Wnt5a–Ror–Dishevelled signaling constitutes a core developmental pathway that controls tissue morphogenesis

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Wnts make up a large family of extracellular signaling molecules that play crucial roles in development and disease. A subset of noncanonical Wnts signal independently of the transcription factor β-catenin by a mechanism that regulates key morphogenetic movements during embryogenesis. The best characterized noncanonical Wnt, Wnt5a, has been suggested to signal via a variety of different receptors, including the Ror family of receptor tyrosine kinases, the Ryk receptor tyrosine kinase, and the Frizzled seven-transmembrane receptors. Whether one or several of these receptors mediates the effects of Wnt5a in vivo is not known. Through loss-of-function experiments in mice, we provide conclusive evidence that Ror receptors mediate Wnt5a-dependent processes in vivo and identify Dishevelled phosphorylation as a physiological target of Wnt5a–Ror signaling. The absence of Ror signaling leads to defects that mirror phenotypes observed in *Wnt5a* null mutant mice, including decreased branching of sympathetic neuron axons and major defects in aspects of embryonic development that are dependent upon morphogenetic movements, such as severe truncation of the caudal axis, the limbs, and facial structures. These findings suggest that Wnt5a–Ror–Dishevelled signaling constitutes a core noncanonical Wnt pathway that is conserved through evolution and is crucial during embryonic development.

noncanonical Wnt signaling | Ror1 | Ror2 | tissue elongation | axon branching

How the complexity of the adult animal arises from the fertilized egg is one of the most fascinating problems in biology. Embryonic development requires the precise coordination of many processes, including cell specification, proliferation, and tissue movements. These processes are controlled by a network of highly conserved signaling pathways, known as core developmental pathways. Dysregulation of these pathways in humans causes birth defects during development and can give rise to cancers in adults. Core pathways that regulate cell fate specification and proliferation have been studied extensively, such as those initiated by the hedgehog, TGF-β, and the Wnt family of secreted signaling proteins. However, the signaling pathways that regulate tissue shape and cell movements are still poorly characterized.

Of the major core developmental pathways, those controlled by Wnts are among the most ancient and versatile. During development, canonical Wnts signal through β-catenin–regulated gene transcription to control processes such as cell proliferation and fate determination (1). Wnts can also signal independently of β-catenin via noncanonical pathways to orchestrate tissue morphogenesis, a fundamental but nebulous process involving the coordination of various cell behaviors such as directed cell movements, changes in cell shape and cell polarization (2, 3). Although the mechanisms of canonical Wnt signaling have been extensively characterized and are relatively well understood, the biochemical basis of noncanonical Wnt signaling remains unclear (2). From cell culture experiments and ectopic expression experiments in *Xenopus* embryos, the regulation of several signaling pathways has been suggested to mediate the effects of noncanonical Wnts, including increased calcium influx, activation of the JNK pathway, inhibition of canonical Wnt signaling, activation of planar cell polarity (PCP) signaling, and phosphorylation of the cytoplasmic scaffolding protein Dishevelled (Dvl) (2–6). Although these proposed signaling mechanisms have the potential to explain aspects of noncanonical Wnt signaling, their relative importance in vivo is not known.

One of the most intensely studied noncanonical Wnts is Wnt5a. Perturbations of Wnt5a signaling in *Xenopus*, zebrafish, and mice all result in similar defects in tissue morphogenesis during embryonic development, strongly suggesting that Wnt5a activates a conserved pathway that controls cell movements and polarity during development (7–9). Frizzled, Ror, and Ryk proteins have all been implicated as putative Wnt5a receptors in various contexts (10–12), but recent studies favor Rors as critical mediators of Wnt5a signaling during development (13). In mice, Ror2 and Wnt5a are spatially and temporally coexpressed during development in many tissues, including the facial primordia, limb mesenchyme, neural crest-derived tissues, and the genital tubercle (9, 14, 15), and mouse mutants of *Rors* and *Wnt5a* exhibit partially overlapping phenotypes (9, 16, 17). Together, these observations suggested that Wnt5a and Rors might function as a signaling unit during development.

The biochemical and genetic evidence implicating Rors as direct Wnt5a receptors, however, remains inconclusive. The physical interaction between Wnt5a and Rors has been difficult to demonstrate convincingly by immunoprecipitation and pull-down experiments in vitro, as Wnts are prone to nonspecific binding (6, 12, 18, 19). In addition, examination of the phenotypes of existing *Wnt5a* and *Ror* mutant mice reveals more severe defects in the *Wnt5a* mutants than in mice lacking both members of the Ror family, calling into question the function of Rors as the primary Wnt5a receptors in vivo (9, 12, 20). At a mechanistic level, Rors have been shown to modulate several Wnt5a-induced noncanonical responses, including inhibition of canonical Wnt signaling, activation of the JNK pathway, and phosphorylation of Dvl proteins (6, 21–23). However, these observations are largely

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based on overexpression of Rors or ectopic application of recombinant Wnt5a to cultured cells. To date, no targets of Ror signaling have been conclusively identified in a physiological context, leaving open the possibility that the previously identified targets of Wnt5a and Ror identified in vitro may not operate in vivo.

In this study, we conduct genetic loss-of-function experiments under physiological conditions to investigate the function of Rors as Wnt5a receptors and to identify in vivo targets of this signaling pathway. We find that disruption of Ror1 and Ror2 expression results in system-wide tissue elongation defects and sympathetic axon innervation deficits, mirroring the phenotypes of the *Wnt5a* KO mouse. These in vivo findings provide compelling evidence that Rors are key mediators of Wnt5a signaling during development. In addition, we identify Dvl2 phosphorylation, but neither the inhibition of β-catenin–dependent Wnt signaling nor c-Jun phosphorylation, as a physiological target of Wnt5a-Ror signaling. Taken together, we propose a revised view of the Wnt5a–Ror pathway that substantially clarifies the molecular logic of noncanonical Wnt signaling.

Results

Generation of Conditional Ror1 and Ror2 Mutant Mice. To determine if Wnt5a signals via Rors in vivo, and if so, to identify the downstream consequences of this signaling, we generated mice that lack both members of the Ror family (Ror1 and Ror2) and examined their in vivo phenotypes. Before development of these conditional *Ror* KO mice, we examined the phenotypes of previously published *Ror* mutants (16, 20). Loss of Ror proteins in these lines had not been confirmed in the original studies, as suitable Ror antibodies were not available, leaving open the possibility that these mice still expressed residual Ror activity. We raised specific Ror1 and Ror2 antibodies that recognize the C-terminal cytoplasmic domains of Ror1 and Ror2, respectively (anti–Ror1-C and anti–Ror2-C; Fig. S1 *A* and *B*). Western blotting with the anti–Ror2-C antibody confirmed the absence of detectable Ror2 protein in the published *Ror2* mutant mice (Fig. S2*B*). Surprisingly, however, Western analysis using the anti–Ror1-C antibody detected a near–full-length Ror1 protein product in the *Ror1* mutant line (Fig. S2*A*), indicating that the previously generated *Ror1* line does not represent a true null mutant.

To gain spatial and temporal control of Ror expression during development and to obtain mice that are true nulls for Ror1, we used homologous recombination techniques to generate new conditional alleles of *Ror1* and *Ror2*. To conditionally target the mouse *Ror1* locus, exons 3 and 4 of the *Ror1* gene were flanked with loxP sequences so that, upon Cre-mediated recombination, a frame-shift mutation is introduced into the expressed Ror1 protein shortly after the signal sequence (Fig. 1*A* and Fig. S3). The *Ror2* conditional allele was similarly generated by flanking exons 3 and 4 of the *Ror2* gene with lox2272 sequences. Lox2272, which can undergo Cre-mediated recombination with itself, but not with loxP sequences, was used to avoid potential interchromosomal recombination with the targeted *Ror1* locus (Fig. 1*B* and Fig. S3). Western analyses of embryo lysates using anti–Ror1-C and anti–Ror2-C antibodies indicated that the expression of Ror1 and Ror2 proteins in the conditional ($Ror1^{f/f}$ and $Ror2^{f/f}$) animals before excision of the Ror alleles is similar to WT levels, whereas no expression of Ror protein was detected when $Ror1^{f/f}$ or $Ror2^{f/f}$ animals were crossed to a ubiquitous Cre deleter (*EIIA-Cre*) to generate germline KO alleles ($Ror1^{-/-}$ and $Ror2^{-/-}$ mice, respectively; Fig. 1 *C* and *D*). Importantly, we did not detect the appearance of any truncated Ror1 or Ror2 protein in the $Ror1^{-/-}$ and $Ror2^{-/-}$ mice, respectively (Fig. 1 *C* and *D*). Thus, these new mutant lines can be used to conditionally disrupt Ror expression in vivo and were used in all subsequent experiments.

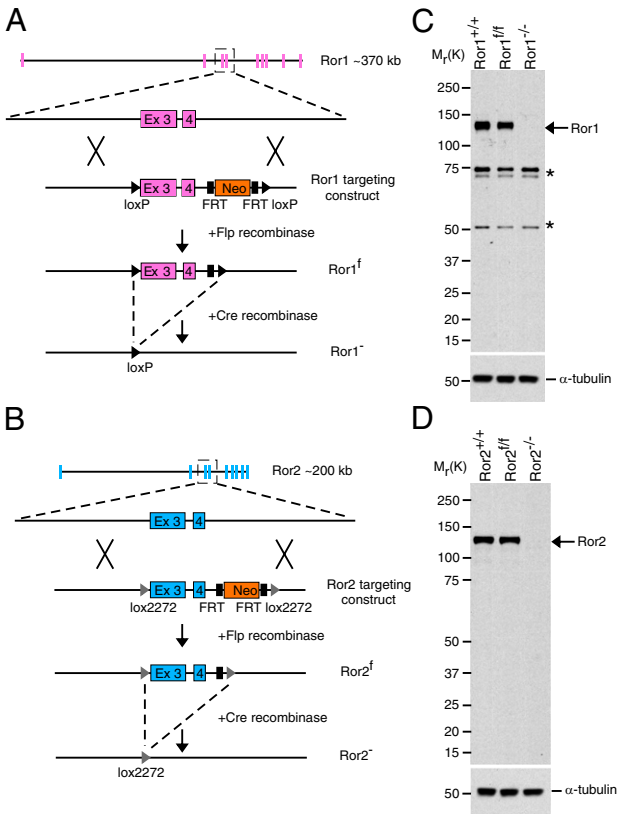

Fig. 1. Generation and characterization of conditional *Ror1* and *Ror2* mutant mice. (*A*) Schematic of the Ror1 gene targeting strategy. The *Ror1* conditional allele ($Ror1^{f}$) was generated by flanking exons 3 and 4 of the *Ror1* genomic locus with loxP sequences. The *Ror1*-null allele ($Ror1^{-}$) was generated by crossing the $Ror1^{f/f}$ mice to the *EIIA-Cre* deleter line. (*B*) Schematic of the *Ror2* genomic locus targeting strategy. The *Ror2* conditional allele was generated by flanking exons 3 and 4 of the *Ror2* gene with lox2272 sequences. Lox2272, which can undergo Cre-mediated recombination with itself, but not with loxP sequences, was used to avoid potential interchromosomal recombination with the targeted *Ror1* locus. The *Ror2* null allele ($Ror2^{-}$) was generated by crossing the $Ror2^{f/f}$ mice to the *EIIA-Cre* deleter line. (*C*) Immunoblot of Ror1 protein in E12.5 embryo lysates from WT ($Ror1^{+/+}$), $Ror1^{f/f}$, and $Ror1^{-/-}$ mice. Protein bands marked by asterisks are proteins unrelated to Ror1 that cross-react with the anti-Ror1-C antibody (Fig. S1). (*D*) Immunoblot of Ror2 protein in E12.5 embryo lysates from WT ($Ror2^{+/+}$), $Ror2^{f/f}$, and $Ror2^{-/-}$ mice.

Disruption of Ror1 and Ror2 Expression During Development Causes Defects in Embryonic Morphogenesis That Phenocopy *Wnt5a*$^{-/-}$ Mice. To determine the relative contribution of Rors as mediators of Wnt5a signaling during early mouse development, and to reveal other Wnt5a-independent functions of Rors, we analyzed the gross morphology of $Ror1^{-/-}$, $Ror2^{-/-}$, and $Ror1^{-/-}$;$Ror2^{-/-}$ (termed *Ror* DKO) embryos. Most $Ror1^{-/-}$ mice are viable at birth and are morphologically indistinguishable from their WT littermates. However, as detailed analysis of $Ror1^{-/-}$ mice is ongoing and outside the scope of the present study, we cannot exclude that subtle abnormalities may be present in these mice similar to those described in the previous *Ror1* mutant (24). $Ror2^{-/-}$ mice exhibit facial malformations and truncation of the limbs and posterior region of the embryo that are consistent with the previously published *Ror2* mutant (Fig. S4 *A* and *B*). However, in contrast to the previously reported *Ror1/2* double mutant, the new *Ror* DKO embryos are not carried to full term, and most die by embryonic day (E) 15.5. Between the ages of E12.5 and E13.5, *Ror* DKO embryos exhibit more severe phenotypes compared with *Ror2* mutants, including increased severity of facial malformations,

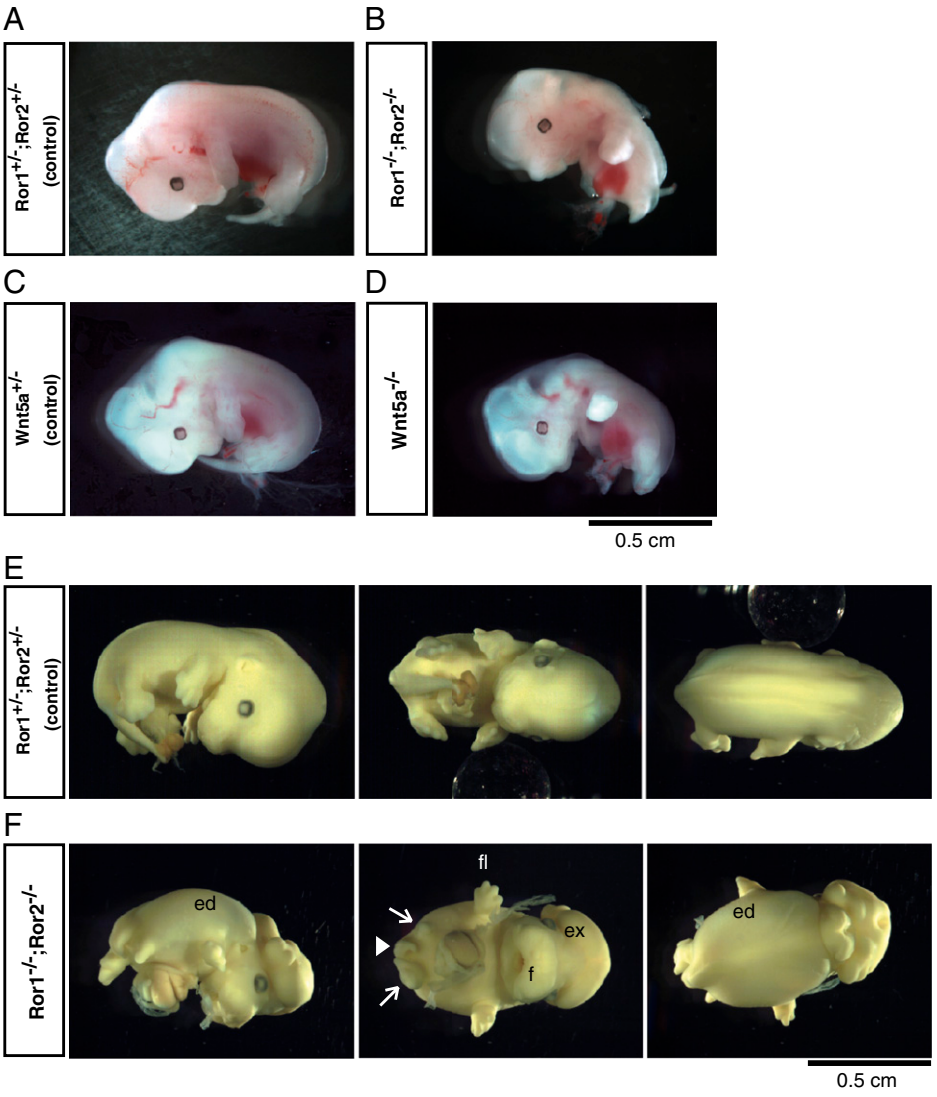

Fig. 2. *Ror1* and *Ror2* double KO embryos exhibit morphogenesis defects. (*A–D*) Representative images of unfixed E12.5 $Ror1^{+/-}$;$Ror2^{+/-}$ embryo (*A*), $Ror1^{-/-}$;$Ror2^{-/-}$ embryo (*B*), $Wnt5a^{+/-}$ mice (*C*), and $Wnt5a^{-/-}$ mice (*D*). Embryos shown in *A* and *B* are littermates. Embryos shown in *C* and *D* are littermates. (*E* and *F*) Images of a Bouin's fixed E12.5 $Ror1^{+/-}$;$Ror2^{+/-}$ embryo (*E*, littermate control of *F*) and *Ror* DKO embryo with exencephaly (*F*). Arrows indicate truncated and asymmetric hindlimbs; arrowhead indicates truncation of the posterior body axis. f, facial malformation; ed, edema; ex, exencephaly; fl, forelimb.

truncation of the caudal axis and limbs, and edema in the trunk (Fig. 2 *A–F*). It is also interesting to note that the right hindlimb of the *Ror* DKO embryos is consistently more underdeveloped than the left hindlimb (Fig. 2*F*), implicating Rors in the maintenance of bilateral limb symmetry (25). In addition, exencephaly is seen occasionally in *Ror* DKO and $Ror1^{-/-}$;$Ror2^{+/+}$ embryos (Fig. 2 *E* and *F* and Fig. S4 *C* and *D*), suggesting that Ror1 may have a role in neural tube closure. With the exception of the hindlimb asymmetry and low-penetrance exencephaly phenotypes, however, *Ror* DKO embryos essentially phenocopy the morphological defects observed in $Wnt5a^{-/-}$ embryos (9) (Fig. 2 *C* and *D*). The concordance of the $Wnt5a^{-/-}$ and *Ror* DKO embryo phenotypes provides strong evidence that Wnt5a and Rors function in the same pathway to regulate embryonic tissue morphogenesis.

Rors Are Required for Wnt5a-Mediated Sympathetic Axon Branching in Vivo. To investigate further if Rors mediate Wnt5a-dependent developmental processes in vivo, we analyzed *Ror* DKO embryos during a second stage of embryonic development in which the role of Wnt5a is well established. During nervous system development, expression of Wnt5a is induced in peripheral sympathetic neurons as their axons enter target organs (26). Wnt5a then signals in an autocrine manner to promote axon branching (26). $Wnt5a^{-/-}$ mice exhibit defective sympathetic innervation of target organs (26). However, the receptors that mediate the effects of Wnt5a on sympathetic innervation are not known. To determine if Rors are expressed in mouse sympathetic neurons, we first examined by in situ hybridization the expression of Ror1 and Ror2 mRNA in the superior cervical ganglion (SCG) at postnatal day (P) 0.5 when their axons actively innervate target tissues. We found that, whereas the expression of *Ror1* mRNA was below the limit of detection under these conditions, *Ror2* mRNA was clearly detected in the SCG (Fig. 3 *A* and *B*). To further establish Ror2 protein expression in the SCG, we exploited a previously generated $Ror2^{LacZ/+}$ knock-in strain in which β-gal staining can be used to track Ror2 expression (17) and confirmed the presence of Ror2-LacZ fusion protein in tyrosine hydroxylase (TH)-positive sympathetic neurons (Fig. 3*C*).

To determine if Rors, like Wnt5a, are required for proper sympathetic neuron target innervation, we conditionally deleted *Ror1* and *Ror2* in sympathetic neurons by using the neural crest-specific Wnt1-Cre deleter line and assessed target innervation by whole-mount TH staining. By using this method, we observed clear sympathetic innervation defects in several target organs at E17.5, when sympathetic axons are initiating peripheral target innervation (Fig. 3 *D–I*). Indeed, the innervation deficits in $Ror1^{f/f}$;$Ror2^{f/f}$;*Wnt1-cre* embryos are similar to those seen in $Wnt5a^{-/-}$ mice (Fig. 3 *D–I* vs. ref. 26). Importantly, sympathetic chain

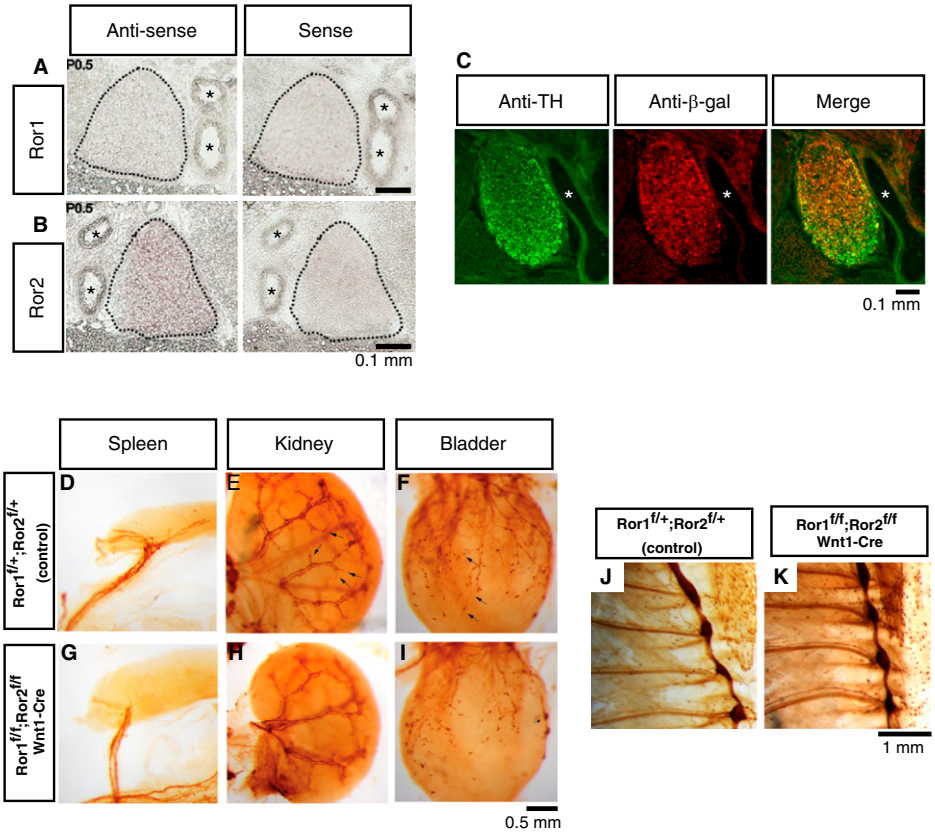

Fig. 3. *Ror1* and *Ror2* double mutant embryos exhibit sympathetic axon branching defects. In situ RNA hybridization of *Ror1* (*A*) and *Ror2* (*B*) in the SCG of P0.5 mice. (*C*) Coimmunostaining of the SCG of a P0.5 $Ror2^{LacZ/+}$ embryo with anti-TH and anti–β-gal antibodies. Asterisks in *A*–*C* denote the carotid arteries used as landmarks during tissue sectioning. (*D*–*I*) TH immunostaining in E17.5 control $Ror1^{f/+}$;$Ror2^{f/+}$ spleen (*D*), kidney (*E*), and bladder (*F*) and littermate E17.5 $Ror1^{f/f}$;$Ror2^{f/f}$;*Wnt1-cre* spleen (*G*), kidney (*H*), and bladder (*I*). Arrows denote axonal branches that are normally seen in control target organs but are compromised in mutant target organs. (*J* and *K*) Sympathetic chain ganglia of the $Ror1^{f/f}$;$Ror2^{f/f}$;*Wnt1-cre* embryos appear grossly intact and show normal coalescence as shown by whole-mount TH staining.

ganglia of the $Ror1^{f/f}$;$Ror2^{f/f}$;*Wnt1-cre* embryos appear grossly intact, suggesting that the observed innervation defects are not a secondary consequence of Ror deletion in an earlier stage of sympathetic nervous system development (Fig. 3 *J* and *K*). These findings provide strong support for the conclusion that Wnt5a signals through Rors to promote axon branching as sympathetic neuron axons innervate their target tissues.

Dvl2 Phosphorylation Is a Physiological Target of Wnt5a–Ror Signaling. Having implicated Rors as mediators of Wnt5a-dependent morphogenetic regulation and axon branching during development, we next turned our attention to the downstream mechanisms of Wnt5a–Ror signaling. A plethora of signaling molecules have been suggested to mediate the effects of Rors in cultured cell lines or in *Xenopus* embryos exposed to ectopic Wnt stimulation (2, 13). However, given that overexpression or ectopic addition of Wnt5a can have nonphysiological effects, ambiguity remains as to which of the previously characterized Wnt5a effector molecules are physiologically relevant (2). To begin to address this issue, we disrupted expression of Wnt5a or Rors in primary mouse embryonic fibroblasts (MEFs) and assessed the effect on a wide range of signaling proteins and pathways (c-Jun phosphorylation, inhibition of canonical Wnt signaling, Dvl phosphorylation, PKCζ phosphorylation, and Vangl2 phosphorylation) that had previously been suggested to mediate Wnt5a or Ror signaling (6, 12, 27–29). Remarkably, of all these previously characterized Wnt5a effectors, the only one found to be affected by the disruption of Wnt5a-Ror signaling in MEFs was Dvl phosphorylation.

For the characterization of Wnt5a-Ror effectors under physiologically relevant conditions, primary MEFs from E12.5 embryos were used, as these cells are derived directly from the mesenchymal tissues that undergo Wnt5a-dependent morphogenetic movements in vivo (30, 31). In addition, these cells express high levels of endogenous Wnt5a, Ror1, and Ror2 in culture (Fig. S5 *A*–*C*), suggesting that MEFs undergo active Wnt5a-Ror signaling in culture. Thus, to identify Wnt5a-Ror effectors within cells through loss-of-function experiments, we compared the phosphorylation and signaling functions of putative Wnt5a/Ror effectors in WT, $Wnt5a^{-/-}$, and *Ror* DKO MEFs.

To measure Dvl phosphorylation, we developed a quantitative Western blotting method to detect a characteristic phosphatase-sensitive motility shift of Dvl2 on SDS/PAGE gels. In WT MEFs unstimulated with exogenous Wnts, Dvl2 is largely present in a highly phosphorylated slow-migrating form [72 ± 3% (SEM) of total Dvl2], indicating that Dvl2 is basally phosphorylated in this culture (Fig. 4*A*). The motility shift of Dvl2 can be reversed by phosphatase treatment (Fig. 4*A*), indicating that the shifted Dvl2 band is a result of Dvl2 phosphorylation, as previously reported in other cell types (22, 32, 33). To determine if Dvl2 phosphorylation is a consequence of endogenous Wnt5a signaling, we assessed Dvl2 phosphorylation in $Wnt5a^{-/-}$ MEFs. Genetic deletion of *Wnt5a* markedly reduced the level of the slowly migrating form of Dvl2 [15 ± 2% (SEM) of total Dvl2], similar to levels observed in MEFs treated with the pan-Wnt antagonist sFRP-3 (Fig. 4*A*), indicating that endogenous Wnt5a activity accounts for most, if not all, of this phosphorylation. Importantly, treatment with the canonical Wnt inhibitor DKK-1 or genetic ablation of *Lrp6*, encoding a required coreceptor in the canonical Wnt pathway, had no effect on this Dvl2 motility shift (Fig. 4*A*), excluding a role for endogenous canonical Wnt signaling in this modification. Together, these experiments establish that the phosphorylation of Dvl2, as detected by the protein motility shift, is a specific target of noncanonical Wnt5a signaling in MEFs. This finding is consistent with the previous observation that RNAi knockdown of Wnt5a in rat fibroblasts leads to a partial loss of Dvl2 phosphorylation (29).

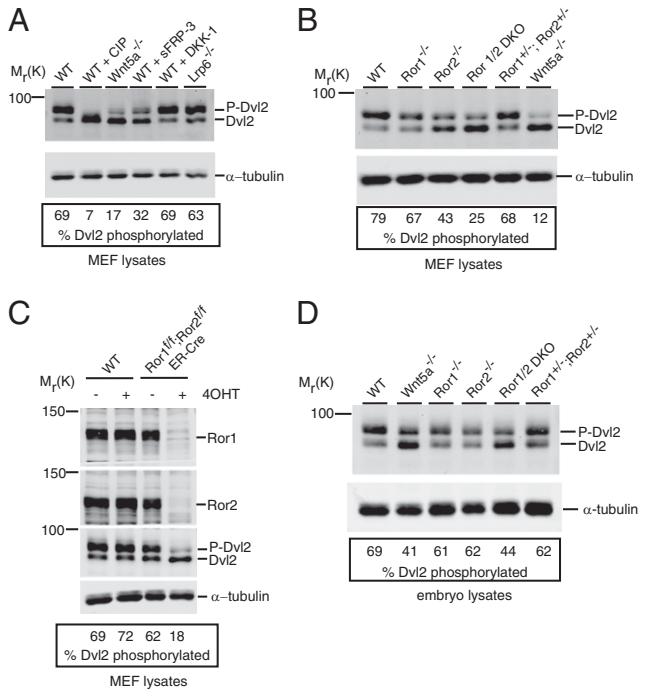

Fig. 4. Dvl2 phosphorylation is a target of noncanonical Wnt5a-Ror signaling in vitro and in vivo. (*A*–*D*) Representative immunoblots showing Dvl2 phosphorylation in protein lysates prepared from MEFs and embryos. (*A*) Dvl2 protein in lysates from WT MEFs, WT MEF lysates treated with calf intestinal phosphatase (CIP), *Wnt5a*$^{-/-}$ MEFs, WT MEFs treated with sFRP-3, WT MEFs treated with DKK-1, and *Lrp6*$^{-/-}$ MEFs. (*B*) Dvl2 protein in lysates from WT MEFs, *Ror1*$^{-/-}$ MEFs, *Ror2*$^{-/-}$ MEFs, *Ror1/2* DKO MEFs, *Ror1*$^{+/-}$;*Ror2*$^{+/-}$ MEFs, and *Wnt5a*$^{-/-}$ MEFs. (*C*) Ror1 protein, Ror2 protein, and Dvl2 protein in WT MEFs and *Ror1*$^{f/f}$;*Ror2*$^{f/f}$;*ER-Cre* MEFs with or without 72 h treatment of 4-OHT. (*D*) Dvl2 protein in E12.5 embryo lysates from WT mice, *Wnt5a*$^{-/-}$ mice, *Ror1*$^{-/-}$ mice, *Ror2*$^{-/-}$ mice, *Ror* DKO mice, and *Ror1*$^{+/-}$;*Ror2*$^{+/-}$ mice. α-Tubulin was used for loading controls in all experiments. Percent Dvl2 phosphorylation was calculated by dividing the upshifted band by total Dvl2 signal.

To investigate the requirement of Rors in Wnt5a-dependent Dvl2 phosphorylation, we compared Dvl2 phosphorylation in WT, *Ror1*$^{-/-}$, *Ror2*$^{-/-}$, and *Ror* DKO MEFs. This analysis revealed that Dvl2 phosphorylation is dependent on the level of Ror expression in these cells, as Dvl2 phosphorylation is increasingly reduced in *Ror1*$^{-/-}$, *Ror2*$^{-/-}$, and *Ror* DKO MEFs (Fig. 4*B*). To ensure that the observed reduction in Dvl2 phosphorylation is not caused by a secondary effect of the chronic loss of Rors during development, we acutely deleted Rors in MEFs by using tamoxifen-inducible Cre-ER. In the absence of 4-hydroxytamoxifen (4-OHT), Cre-ER is sequestered in the cytoplasm and is inactive. However, upon exposure to 4-OHT, Cre-ER translocates to the nucleus and induces recombination within the *Ror1* and *Ror2* genomic loci. Treatment of *Ror1*$^{f/f}$;*Ror2*$^{f/f}$;*Cre-ER* MEFs with 4-OHT eliminated detectable Ror protein expression and reduced Dvl2 phosphorylation to a level similar to *Ror* DKO MEFs (Fig. 4*C*). We thus conclude that Rors are required for Wnt5a to induce Dvl2 phosphorylation in MEFs.

To determine if Wnt5a-Ror signaling triggers Dvl2 phosphorylation in vivo during embryonic development, we compared Dvl2 phosphorylation in protein lysates prepared from E12.5 WT, *Wnt5a*$^{-/-}$, *Ror1*$^{-/-}$, *Ror2*$^{-/-}$, and *Ror* DKO embryos. Consistent with our findings in MEFs, Dvl2 phosphorylation was substantially attenuated in *Wnt5a*$^{-/-}$ and *Ror* mutant embryos compared with WT samples (Fig. 4*D*), demonstrating a requirement of Wnt5a-Ror signaling for proper Dvl2 phosphorylation in vivo during embryonic development. These findings indicate that the Wnt5a–Ror–Dvl pathway operates broadly in the developing mouse embryo and suggests a critical function for this pathway in controlling embryonic morphogenesis.

Rors Function as Receptors for Wnt5a-Dependent Dvl2 Phosphorylation. To test more directly whether Rors act as receptors for Wnt5a in mediating Dvl2 phosphorylation, we developed reagents that physically block the extracellular domains of Rors. High-affinity function-blocking antibodies were generated against the ectodomains (ECDs) of Ror1 and Ror2 (Fig. S6 *A* and *B*). Addition of these anti-Ror1 and anti-Ror2 ECD antibodies, but not control rabbit IgG, blocked Dvl2 phosphorylation in a dose-dependent manner (Fig. 5*A*). Importantly, loss of Dvl2 phosphorylation cannot be attributed to Ror receptor clustering by the antibodies, as these antibodies were still effective at blocking Dvl2 phosphorylation when they were rendered monovalent by papain cleavage (Fig. 5 *B* and *C*). This finding, taken together with the Wnt5a and Ror1/Ror2 genetic loss-of-function studies, strongly supports the idea that Rors function as Wnt5a receptors that signal to phosphorylate Dvl2.

Rors Are Not Required for Wnt5a-Dependent Inhibition of Canonical Wnt Signaling or c-Jun Phosphorylation. We next asked if Rors are required for Wnt5a-dependent inhibition of canonical Wnt

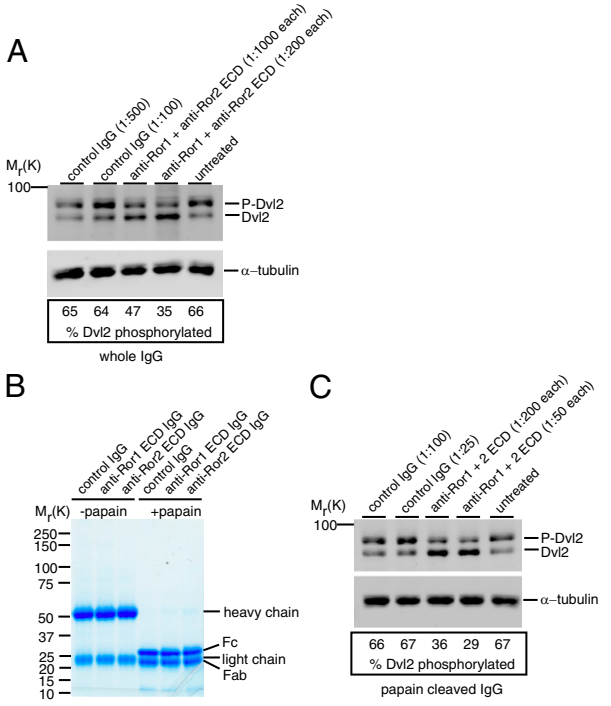

Fig. 5. Rors function as receptors for Wnt5a. (*A*) Dvl2 protein in lysates from WT MEFs treated for 18 h with native control rabbit IgG at 1:500 or 1:100 dilutions, WT MEFs treated for 18 h with both native anti-Ror1 ECD antibodies and anti-Ror2 ECD antibodies at 1:1,000 or 1:200 dilutions of each antibody, and untreated WT MEFs. The stock concentration of control rabbit IgG, anti-Ror1 ECD, and anti-Ror2 ECD antibodies are all 12 mg/mL (total IgG fraction purified from whole serum). (*B*) Coomassie G-250 stained gel showing control rabbit IgG, anti-Ror1 ECD antibodies, and anti-Ror2 ECD antibodies with or without pretreatment with papain. (*C*) Dvl2 protein in lysates from WT MEFs treated for 18 h with papain-cleaved control rabbit IgG at 1:100 or 1:25 dilutions, WT MEFs treated for 18 h with papain-cleaved anti-Ror1 ECD antibodies and papain-cleaved anti-Ror2 ECD antibodies at 1:200 or 1:50 dilutions of each antibody, and untreated WT MEFs. The stock concentration of papain-cleaved control rabbit IgG, anti-Ror1 ECD, and anti-Ror2 ECD antibodies are all 7 mg/mL (cleaved total IgG fraction purified from whole serum).

signaling in MEFs. Multiple studies in which Wnt5a is ectopically applied to cultured cells, or Rors are overexpressed in cell lines, have suggested a role for Ror proteins in Wnt5a-dependent inhibition of canonical Wnt signaling (6, 23, 27, 34). Given the negligibly low levels of canonical Wnt signaling observed in WT and *Ror* DKO MEFs, we used a previously described protocol to induce canonical Wnt signaling, adding purified Wnt3a to the culture media and monitoring β-catenin–dependent gene transcription by using a β-catenin–responsive luciferase-based reporter (6, 34). Wnt3a treatment induced expression from the luciferase reporter gene equally well in WT and *Ror* DKO cells (Fig. 6*A*), indicating that Ror expression is not required for canonical Wnt signaling. We also observed a dose-dependent inhibition of canonical signaling by Wnt5a in WT MEFs, consistent with previous reports (6, 34) (Fig. 6*A*). Surprisingly, however, the ability of Wnt5a to antagonize canonical Wnt signaling was not affected by the disruption of Ror expression in *Ror* DKO MEFs (Fig. 6*A*), indicating that Rors are not required for Wnt5a-induced inhibition of canonical Wnt signaling in MEFs. Thus, we conclude that Wnt5a inhibits canonical signaling through Ror-independent mechanisms, possibly by competing with canonical Wnts for binding to receptors such as LRP5/6 and Frizzleds, as previously suggested (35).

Similarly, our findings with the use of *Wnt5a*$^{-/-}$ or *Ror* DKO MEFs do not support previous reports that Wnt5a signals noncanonically through Rors to activate the JNK pathway (12, 21, 36, 37). Western blotting with an antibody specific for the phosphorylated form of the JNK substrate c-Jun (anti–phospho-c-Jun S63) showed no change in levels of c-Jun phosphorylation among WT, *Wnt5a*$^{-/-}$, or *Ror* DKO MEFs (Fig. 6*B*). Moreover, c-Jun phosphorylation was induced to similar levels in MEFs by the application of exogenous Wnt3a or Wnt5a over a range of concentrations (Fig. 6 *C* and *D*), suggesting that c-Jun phosphorylation is not a specific consequence of noncanonical signaling by Wnt5a. It is possible that, when ectopically applied to cells, Wnts act nonphysiologically to activate c-Jun phosphorylation. Together, our loss-of-function analyses in MEFs do not support previous models in which Wnt5a signals through Rors to inhibit canonical Wnt signaling or to trigger c-Jun phosphorylation, although it remains formally possible that Rors might mediate these responses in other cell types.

Discussion

In this study, we have used mouse loss-of-function experiments to provide conclusive evidence that Ror receptors mediate diverse Wnt5a-dependent processes in vivo, as the absence of Ror signaling leads to widespread tissue morphogenesis defects during embryogenesis and sympathetic axon branching defects that phenocopy previously characterized mutants of Wnt5a. We have also identified Dvl phosphorylation as a physiological target of Wnt5a-Ror signaling, as Dvl phosphorylation is strongly reduced in the absence of this signaling in vitro and in vivo.

Core Noncanonical Wnt Pathway That Controls Tissue Morphogenesis. Taken together with the large body of evidence supporting the biological importance of Wnt5a and Dvl in regulating cell movement, shape, and polarity (2, 5, 29, 38, 39), our findings establish Wnt5a–Ror–Dvl signaling as a core developmental signaling pathway that orchestrates embryonic morphogenesis during development. Consistent with this view, Wnt5a, Rors, and Dvls are broadly and dynamically expressed during development, and are highly conserved through evolution, with homologs in animals as diverse as humans, worms, and sponges (9, 14, 15, 40). These observations strongly suggest that the Wnt–Ror–Dvl pathway is used reiteratively in diverse developmental contexts and has been functionally conserved through the past 600 million years of animal evolution.

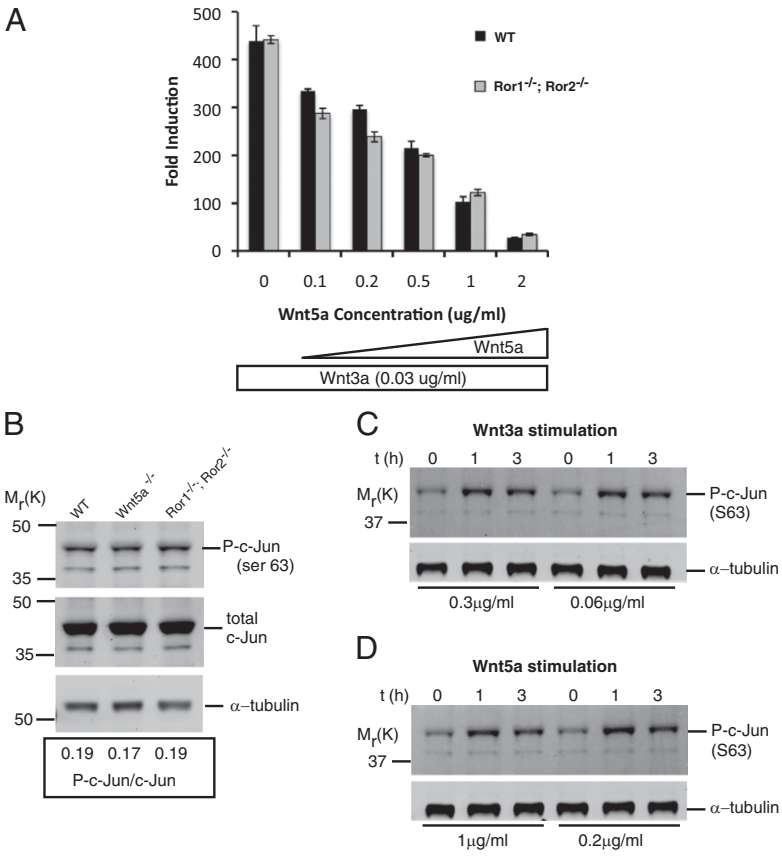

Fig. 6. Rors are not required for Wnt5a-dependent inhibition of canonical Wnt signaling or c-Jun phosphorylation, and Wnt5a and Wnt3a both induce c-Jun phosphorylation in MEFs. (*A*) Wnt5a-induced inhibition of Wnt3a-stimulated β-catenin–responsive luciferase reporter activity in WT MEFs and *Ror* DKO (*Ror1*$^{-/-}$;*Ror2*$^{-/-}$) MEFs. (*B*) Immunoblot showing levels of phospho-c-Jun (ser 63) and total c-Jun proteins in lysates from WT MEFs, *Wnt5a*$^{-/-}$ MEFs, and *Ror* DKO (*Ror1*$^{-/-}$;*Ror2*$^{-/-}$) MEFs. Phospho-c-Jun to total c-Jun ratio was calculated by using quantitative Western blotting. (*C* and *D*) E12.5 MEFs were stimulated with Wnt3a (*C*) or Wnt5a (*D*) at the indicated concentrations. Protein samples were collected at 0, 1, and 3 h after stimulation with Wnt proteins and analyzed by Western blotting using the anti–phospho-c-Jun (S63) antibody. α-Tubulin was used for loading controls.

The identification of Dvl2 as a physiological target of Wnt5a-Ror signaling is consistent with previous loss-of-function analyses of Dvl proteins in mice. *Dvl* KO mice exhibit complex phenotypes, as Dvl proteins are known to be functionally redundant and are also involved in canonical Wnt and PCP signaling (5, 41). Importantly, $Dvl2^{-/-}$, $Dvl2^{-/-}$;$Dvl3^{+/-}$, and $Dvl2^{+/-}$;$Dvl3^{-/-}$ mutants share a number of specific phenotypes with *Ror* DKO animals, such as truncation of posterior body axis and snout (41), consistent with our hypothesis that Wnt5a, Rors, and Dvls function in a common pathway to control tissue elongation in vivo.

The widespread tissue elongation phenotypes we observed in *Ror* DKO mice can be explained by dysregulation of Wnt5a-dependent processes such as directed cell movement and cell polarization. For example, during avian gastrulation, Wnt5a and other noncanonical Wnts at the primitive streak direct morphogenetic cell movements that are required for axis extension (42–44). Given that both Wnt5a and Ror2 are highly expressed in the primitive streak of mouse embryos (9, 15), precisely at the time when the posterior axis undergoes elongation (45), the posterior truncation phenotypes of the *Ror* DKO and the $Wnt5a^{-/-}$ embryos likely result from defects in morphogenetic cell movements at the primitive streak. Likewise, recent studies using in vivo two-photon imaging of live chick and mouse embryos demonstrated a critical role of Wnt5a in regulating the orientation of cell division, cell movement, and cell shape in limb bud mesenchymal cells, processes thought to drive elongation of the developing limb buds (30, 31).

These observations, together with the finding that *Ror* DKO and $Wnt5a^{-/-}$ embryos have very similar defects in limb development and axis elongation, strongly suggest that Wnt5a signals through Rors to control tissue extension by regulating aspects of morphogenetic cell movements or cell polarization. Nevertheless, as cell movements, cell polarization, and cell shape changes occur simultaneously and are often interdependent during tissue morphogenesis, the precise cell behaviors that are directly regulated by the Wnt5a–Ror–Dvl pathway remain unknown. Identification of these behaviors represents an important direction of future investigation.

Role of Wnt–Ror Signaling in PCP Regulation. The embryonic phenotypes of the *Ror* DKO mice also clarify a major controversy in Wnt signaling and development regarding whether noncanonical Wnt-Ror signaling and PCP signaling are regulatory components of the same pathway or whether they regulate independent pathways that control embryonic tissue morphogenesis (2, 3). PCP is a process in which a field of cells polarizes with respect to the plane of the associated epithelial tissue, and is thought to require the asymmetric segregation of several core PCP determinants, such as the Vangl family of proteins (3).

Recent studies have hypothesized that Wnt5a signals through Rors to regulate the PCP pathway, as mouse *Ror2* and *Wnt5a* single mutants exhibit mild or low-penetrance PCP-like phenotypes (46, 47). However, we observed that *Ror* DKO mutants lacking all Ror signaling capacity have substantially nonoverlapping phenotypes compared with mice with mutations in both members of the Vangl family (48), a finding that is inconsistent with Rors functioning as crucial mediators of PCP signaling. For example, $Vangl1^{-/-}$;$Vangl2^{-/-}$ double mutants display the characteristic PCP phenotype craniorachischisis, a failure to close the neural tube from hindbrain to tail, whereas *Ror* DKO embryos do not display this phenotype (48). Conversely, all *Ror* DKO embryos display truncation of the face and limbs, whereas these phenotypes in the $Vangl1^{-/-}$;$Vangl2^{-/-}$ double mutants are substantially milder (48). Thus, these observations suggest that the core functions and signaling mechanisms of the Wnt5a-Ror and PCP pathways are fundamentally distinct. This view, however, neither excludes a model in which Wnts signal through other receptors, such as Frizzleds, to impinge on PCP signaling (49), nor a model in which Wnt–Ror signaling cooperates with components of the PCP pathway in specific developmental contexts (27, 50).

Physiological Mode of Wnt5a–Ror Interaction. Our preliminary observations indicate that, in primary MEF cultures, endogenous Wnt5a is not readily released into the culture media and may require direct cell–cell contact or limited diffusion for proper signaling to Rors. For example, conditioned media collected from WT MEF cultures are unable to rescue Dvl2 phosphorylation in $Wnt5a^{-/-}$ MEFs (Fig. S7*A*). Even when WT and $Wnt5a^{-/-}$ MEFs cultured on separate coverslips are placed in close proximity within the same culture dish, WT MEFs are still unable to recue Dvl2 phosphorylation in $Wnt5a^{-/-}$ MEFs (Fig. S7*B* shows experimental details). These observations also raise the possibility that the Wnt5a–Ror interaction may involve an autocrine mode of Wnt5a signaling or may require other components present on the cell surface, such as ECM components or coreceptors (35). The intricate interaction between endogenous Wnt5a and Rors observed in these experiments highlights the importance of investigating noncanonical Wnt signaling under physiological conditions, without the ectopic application of Wnts. In this study, we have relied on endogenous Wnt5a expressed by MEFs to investigate downstream signaling mechanisms that are activated by noncanonical Wnts. This MEF system and the loss-of-function strategy used here should be useful for identifying other physiologically relevant effectors of noncanonical Wnt5a–Ror signaling.

The idea that Wnt5a signals in an autocrine manner was originally suggested by the findings that, in cultured sympathetic neurons, Wnt5a signals cell-autonomously to regulate axon branching (26), and is further supported by the observation that conditional deletion of *Wnt5a* in sympathetic neurons in vivo phenocopies the sympathetic axon branching defects of $Wnt5a^{-/-}$ embryos (Y.K.R. and R.K., unpublished observations). Autocrine Wnt5a–Ror signaling may be particularly critical in this context to ensure that axon branching does not occur until the axon reaches the appropriate target field, as sympathetic axons must not respond to external sources of Wnt5a while navigating their way to the target field. Previous studies have shown that, when the sympathetic neuron axon terminal has reached the peripheral target organ, NGF, expressed specifically in target organs, induces expression of Wnt5a in sympathetic neurons to trigger axon branching (26). It will be interesting to determine whether an autocrine mode of Wnt5a–Ror signaling is also essential for proper morphogenetic movements in which the spatial coordination of cells is critical to shape developing tissues.

Significance of Dvl Phosphorylation. The pleiotropic role of Dvl in canonical and noncanonical Wnt signaling is well established (5), but how pathway specificity is achieved at the level of Dvl regulation remains unknown. Our demonstration that Dvl phosphorylation is a direct and specific consequence of noncanonical Wnt5a–Ror signaling, along with previous reports that Dvl phosphorylation can be uncoupled from β-catenin–dependent Wnt signaling (29, 32, 33, 51), raises the intriguing possibility that Dvl phosphorylation functions as a molecular switch to specify the canonical or noncanonical functionality of the protein. Phosphorylation of Dvl may induce changes in subcellular localization, protein–protein interactions (39, 52), or allosteric conformations that are relevant to the function of Dvl in noncanonical signaling and morphogenesis (23). Determining the sites of Dvl phosphorylation, the precise mechanism by which Wnt5a–Ror signaling modulates Dvl phosphorylation, and the impact of Dvl phosphorylation on various aspects of Wnt signaling represent important areas of

future investigation. We expect the conceptual framework and molecular tools presented here to be essential in many future studies of noncanonical Wnt signaling.

Materials and Methods

In brief, the *Ror1*^{f} and *Ror2*^{f} alleles were generated by homologous recombination in mouse embryonic stem cells. All mice used in the study were derived from a sv129/C57BL/6 hybrid genetic background. Experiments involving animals were conducted according to protocols and guidelines approved by the institutional animal care and use committee at Harvard Medical School. Primary MEF cultures were derived from E12.5 embryos and used within five passages. Quantitative Western blotting was performed using the Odyssey infrared imaging system (Li-Cor Biosciences) according to the manufacturer's instructions. Detailed methods are provided in *SI Materials and Methods*.

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1. Angers S, Moon RT (2009) Proximal events in Wnt signal transduction. *Nat Rev Mol Cell Biol* 10:468–477.
2. Veeman MT, Axelrod JD, Moon RT (2003) A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev Cell* 5:367–377.
3. Goodrich LV, Strutt D (2011) Principles of planar polarity in animal development. *Development* 138:1877–1892.
4. Kohn AD, Moon RT (2005) Wnt and calcium signaling: Beta-catenin-independent pathways. *Cell Calcium* 38:439–446.
5. Boutros M, Mlodzik M (1999) Dishevelled: At the crossroads of divergent intracellular signaling pathways. *Mech Dev* 83:27–37.
6. Mikels AJ, Nusse R (2006) Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. *PLoS Biol* 4:e115.
7. Moon RT, et al. (1993) Xwnt-5A: A maternal Wnt that affects morphogenetic movements after overexpression in embryos of Xenopus laevis. *Development* 119:97–111.
8. Rauch GJ, et al. (1997) Wnt5 is required for tail formation in the zebrafish embryo. *Cold Spring Harb Symp Quant Biol* 62:227–234.
9. Yamaguchi TP, Bradley A, McMahon AP, Jones S (1999) A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development* 126: 1211–1223.
10. He X, et al. (1997) A member of the Frizzled protein family mediating axis induction by Wnt-5A. *Science* 275:1652–1654.
11. Yoshikawa S, McKinnon RD, Kokel M, Thomas JB (2003) Wnt-mediated axon guidance via the Drosophila Derailed receptor. *Nature* 422:583–588.
12. Oishi I, et al. (2003) The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. *Genes Cells* 8:645–654.
13. Green JL, Kuntz SG, Sternberg PW (2008) Ror receptor tyrosine kinases: Orphans no more. *Trends Cell Biol* 18:536–544.
14. Al-Shawi R, Ashton SV, Underwood C, Simons JP (2001) Expression of the Ror1 and Ror2 receptor tyrosine kinase genes during mouse development. *Dev Genes Evol* 211: 161–171.
15. Matsuda T, et al. (2001) Expression of the receptor tyrosine kinase genes, Ror1 and Ror2, during mouse development. *Mech Dev* 105:153–156.
16. Takeuchi S, et al. (2000) Mouse Ror2 receptor tyrosine kinase is required for the heart development and limb formation. *Genes Cells* 5:71–78.
17. DeChiara TM, et al. (2000) Ror2, encoding a receptor-like tyrosine kinase, is required for cartilage and growth plate development. *Nat Genet* 24:271–274.
18. Hsieh JC, Rattner A, Smallwood PM, Nathans J (1999) Biochemical characterization of Wnt-frizzled interactions using a soluble, biologically active vertebrate Wnt protein. *Proc Natl Acad Sci USA* 96:3546–3551.
19. van Amerongen R, Mikels A, Nusse R (2008) Alternative wnt signaling is initiated by distinct receptors. *Sci Signal* 1:re9.
20. Nomi M, et al. (2001) Loss of mRor1 enhances the heart and skeletal abnormalities in mRor2-deficient mice: redundant and pleiotropic functions of mRor1 and mRor2 receptor tyrosine kinases. *Mol Cell Biol* 21:8329–8335.
21. Nomachi A, et al. (2008) Receptor tyrosine kinase Ror2 mediates Wnt5a-induced polarized cell migration by activating c-Jun N-terminal kinase via actin-binding protein filamin A. *J Biol Chem* 283:27973–27981.
22. Nishita M, et al. (2010) Ror2/Frizzled complex mediates Wnt5a-induced AP-1 activation by regulating Dishevelled polymerization. *Mol Cell Biol* 30:3610–3619.
23. Witte F, et al. (2010) Negative regulation of Wnt signaling mediated by CK1-phosphorylated Dishevelled via Ror2. *FASEB J* 24:2417–2426.
24. Lyashenko N, et al. (2010) Mice lacking the orphan receptor ror1 have distinct skeletal abnormalities and are growth retarded. *Dev Dyn* 239:2266–2277.
25. Allard P, Tabin CJ (2009) Achieving bilateral symmetry during vertebrate limb development. *Semin Cell Dev Biol* 20:479–484.
26. Bodmer D, et al. (2009) Wnt5a mediates nerve growth factor-dependent axonal branching and growth in developing sympathetic neurons. *J Neurosci* 29:7569–7581.
27. Gao B, et al. (2011) Wnt signaling gradients establish planar cell polarity by inducing Vangl2 phosphorylation through Ror2. *Dev Cell* 20:163–176.
28. Zhang X, et al. (2007) Dishevelled promotes axon differentiation by regulating atypical protein kinase C. *Nat Cell Biol* 9:743–754.
29. Schlessinger K, McManus EJ, Hall A (2007) Cdc42 and noncanonical Wnt signal transduction pathways cooperate to promote cell polarity. *J Cell Biol* 178:355–361.
30. Wyngaarden LA, et al. (2010) Oriented cell motility and division underlie early limb bud morphogenesis. *Development* 137:2551–2558.
31. Gros J, et al. (2010) WNT5A/JNK and FGF/MAPK pathways regulate the cellular events shaping the vertebrate limb bud. *Curr Biol* 20:1993–2002.
32. Rothbächer U, et al. (2000) Dishevelled phosphorylation, subcellular localization and multimerization regulate its role in early embryogenesis. *EMBO J* 19:1010–1022.
33. González-Sancho JM, Brennan KR, Castelo-Soccio LA, Brown AM (2004) Wnt proteins induce dishevelled phosphorylation via an LRP5/6- independent mechanism, irrespective of their ability to stabilize beta-catenin. *Mol Cell Biol* 24:4757–4768.
34. Mikels A, Minami Y, Nusse R (2009) Ror2 receptor requires tyrosine kinase activity to mediate Wnt5A signaling. *J Biol Chem* 284:30167–30176.
35. Grumolato L, et al. (2010) Canonical and noncanonical Wnts use a common mechanism to activate completely unrelated coreceptors. *Genes Dev* 24:2517–2530.
36. Schambony A, Wedlich D (2007) Wnt-5A/Ror2 regulate expression of XPAPC through an alternative noncanonical signaling pathway. *Dev Cell* 12:779–792.
37. Yamanaka H, et al. (2002) JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Rep* 3:69–75.
38. Wallingford JB, et al. (2000) Dishevelled controls cell polarity during Xenopus gastrulation. *Nature* 405:81–85.
39. Narimatsu M, et al. (2009) Regulation of planar cell polarity by Smurf ubiquitin ligases. *Cell* 137:295–307.
40. Adamska M, et al. (2010) Structure and expression of conserved Wnt pathway components in the demosponge Amphimedon queenslandica. *Evol Dev* 12:494–518.
41. Etheridge SL, et al. (2008) Murine dishevelled 3 functions in redundant pathways with dishevelled 1 and 2 in normal cardiac outflow tract, cochlea, and neural tube development. *PLoS Genet* 4:e1000259.
42. Chuai M, et al. (2006) Cell movement during chick primitive streak formation. *Dev Biol* 296:137–149.
43. Voiculescu O, Bertocchini F, Wolpert L, Keller RE, Stern CD (2007) The amniote primitive streak is defined by epithelial cell intercalation before gastrulation. *Nature* 449:1049–1052.
44. Hardy KM, et al. (2008) Non-canonical Wnt signaling through Wnt5a/b and a novel Wnt11 gene, Wnt11b, regulates cell migration during avian gastrulation. *Dev Biol* 320:391–401.
45. Tam PP, Loebel DA, Tanaka SS (2006) Building the mouse gastrula: Signals, asymmetry and lineages. *Curr Opin Genet Dev* 16:419–425.
46. Forrester WC, Dell M, Perens E, Garriga G (1999) A C. elegans Ror receptor tyrosine kinase regulates cell motility and asymmetric cell division. *Nature* 400:881–885.
47. Yamamoto S, et al. (2008) Cthrc1 selectively activates the planar cell polarity pathway of Wnt signaling by stabilizing the Wnt-receptor complex. *Dev Cell* 15:23–36.
48. Song H, et al. (2010) Planar cell polarity breaks bilateral symmetry by controlling ciliary positioning. *Nature* 466:378–382.
49. Shafer B, Onishi K, Lo C, Colakoglu G, Zou Y (2011) Vangl2 promotes Wnt/planar cell polarity-like signaling by antagonizing Dvl1-mediated feedback inhibition in growth cone guidance. *Dev Cell* 20:177–191.
50. Wen J, et al. (2010) Loss of Dact1 disrupts planar cell polarity signaling by altering dishevelled activity and leads to posterior malformation in mice. *J Biol Chem* 285: 11023–11030.
51. Bryja V, Schulte G, Rawal N, Grahn A, Arenas E (2007) Wnt-5a induces Dishevelled phosphorylation and dopaminergic differentiation via a CK1-dependent mechanism. *J Cell Sci* 120:586–595.
52. Yokoyama N, Golebiewska U, Wang HY, Malbon CC (2010) Wnt-dependent assembly of supermolecular Dishevelled-3-based complexes. *J Cell Sci* 123:3693–3702.