Drosophila* genetic screens did not provide many clues about the WNT5A-ROR pathway, the large number of naturally occurring RS mutations that exist in humans and the related mutations in animal populations can be leveraged as a powerful

discovery tool. Importantly, some RS patients may carry mutations in genes that have not yet been linked to the WNT5A-ROR pathway. Mapping these genes could help identify novel players in the pathway. Similarly, many of the known RS mutations are likely to be dominant interfering in nature. Illuminating which specific steps of WNT5A-ROR signaling are being "interfered" with should teach us about the normal regulatory mechanisms of the pathway.

5. *How are specificities between canonical and non-canonical WNT signaling achieved?* It is perhaps not far-fetched to speculate that the canonical and non-canonical WNT pathways evolved from a common ancestral pathway. After all, they share several key constituents, including WNTs, FZDs and DVLs. However, each of these pathways exhibits exquisite specificity, and we are only beginning to comprehend how these specificities are achieved (Sonavane and Willert, 2021). One level of specificity can be achieved through non-promiscuous WNT-FZD pairing, but among the 19 WNTs and 10 FZDs present in mammals, we do not yet fully understand which of these are selectively involved in canonical versus non-canonical signaling, and which WNT-FZD combinations occur in the *in vivo* milieu. Another level of specificity can be achieved through the utilization of pathway-specific co-factors, such LRP5/6 in canonical and ROR1/2 in non-canonical signaling. However, much remains to be learned about how LRPs and RORs differentially interact and synergize with FZDs to generate unique signaling outcomes. Likewise, DVLs might have also evolved to recruit different cohorts of interacting partners during canonical versus nonconical WNT signaling to specify the signals that flow through each pathway. Understanding WNT5A-ROR signaling thus represents a critical step toward understanding how WNT pathway specificities are achieved and how the WNT signaling system evolved.

It is our hope that with recent advances in experimental methodologies, such as high-throughput genome sequencing, quantitative proteomics, CRISPR/Cas9 and super-resolution microscopy, we are better positioned than ever before to access the wealth of knowledge about WNT5A-ROR signaling that still awaits to be discovered. It is also our belief that by better understanding WNT5A-ROR SIGNALING, we will gain broad new perspectives on the mechanisms of development, cytoskeletal regulation and pathogenesis that it controls.

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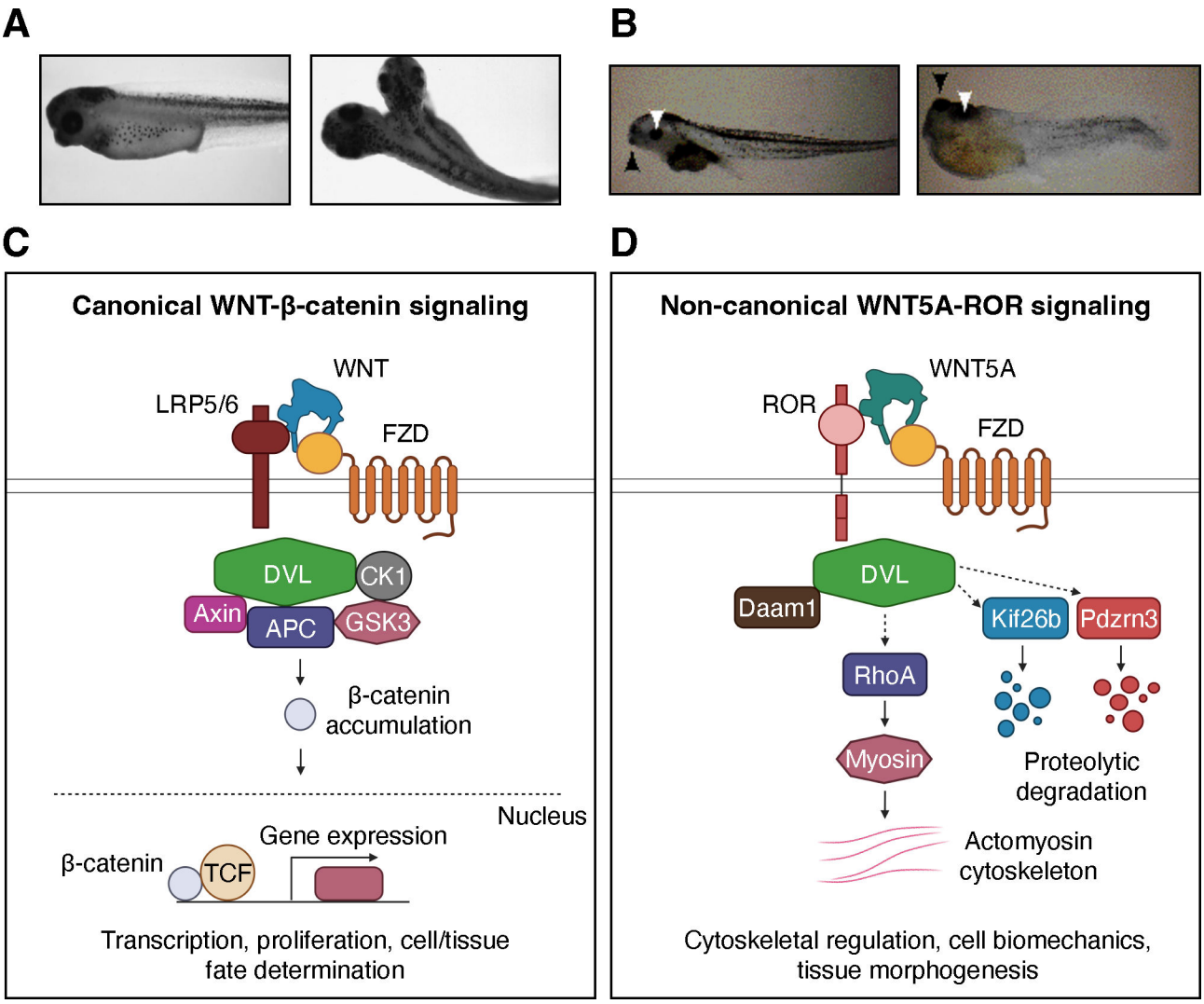

Figure 1. Overview of canonical WNT-β-catenin and non-canonical WNT5A-ROR signaling.

WNT signaling was initially classified into two major branches based on the distinct phenotypes induced by overexpression of different WNT ligands in *Xenopus laevis*. **(A)** The axis duplication phenotype induced by overexpression of canonical WNTs. Image from (Cui et al., 1995) in which *Xenopus* WNT8B was overexpressed. **(B)** The gross tissue truncation/malformation phenotype induced by overexpression of noncanonical WNTs. Image from (Moon et al., 1993) in which *Xenopus* WNT5A was overexpressed. **(C and D)** Molecular schematics of the canonical WNT-β-catenin (C) and non-canonical WNT5A-ROR (D) signaling cascades. See main text for details. Created using www.BioRender.com

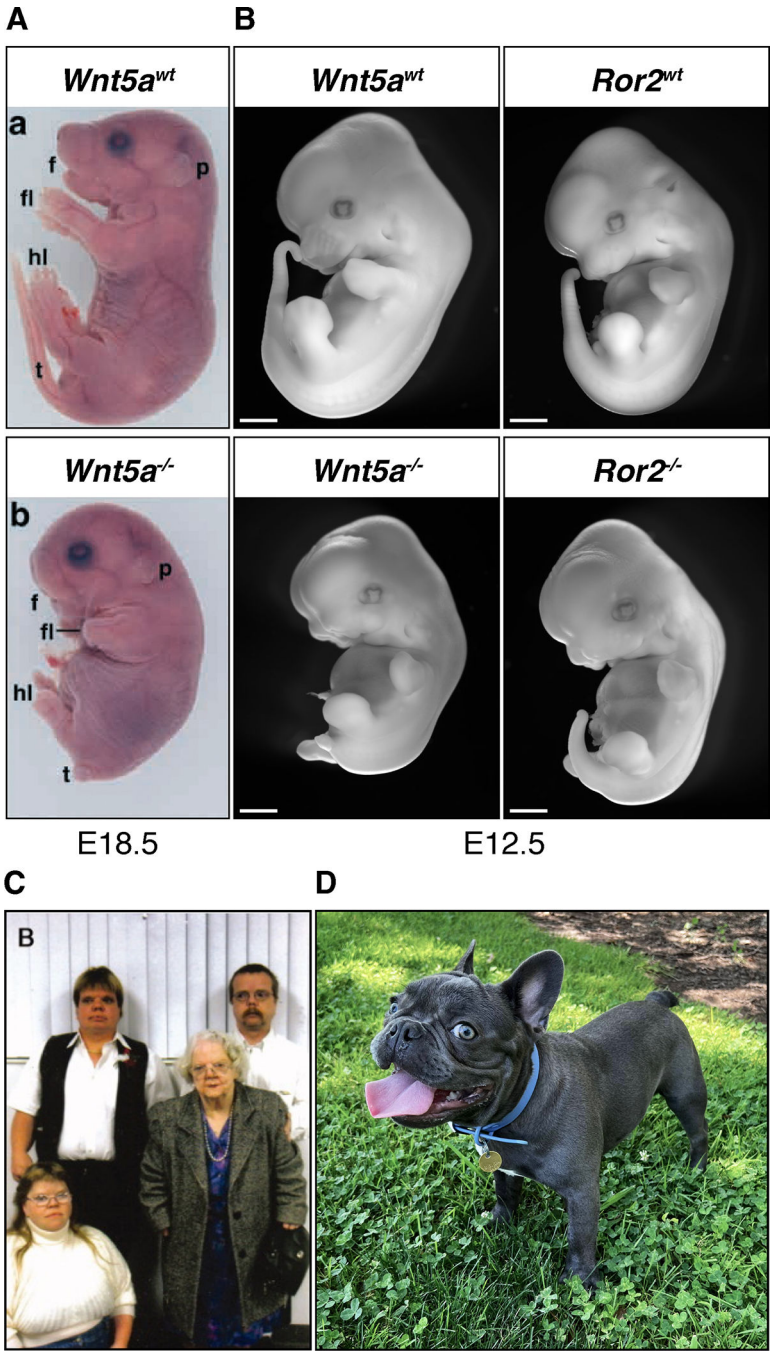

Figure 2. Robinow syndrome and Robinow-like disorders arise from mutations in components of WNT5A-ROR signaling.

(A) *Wnt5a* knockout mouse embryos (E18.5, images from (Yamaguchi et al., 1999)) exhibit body axis, limb, and tail truncations in addition to underdeveloped external genitalia and craniofacial malformations, compared to their wild-type littermates. These physical features are phenocopied in *Ror2* knockout mouse embryos (**B;** E12.5 embryos derived from previously described *Wnt5a* (Yamaguchi et al., 1999) and *Ror2* (Ho et al., 2012) mutant

lines, photographed by S. Srinivasan. Scale bar = 1mm), as well as in RS patients (**C;** image from (Person et al., 2010), photographed by J. Lohr**)** and bulldogs (**C**; French bulldog).

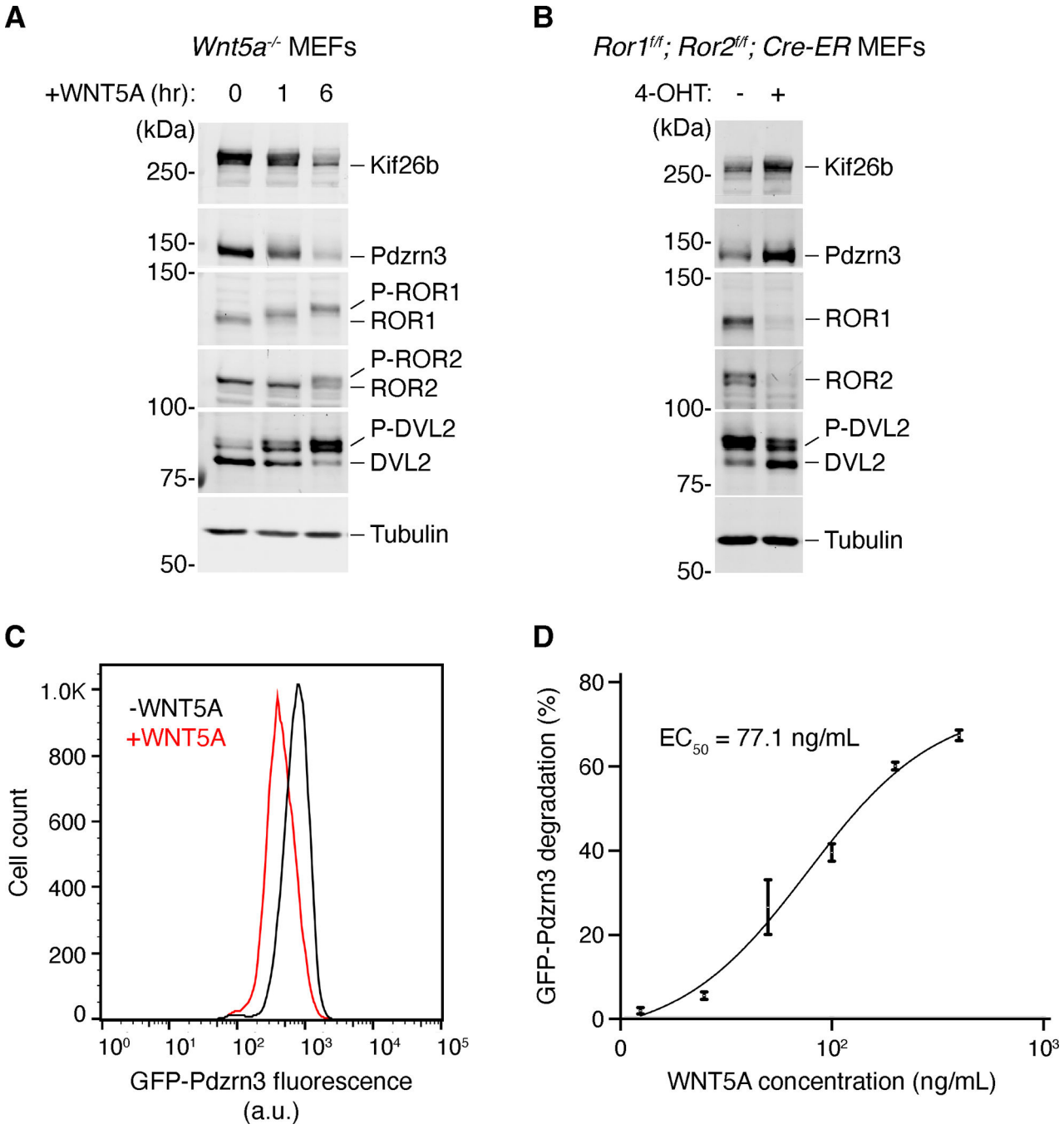

Figure 3. Molecular responses to WNT5A-ROR signaling.

Several downstream targets of WNT5A-ROR signaling have recently been identified, enabling molecular examination of the WNT5A-ROR signaling pathway. **(A)** Western blot analysis of *Wnt5a* knockout MEFs stimulated with recombinant WNT5A. WNT5A induces the phosphorylation of DVL2, ROR1 and ROR2 (P-DVL2, P-ROR1, P-ROR2), as well as the degradation of Kif26b and Pdzrn3 (Ho et al., 2012; Konopelski Snavely et al., 2021; Susman et al., 2017). **(B)** Combined genetic deletion of *Ror1* and *Ror2* results in reduction of DVL2 phosphorylation, as well as stabilization of Kif26b and Pdzrn3 (Ho et al., 2012;

Konopelski Snavely et al., 2021; Susman et al., 2017). Genenetic deletion of *Ror1* and *Ror2* was achieved by treating MEFs carrying *Ror1* and *Ror2* conditional ($^{f/f}$) alleles as well as a transgene encoding the tamoxifen-inducible CRE-ER, with the tamoxifen analog 4-hydroxy-tamoxifen (4-OHT). **(C)** WNT5A-ROR signaling can also be quantitatively assayed by monitoring the fluorescence of a GFP-Pdzrn3 degradation reporter via flow cytometry (Konopelski Snavely et al., 2021). Histograms of a representative flow cytometry experiment showing the fluorescence decrease in MEFs expressing the GFP-Pdzrn3 reporter following WNT5A stimulation. **(D)** Dose-response curve showing GFP-Pdzrn3 degradation as a function of WNT5A concentration (Konopelski Snavely et al., 2021).

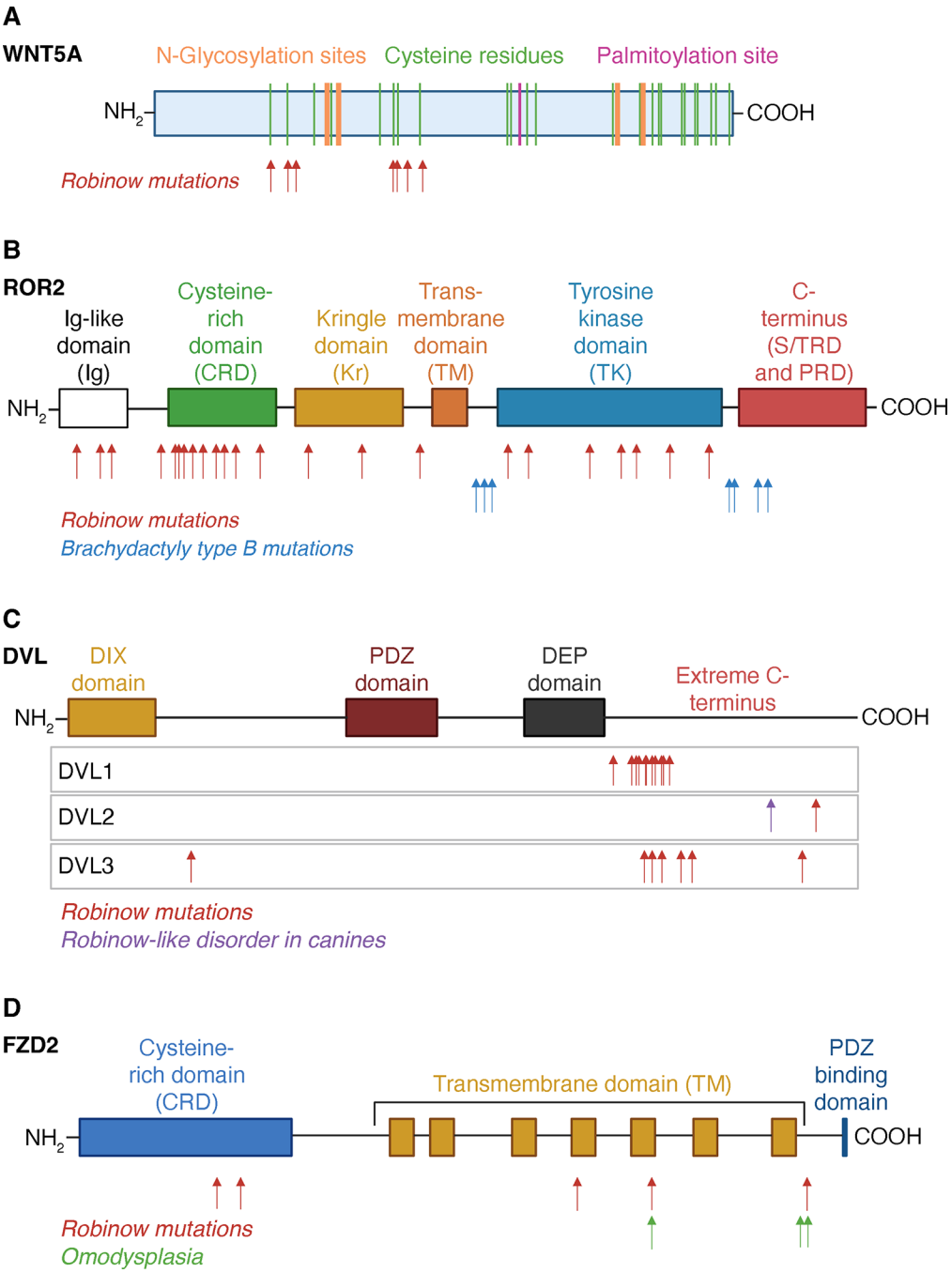

Figure 4. Schematics of protein structures for WNT5A, ROR2, DVLs and FZD2, and the locations of mutations that cause Robinow syndrome and Robinow syndrome-like disorders.

(A) WNT5A possesses an N-terminal signal peptide sequence and 24 highly conserved cysteine residues, 20 of which are involved in the formation of 10 disulfide bonds. WNT5A is also post-translationally modified by N-glycosylation and palmitoylation. Locations of known mutations that cause RS are noted. **(B)** ROR2 possesses an N-terminal signal peptide, extracellular immunoglobulin (Ig), cysteine-rich (CRD), and kringle (Kr) domains, a transmembrane (TM) domain, and intracellular tyrosine kinase (TK), C-terminal serine/

threonine-rich (S/TR) and proline-rich (PR) domains. Locations of known mutations that cause RS and BDB are noted. **(C)** DVL proteins possess three modular domains, an N-terminal DIX domain, internal PDZ and DEP domains, and a highly conserved extreme C-terminus. Locations of known mutations that cause RS in humans and a RS-like disorder in canine are noted. **(D)** FZD2 is a seven-pass transmembrane receptor with an extracellular N-terminus that includes the cysteine-rich domain (CRD) that binds to WNTs, followed by the seven-pass transmembrane domain and an intracellular C-terminus that includes a DVL PDZ binding domain. The seven transmembrane helecies are linked by three extracellular loops and three intracellular loops. Locations of known mutations that cause RS and ADO are noted. Created using www.BioRender.com