

The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway

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Abstract

Background: Ror2 is an orphan receptor, belonging to the Ror family of receptor tyrosine kinases. Although Ror2 has been shown to play crucial roles in developmental morphogenesis, the precise signalling events that Ror2 mediates remain elusive. Since Ror2 possesses an extracellular cysteine-rich domain (CRD) that resembles the Wnt-binding sites of the Frizzled (Fz) proteins, it is conceivable that Ror2 interacts with members of the Wnt family.

Results: Both Ror2^{-/-} and Wnt5a^{-/-} mice exhibit dwarfism, facial abnormalities, short limbs and tails, dysplasia of lungs and genitals, and ventricular

septal defects. *In vitro* binding assay revealed that Wnt5a binds to the CRD of Ror2. Furthermore, Ror2 associates via its CRD with rFz2, a putative receptor for Wnt5a. Interestingly, Wnt5a and Ror2 activate the non-canonical Wnt pathway, as assessed by activation of JNK in cultured cells and inhibition of convergent extension movements in *Xenopus*.

Conclusions: Our findings indicate that Wnt5a and Ror2 interact physically and functionally. Ror2 may thus act as a receptor for Wnt5a to activate non-canonical Wnt signalling.

Introduction

Receptor tyrosine kinases (RTKs) play crucial roles in developmental morphogenetic processes by regulating cellular proliferation, differentiation, migration, and death (Schlessinger 2000). Ror2 is a member of the Ror family of RTKs, characterized by the presence of intracellular tyrosine kinase domains related to those of the Trk-family RTKs, and by the presence of extracellular Frizzled-like cysteine-rich domains (CRDs) and

membrane-proximal Kringle domains, that are assumed to mediate protein-protein interactions (Masiakowski & Carroll 1992; Oishi *et al.* 1999; Forrester 2002; Yoda *et al.* 2003). Ror2 is expressed in the face, limbs, heart, and lungs during mouse embryogenesis (Matsuda *et al.* 2001). Mice lacking Ror2 expression exhibit dwarfism, facial abnormalities, shortened limbs and tails (due to skeletal abnormalities with foreshortened or misshapen bones), abnormalities in axial skeletons, ventricular septal defects (VSD), and respiratory dysfunction, resulting in neonatal lethality (DeChiara *et al.* 2000; Takeuchi *et al.* 2000). Furthermore, it has recently been reported that in humans, mutations within Ror2 cause brachydactyly type B (BDB), a dominant skeletal disorder characterized by hypoplasia/aplasia of distal phalanges (Oldridge *et al.* 2000; Schwabe *et al.* 2000), and Robinow syndrome, a recessive

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condition characterized by short stature, limb bone shortening, segmental defects of the spine, and a dysmorphic facial appearance (reviewed in Patton & Afzal 2002). These findings further emphasize the crucial role of Ror2 in developmental morphogenesis. Currently, the ligands of Ror-family RTKs, including Ror2, are unknown. Since Ror2 possesses an extracellular CRD that closely resembles the Wnt-binding sites of the Frizzled (Fz) proteins (Masiakowski & Yancopoulos 1998; Rehn *et al.* 1998), it can be assumed that Ror2 may interact with a member(s) of the Wnt family of proteins.

Members of the Wnt family have also been implicated in a variety of developmental morphogenetic processes (Wodarz & Nusse 1998). Previous studies indicate that Wnt family proteins can be classified into at least two subfamilies (Kühl *et al.* 2000); one is the Wnt1 class (e.g. Wnt1, Wnt3a, Wnt8) that activates the canonical Wnt/ β -catenin pathway to regulate cell proliferation and cell fate (Cadigan & Nusse 1997; Sokol 1999), and the other is the Wnt5a class (e.g. Wnt5a, Wnt11) that activates a non-canonical Wnt pathway to regulate cell polarity in *Drosophila* and convergent extension movements in *Xenopus* and *zebrafish* (Moon *et al.* 1993; Heisenberg *et al.* 2000; Sokol 2000; Tada & Smith 2000; Wallingford *et al.* 2000). Recent studies have identified a series of proteins that are involved in this non-canonical Wnt signalling pathway in *Drosophila*, *Xenopus*, and *zebrafish*, including Frizzled 7 (Xfz7), Strabismus (Stbm), Dishevelled (Dsh), and JNK (Boutros *et al.* 1998; Djiane *et al.* 2000; Heisenberg *et al.* 2000; Tada & Smith 2000; Wallingford *et al.* 2000; Jessen *et al.* 2002; Park & Moon 2002). However, the molecules involved in non-canonical Wnt signalling in mammals remain unknown, with the exception of mouse Wnt5a, which activates JNK in cultured cells (Yamanaka *et al.* 2002). Among the Wnt family genes in mammals, mouse Wnt5a exhibits a remarkably similar developmental expression pattern to mouse Ror2. Expression of Wnt5a is detected in the developing face, limbs and tail, lungs, and genitals (Yamaguchi *et al.* 1999; Li *et al.* 2002). Mice with a disruption in Wnt5a exhibit dwarfism, facial abnormalities, shortened limbs and tails (foreshortened along the proximodistal (P-D) axis), dysmorphic ribs and vertebrae, absence of the genital tubercle, and abnormalities in distal lung morphogenesis (Yamaguchi *et al.* 1999; Li *et al.* 2002). RNA injection experiments in *zebrafish* and *Xenopus* have indicated that rat Frizzled2 (rFz2) and human Frizzled5 (hFz5) can function as receptor(s) for *Xenopus* Wnt5a (Xwnt5a), which is 95% identical with the mouse Wnt5a (He *et al.* 1997; Slusarski *et al.* 1997). However the actual receptor for mouse Wnt5a has not

been definitively identified, and its signalling pathway remains to be elucidated.

In this study, we compared the developmental phenotypes of Ror2^{-/-} and Wnt5a^{-/-} mice in detail, and examined the possible physical and functional interactions between Ror2 and Wnt5a. Previous reports have described some overlap in the developmental phenotypes of Ror2^{-/-} and Wnt5a^{-/-} mice (Yamaguchi *et al.* 1999; Takeuchi *et al.* 2000), and these similarities were further strengthened by our observations, showing that; (i) similar to Wnt5a^{-/-} mice, Ror2^{-/-} mice exhibit smaller somites and shortened presomitic mesoderm (PSM), abnormalities in the lungs with the foreshortened trachea, and dysplasia of genitals; and (ii) similar to Ror2^{-/-} mice, Wnt5a^{-/-} mice exhibit ventricular septal defects (VSD). Importantly, we demonstrate that Ror2 is involved in the non-canonical Wnt5a/JNK signalling pathway and interacts both physically and functionally with Wnt5a, as demonstrated by the following results: (1) Wnt5a, but not Wnt3a, binds to the extracellular CRD of Ror2 *in vitro*; (2) Ror2 forms a complex with rFz2, a putative receptor for Wnt5a (He *et al.* 1997), via its extracellular CRD when both molecules are expressed in HEK293T cells; (3) JNK, a crucial mediator of the non-canonical Wnt signalling pathway, is activated when Ror2 or Wnt5a is expressed singly in NIH3T3 cells, and activated additively when the two are coexpressed; and (4) convergent extension in *Xenopus* embryos is inhibited when Ror2 mRNA or Wnt5a mRNA is injected, and is inhibited synergistically when both mRNAs are injected. We also discuss the possible molecular nature of the receptor complex for Wnt5a.

Results

Similar overall phenotypes were observed in Ror2- and Wnt5a-deficient mice

Previous studies indicated that Ror2^{-/-} and Wnt5a^{-/-} mice share similar developmental phenotypes (Yamaguchi *et al.* 1999; Takeuchi *et al.* 2000). Both Ror2^{-/-} and Wnt5a^{-/-} newborns exhibited dwarfism, facial abnormalities, short limbs and tails, and respiratory dysfunction (Fig. 1A,C), and died shortly after birth. To compare the phenotypes of Ror2^{-/-} and Wnt5a^{-/-} mice in more detail, we extended the analysis to the heart of Wnt5a^{-/-} mice, the lungs and genitals of Ror2^{-/-} mice, and to the somites of Ror2^{-/-} embryos. Similar to Ror2^{-/-} mice, Wnt5a^{-/-} mice exhibited ventricular septal defects (VSD) (Fig. 1B). In addition, Wnt5a^{-/-} mice exhibited complete transposition of the great arteries (data not shown), a phenotype reminiscent of Ror1/Ror2 double mutant mice (Nomi *et al.*

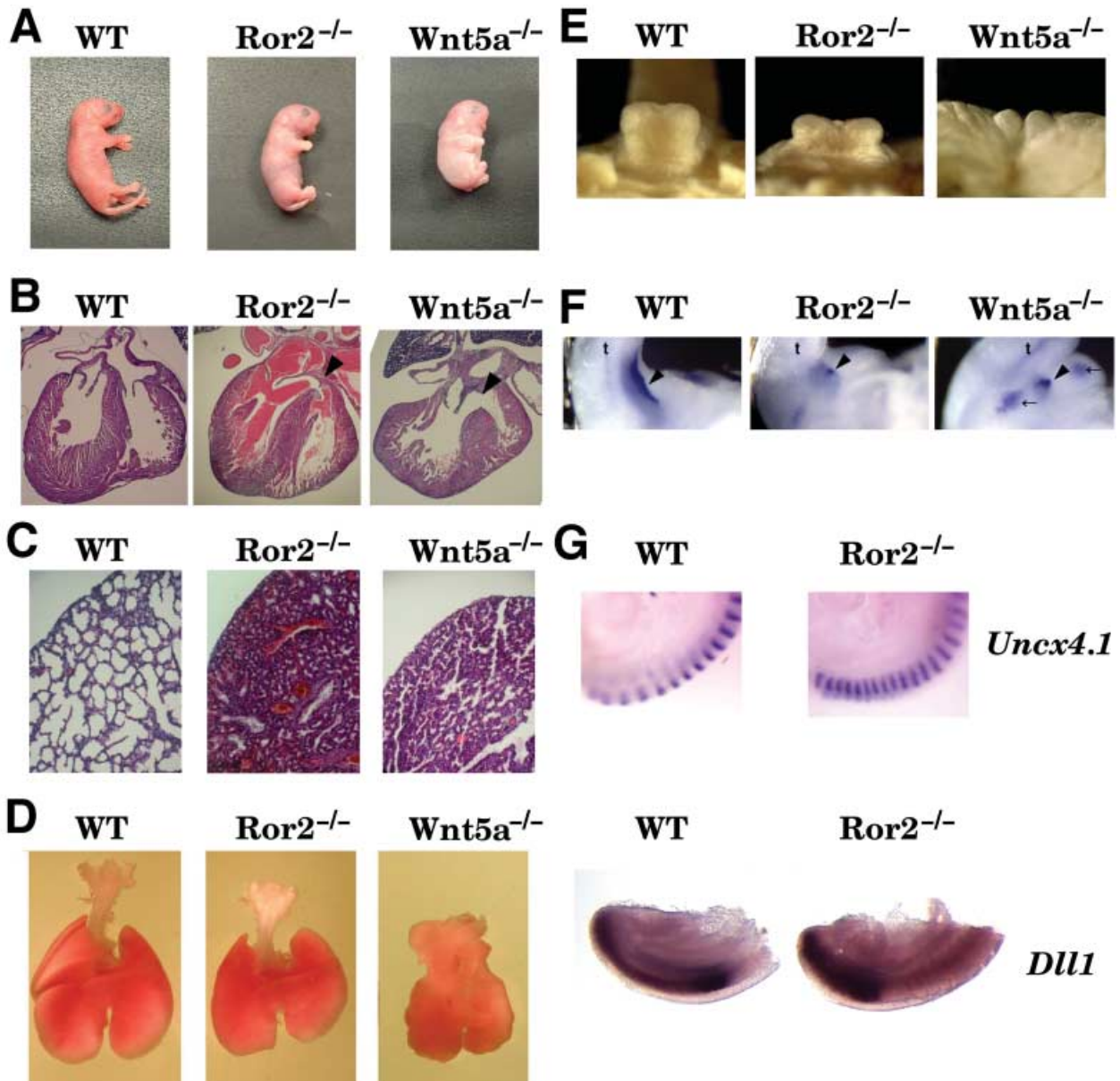


Figure 1 Comparison of developmental phenotypes in *Ror2*^{-/-} and *Wnt5a*^{-/-} mice. (A) Gross appearance of wild-type (WT), *Ror2*^{-/-}, and *Wnt5a*^{-/-} newborns. Both *Ror2*^{-/-} and *Wnt5a*^{-/-} newborns exhibit dwarfism, facial abnormalities, and foreshortened limbs and tail. (B) Histological analysis of the Haematoxylin and Eosin (H&E)-stained longitudinal sections of the hearts. Arrowheads indicate ventricular septal defects (VSD) in *Ror2*^{-/-} and *Wnt5a*^{-/-} newborns. (C) Histological analysis of the H&E-stained horizontal sections of the lungs. Respiratory dysfunction is indicated in *Ror2*^{-/-} and *Wnt5a*^{-/-} newborns. (D) E18.5 WT, *Ror2*^{-/-}, and *Wnt5a*^{-/-} lungs. The foreshortened phenotype of the *Ror2*^{-/-} and *Wnt5a*^{-/-} trachea is indicated. (E) Dorsal views of the external genitals. Hypoplasia or absence of the genital tubercle was observed in the *Ror2*^{-/-} and *Wnt5a*^{-/-} mice, respectively. (F) Whole mount *in situ* hybridization analysis of WT, *Ror2*^{-/-}, and *Wnt5a*^{-/-} embryos (E10.5). In both *Ror2*^{-/-} and *Wnt5a*^{-/-} mice, the expression of *Shh* is reduced compared with that of control (arrowheads). *Shh* expression in the limb is indicated by arrows. (G) Whole mount *in situ* hybridization analysis of WT and *Ror2*^{-/-} embryos (E9.5). Expression of *Uncx 4.1* and *Dll1* in WT and *Ror2*^{-/-} mice (lateral views) are indicated. Note the shortened PSM and the smaller somites in *Ror2*^{-/-} mice. t, tail.

2001). On the other hand, *Ror2*^{-/-} embryos (E18.5) exhibited abnormalities in the lungs with foreshortened trachea along the P-D axis and a reduced number of cartilage rings (Fig. 1D), similar to *Wnt5a*^{-/-} mice. *Ror2*^{-/-} mice also exhibited outgrowth defects in the genitals (GT), although hypoplasia of the genitals was somewhat modest compared to *Wnt5a*^{-/-} mice (Fig. 1E). It has been reported that sonic hedgehog (Shh) is required for the initiation of GT outgrowth in mice (Haraguchi *et al.* 2001). *Shh* was normally expressed in the outermost part of the urogenital sinus epithelium at E10.5, before the onset of GT outgrowth (Fig. 1F). Consistent with the genital outgrowth reduction, in both *Ror2* and *Wnt5a* mutant mice, the expression of *Shh* was reduced compared with that of control (Fig. 1F). Furthermore, compared with the wild-type embryos, *Ror2*^{-/-} embryos exhibited more compressed somites and presomitic mesoderm (PSM) in the anterior-posterior axis as assessed by expression of *uncx4.1* and *dll1* (Fig. 1G). The abnormalities are characteristic of those observed in *Wnt5a* mutants (Yamaguchi *et al.* 1999). Thus, these results indicate that the overall phenotypes of *Ror2*^{-/-} and *Wnt5a*^{-/-} mice are remarkably similar, suggesting a possible interaction of Ror2 with Wnt5a during mouse development.

Ror2 associates with Wnt5a via its CRD *in vitro*

Next, we addressed the question of whether Wnt5a can associate physically with the extracellular region of Ror2. To this end, Ror2-Fc fusion proteins (Fig. 2A), consisting of the extracellular region of Ror2 fused to the Fc portion of human IgG1, were coupled with protein G-sepharose, and were mixed with either HA-tagged Wnt5a (Wnt5a-HA)-containing medium or Wnt3a-HA-containing medium. After extensive washing, Wnt proteins bound to the Ror2-Fc-coupled protein G-sepharose were detected by anti-HA immunoblotting (see Experimental procedures). As shown in Fig. 2B, Wnt5a-HA, but not Wnt3a-HA, bound to the extracellular region of Ror2 *in vitro*. Since the CRD of Ror2 resembles the Wnt-binding sites of Frizzled proteins, we next examined whether or not the CRD of Ror2 is required for Wnt5a binding to Ror2. As shown in Fig. 2C, Ror2ΔCRD-Fc (Fig. 2A), a version of Ror2-Fc that lacks the CRD, failed to associate with Wnt5a-HA. These results indicate that Wnt5a selectively binds to Ror2, presumably via the CRD.

Ror2 forms a complex with rFz2

Considering the previous findings suggesting that rFz2 and hFz5 may be receptors for Wnt5a (He *et al.* 1997;

Slusarski *et al.* 1997), we considered the possibility that Ror2 may associate with the mouse ortholog of rFz2 (or hFz5) to form a receptor complex that recognizes Wnt5a. In order to test this possibility, HA-tagged Ror2 alone, Flag-tagged soluble form of rFz2 (rFz2CRD) alone, or both were expressed in HEK293T cells. Their association was evaluated by anti-HA immunoprecipitation followed by anti-Flag immunoblotting. It was found that rFz2CRD co-immunoprecipitated with Ror2 (Fig. 3B). To verify the specificity of this association between Ror2 and rFz2CRD, and to identify a region(s) within Ror2 that is required for this association, we generated a series of Ror2 mutants (see Fig. 3A) and evaluated their abilities to associate with rFz2CRD in HEK293T cells. Ror2ΔCRD and Ror2ΔCK, Ror2 mutants that lack the CRD, failed to associate with rFz2CRD (Fig. 3B). These results suggest that Ror2 forms a complex with rFz2 via its CRD. We further examined the selectivity of the physical association between Ror2 and Frizzled proteins. It was found that Ror2 can also associate with hFz5CRD, but not mFz8CRD (Fig. 3C).

Ror2 and Wnt5a activate JNK

It has recently been reported that mouse Wnt5a is capable of activating JNK in cultured cells (Yamanaka *et al.* 2002). To assess the functional significance of the physical interaction between Ror2 and Wnt5a described above, we examined the effects of Wnt5a and/or Ror2 expression on JNK activities in NIH3T3 cells (see Experimental procedures). As expected, expression of Wnt5a alone resulted in about a threefold increase in JNK activity compared with the basal activity (Fig. 4A,B). Interestingly, expression of Ror2 alone also resulted in JNK activation (about 2.5-fold increase in JNK activity compared with the basal activity) (Fig. 4A,B). As shown in Fig. 4, coexpression of Wnt5a and Ror2 had an additive effect on JNK activity. The results suggest that, like Wnt5a, Ror2 is also involved in JNK activation.

Ror2 and Wnt5a synergistically inhibit convergent extension movement

It has been well documented that Wnt5a is capable of regulating convergent extension movements in *Xenopus* (Moon *et al.* 1993; Yamanaka *et al.* 2002). Therefore, we next assessed whether the Wnt5a and Ror2 interaction contributes to convergent extension by monitoring changes in the morphology of ectodermal explants stimulated by BVg1. Control explants elongated significantly and exhibited typical morphological changes (Tada &

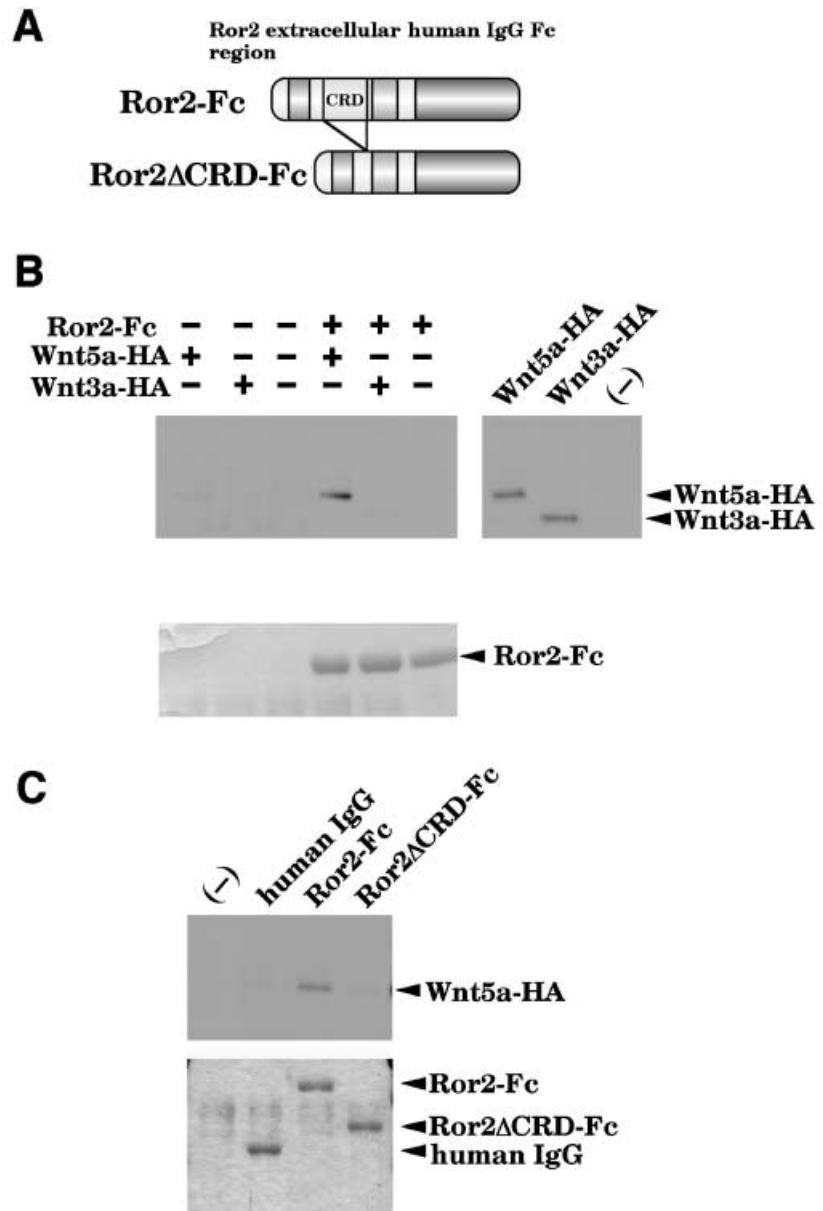


Figure 2 Binding of Wnt5a to the extracellular CRD of Ror2 *in vitro*. (A) Schematic representation of Ror2-Fc and Ror2 Δ CRD-Fc fusion proteins. (B) Wnt5a, but not Wnt3a, binds to the extracellular domain of Ror2. Ror2-Fc fusion proteins, adsorbed to protein G-Sepharose, were mixed with conditioned media containing either Wnt5a-HA or Wnt3a-HA. *In vitro* binding analyses was performed as described in Experimental procedures. Amounts of Ror2-Fc and HA-tagged Wnt proteins utilized in analysis were assessed by CBBR staining (lower panel) and anti-HA immunoblotting (right panel), respectively. (C) Wnt5a binds to the CRD of Ror2. Ror2-Fc, Ror2 Δ CRD-Fc fusion proteins, or human IgG (as a control), adsorbed to protein G-Sepharose, were mixed with conditioned media containing Wnt5a-HA. *In vitro* binding analyses was performed as described above. Amounts of Wnt5a-HA bound to the respective fusion proteins or human IgG were evaluated by anti-HA immunoblotting (upper panel). Note that Wnt5a-HA binds to Ror2-Fc, but not Ror2 Δ CRD-Fc. Amounts of human IgG, Ror2-Fc, and Ror2 Δ CRD-Fc were assessed by CBBR staining (lower panel).

Smith 2000) (Fig. 5A,B,BVg1). Expression of Wnt5a or Ror2 alone inhibited both elongation of and morphological change in the explants (Fig. 5A,B), showing that both Wnt5a and Ror2 function to regulate convergent extension movements. Intriguingly, coexpression of Wnt5a and Ror2 synergistically inhibited convergent extension (Fig. 5B), indicating that Wnt5a and Ror2 interact functionally. Expression of Ror2Tc (see Fig. 3A), a Ror2 mutant lacking the cytoplasmic region, exhibited only a marginal effect on convergent extension (Fig. 5A,B). However, coexpression of Wnt5a and Ror2Tc synergistically inhibited convergent extension, although not as

much as coexpression of Wnt5a and wild-type Ror2 (Fig. 5B panels B, E, F). This observation raises the possibility that the extracellular region of Ror2 possesses weak activities independent of its cytoplasmic tyrosine kinase domain.

Discussion

Our present data, together with the previous reports (Yamaguchi *et al.* 1999; Takeuchi *et al.* 2000; Li *et al.* 2002), indicate that the overall phenotypes of Ror2^{-/-} and Wnt5a^{-/-} mice are remarkably similar, suggesting a

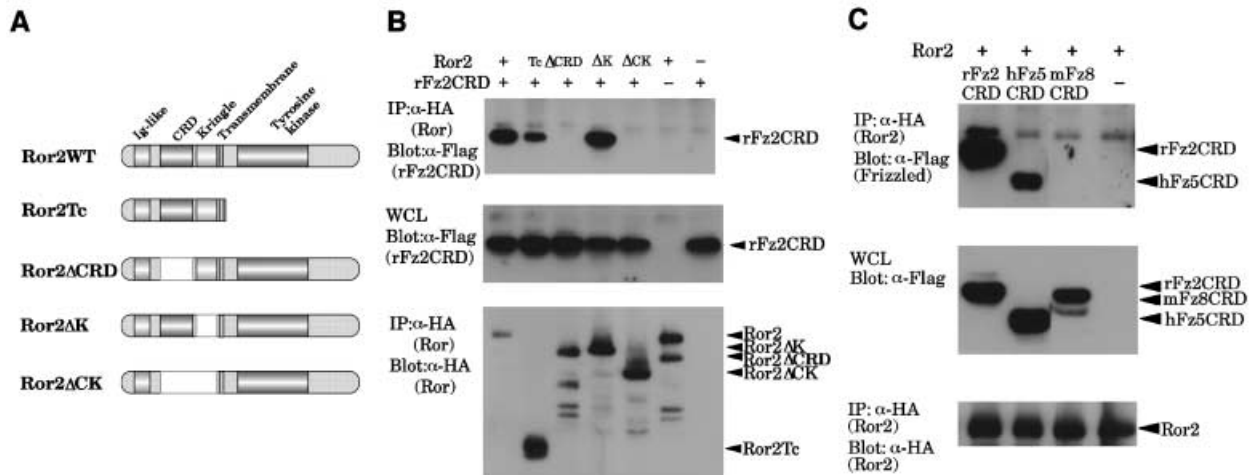


Figure 3 Ror2 associates with rFz2 via its CRD. (A) Schematic representation of Ror2 and its mutant derivatives. (B) Failure of Ror2 mutants (Δ CRD, Δ CK), lacking the CRD, to associate with rFz2. Ror2 or the respective mutants were coexpressed with rFz2 (rFz2CRD) in HEK293T cells. Association of rFz2 (rFz2CRD) with wild-type or the respective Ror2 mutants was monitored by anti-Flag immunoblotting of anti-HA immunoprecipitates (upper panel). Expression levels of the respective Ror2 proteins and of rFz2CRD were determined by anti-HA immunoblotting of anti-HA immunoprecipitates (lower panel) or anti-Flag immunoblotting of whole cell lysates (middle panel). (C) Ror2 binds to rFz2 and hFz5, but not mFz8. Ror2 was coexpressed with rFz2 (rFz2CRD), hFz5 (hFz5CRD), or mFz8 (mFz8CRD) in 293T cells. Immunoprecipitation and immunoblotting analyses were performed as described in Fig. 3B.

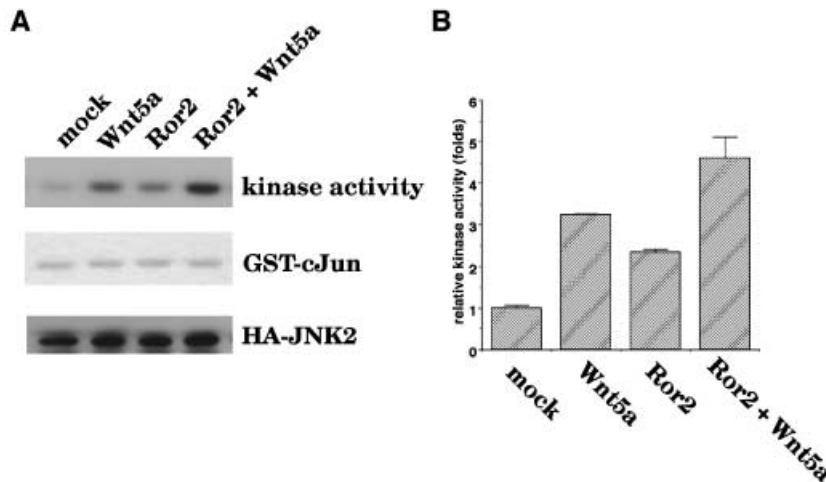
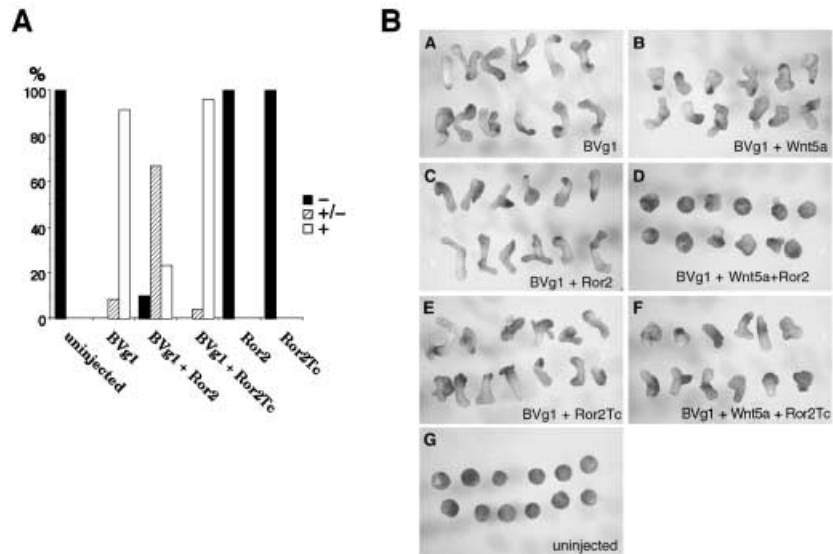


Figure 4 Activation of JNK by Wnt5a and/or Ror2. (A) HA-tagged JNK2 was coexpressed with Wnt5a, Ror2, or both in NIH3T3 cells, and immunoprecipitated with anti-HA antibodies. JNK activities in the respective immunoprecipitates were determined by *in vitro* phosphorylation of GST-cJun (upper panel). Amounts of GST-cJun and of HA-JNK2 in the anti-HA immunoprecipitates were estimated by CBBR staining and anti-HA immunoblotting, respectively (middle and lower panels). (B) The histogram indicates relative JNK activities (means) with standard error bars (SD). Relative JNK activities were determined by normalizing the amounts of GST-cJun and HA-JNK2 in each reaction. Essentially identical results were obtained in three independent experiments.

possible interaction of Ror2 with Wnt5a. In fact, we have shown that physical and functional interaction between Wnt5a and Ror2. *In vitro* binding analyses showed that Wnt5a, but not Wnt3a, binds to the extracellular CRD of Ror2 (Fig. 2). This result suggests that Ror2 exhibits selectivity for association with members of Wnt family. It has recently been reported that *Xenopus* Wnt proteins (XWnt5a, XWnt11, and XWnt8) can co-immunoprecipitate with the *Xenopus* ortholog of

mammalian Ror2 (Xror2) when the latter is expressed in *Xenopus* embryos (Hikasa *et al.* 2002). Further study is required to clarify the structural basis of the selective binding between Wnt family proteins and Ror2. We have also shown that Ror2 forms a complex with rFz2 or hFz5, putative receptors for Wnt5a, but not with mFz8 (Fig. 3). It has been shown that LRP6, a member of the LDLR-related protein (LRP) family, associates with mFz8 in the presence of Wnt1, and that LRP6

Figure 5 Inhibition of convergent extension in *Xenopus* embryos by Wnt5a and/or Ror2. (A) Animal caps expressing the indicated constructs with *BVg1*. Each mRNA (*Ror2* 500 pg; *Ror2Tc* 500 pg) was injected with *BVg1* mRNA (10 pg) into the animal pole region of two cells of 4-cell stage embryos. Animal caps were excised from stage 8.5 embryos and cultured in 1 × Steinberg solution until stage 19. The percentages of elongated animal caps are indicated. – no elongation; +/- weak elongation; + strong elongation. $n = 20$ –35 for each condition. (B) Each combination of mRNAs (*Wnt5a* 500 pg; *Ror2* 250 pg; *Ror2Tc* 250 pg) with *BVg1* mRNA (15 pg) was injected into the animal pole region of two cells of 4-cell stage embryos. Animal caps were excised from stage 8.5 embryos and cultured in 1 × Steinberg solution until stage 19.



functions as a co-receptor that initiates canonical Wnt signalling (Tamai *et al.* 2000). In this respect, it will be of interest to examine whether or not Ror2 can function as a co-receptor to initiate non-canonical Wnt5a signalling in mammals.

It was found that expression of Wnt5a or Ror2 alone results in activation of JNK in NIH3T3 cells and inhibition of convergent extension movements in *Xenopus* embryos (Figs 4 and 5A). In addition, coexpression of Wnt5a and Ror2 synergistically inhibited convergent extension movements in *Xenopus* (Fig. 5B). These results indicate that both Ror2 and Wnt5a interact functionally. Co-expression of Wnt5a and Ror2Tc, which lacks the cytoplasmic region of Ror2, also synergistically inhibited convergent extension movements to some extents, raising the possibility that Ror2 contributes partly to Wnt5a signalling irrespective of its cytoplasmic region. With this respect, it is worth noting that the *C. elegans* ortholog of Ror (CAM-1) exhibits both tyrosine kinase-dependent and -independent functions (Forrester *et al.* 1999; Forrester 2002; Yoda *et al.* 2003).

Gene disruption studies of Wnt5a and Ror2 in mice have also pointed out an important finding that phenotypes of *Wnt5a*^{-/-} and *Ror2*^{-/-} mice during somitogenesis and possibly cardiogenesis (the smaller somites and shortened PSM, VSD and complete transposition of the great arteries, see Fig. 1 panels B, G) are highly related to those observed in loss-of-function mutations (analyses) of genes (e.g. *XWnt11*, *Xdsh*, *XJNK*, *tri/stbm*) involved in non-canonical Wnt signalling in *Xenopus* or *zebrafish* (Heisenberg *et al.* 2000; Wallingford *et al.* 2000; Jessen *et al.* 2002; Pandur *et al.* 2002; Yamanaka

et al. 2002). Taken together with our results showing that Wnt5a interacts with Ror2 both physically and functionally, these observations suggest that Ror2 acts as a receptor for Wnt5a to activate the non-canonical Wnt5a/JNK pathway during developmental morphogenesis in mammals.

Recently, it has been reported that in *Drosophila* Derailed (Drl), a member of RYK (receptor-like tyrosine kinase) family, can be a receptor for Wnt5, an ortholog of mammalian Wnt5a (Yoshikawa *et al.* 2003). RYK family is an atypical receptor tyrosine kinase and appears to lack catalytic activity. It has also been suggested that members of RYK family transduce a signal together with another catalytically active tyrosine kinase (Yoshikawa *et al.* 2001). In this respect, it is interesting to consider a possibility that RYK and Ror2 also interact physically and functionally, and contribute to form a receptor complex for Wnt5a. With this respect it is of importance to note that the phenotypes of *RYK*^{-/-} mice, *Ror2*^{-/-} mice, and *Wnt5a*^{-/-} are quite similar, including craniofacial abnormalities and shortened limbs (Halford *et al.* 2002). Further study will be required to clarify the possible interaction among RYK, Ror2, and rFz2 in Wnt5a signalling.

Experimental procedures

Plasmid construction

Mouse Ror2 cDNA was subcloned into pcDNA3 together with a Flag or HA epitope tag at its C-terminus (pcDNA-Ror2-Flag and pcDNA-Ror2-HA). Mouse Wnt5a cDNA was subcloned into

pCS2 (pCS2-*Wnt5a*). The following deletion mutants were derived from pcDNA-Ror2-HA: pcDNA-Ror2Δc-HA deletes amino acids (a.a.) 435–944 (most part of the cytoplasmic region), pcDNA-Ror2ΔCRD-HA deletes a.a. 174–300 (corresponding to the CRD), pcDNA-Ror2ΔK-HA deletes a.a. 316–395 (corresponding to the Kringle domain), and pcDNA-Ror2ΔCK-HA deletes a.a. 174–394 (containing both the CRD and Kringle domains). The pCS2-Ror2 construct was generated by ligating *Xho*I-digested pCS2 with a *Sal*I-digested DNA fragment derived from pcDNA-Ror2-FLAG. pCS2-Ror2Tc encodes Ror2, lacking a.a. 435–944. Ror2-Fc and Ror2ΔCRD-Fc fusion proteins consist of the extracellular region (residues 1–400) of Ror2 or its deletion derivative (lacking a.a. 174–300) fused to the Fc portion of human IgG1 (a.a. 247–477), respectively. rFz2CRD, hFz5CRD, and mFz8CRD consist of a.a. 1–253 of rFz2, a.a. 1–219 of hFz5, and a.a. 1–235 of mFz8, respectively.

***In vitro* binding assay**

Recombinant Ror2-Fc and Ror2ΔCRD-Fc fusion proteins were overproduced using the BAC-to-BAC Baculovirus Expression System kit (Gibco BRL), according to the manufacturer's instructions. Recombinant fusion proteins or control human IgG protein (Cappel) were adsorbed to protein G-sepharose (Amersham Pharmacia Biotech) for 2 h at 4 °C. After removal of unbound materials, protein G-sepharose coupled with Ror2-Fc, Ror2ΔCRD-Fc, or with control human IgG were washed once with TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% (v/v) Tween 20), and then mixed with medium containing Wnt3a or Wnt5a (Shibamoto *et al.* 1998; Yamanaka *et al.* 2002). Two hours after incubation at 4 °C, Sepharose beads were washed four times with TBST, and bound proteins were subsequently eluted with Laemmli buffer. Eluted proteins were subjected to SDS-PAGE (10% PAG), and analysed by immunoblotting procedure (see below).

Kinase assays

Determination of JNK kinase activity was performed as previously described with a minor modification (Yamanaka *et al.* 2002). Eighteen hours after transfection, NIH3T3 cells were solubilized with lysis buffer A (20 mM HEPES (pH 7.4), 0.5% (v/v) Triton-X 100, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 2 mM dithiothreitol, 10 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulphonyl fluoride (PMSF), 20 μg/ml leupeptin and 10 μg/ml aprotinin), and HA-tagged JNK protein was immunoprecipitated with anti-HA antibody (16B12, BAbCO). Immunoprecipitates were washed three times with lysis buffer A, then once with kinase reaction buffer (20 mM HEPES (pH 7.4), 2 mM EGTA, 15 mM MgCl₂). The immunoprecipitates were resuspended in 30 μl of kinase reaction buffer containing 2 μg of GST-cJun protein and 10 μCi γ³²P-ATP (3000 Ci/mmol, Amersham Pharmacia Biotech), and incubated for 5 min at 30 °C. The reaction was terminated by the addition of Laemmli sample buffer and samples were separated by SDS-PAGE (10% PAG). Subsequently, the gels were subjected to autoradiography, and band intensities were quantified using an imaging analyser (BAS2000; Fujix).

Immunoprecipitation and immunoblotting

Cells were solubilized with lysis buffer B (50 mM Tris-HCl (pH 7.4), 0.5% (v/v) NP-40, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 10 μg/mL leupeptin and 10 μg/mL aprotinin), and cell lysates were prepared by centrifugation at 12 000 g for 15 min to remove insoluble materials. Cell lysates were precleared for 1 h at 4 °C with protein A-sepharose (Amersham Pharmacia Biotech). The precleared supernatants were then immunoprecipitated with anti-HA antibody conjugated to protein A-sepharose beads for 2 h at 4 °C. The immunoprecipitates were washed five times with 1 ml of the above lysis buffer B, and eluted with Laemmli sample buffer. The immunoprecipitates or whole cell lysates were separated by SDS-PAGE (10% PAG), and transferred to PVDF membrane filters (Immobilon, Millipore). The membranes were immunoblotted with anti-HA or anti-Flag antibodies (M2, Sigma), and bound antibodies were visualized with HRP-conjugated goat anti-mouse IgG antibodies (Bio-Rad), using chemiluminescence reagent (Renaissance, NEN).

***In situ* hybridization**

In situ hybridization analyses were performed essentially as previously described (Matsuda *et al.* 2001). Single strand RNA probes for *shh*, *Uncx4.1* and *Dll1* were synthesized as previously described (Barrantes *et al.* 1999; Haraguchi *et al.* 2001; Matsuda *et al.* 2001).

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