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RESEARCH ARTICLE

Ror2 signaling is required for local upregulation of GDF6 and activation of BMP signaling at the neural plate border

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ABSTRACT

The receptor tyrosine kinase Ror2 is a major Wnt receptor that activates β-catenin-independent signaling and plays a conserved role in the regulation of convergent extension movements and planar cell polarity in vertebrates. Mutations in the *ROR2* gene cause recessive Robinow syndrome in humans, a short-limbed dwarfism associated with craniofacial malformations. Here, we show that Ror2 is required for local upregulation of *gdf6* at the neural plate border in *Xenopus* embryos. Ror2 morphant embryos fail to upregulate neural plate border genes and show defects in the induction of neural crest cell fate. These embryos lack the spatially restricted activation of BMP signaling at the neural plate border at early neurula stages, which is required for neural crest induction. Ror2-dependent planar cell polarity signaling is required in the dorsolateral marginal zone during gastrulation indirectly to upregulate the BMP ligand Gdf6 at the neural plate border and Gdf6 is sufficient to rescue neural plate border specification in Ror2 morphant embryos. Thereby, Ror2 links Wnt/planar cell polarity signaling to BMP signaling in neural plate border specification and neural crest induction.

KEY WORDS: Ror2, Gdf6, BMP, Wnt, Neural crest, Neural plate border

INTRODUCTION

The receptor tyrosine kinase Ror2 is a major regulator of β-catenin-independent Wnt signaling. In humans, the rare recessive Robinow syndrome is caused by a loss-of-function mutation in the *ROR2* gene. Affected individuals are of short stature with short limbs and hemivertebrae. In addition, affected individuals show defects in cardiac and craniofacial morphogenesis (van Bokhoven et al., 2000), with the craniofacial cartilage and bone as well as parts of the cardiac outflow tract deriving from the neural crest.

The neural crest is a vertebrate-specific, multipotent and migratory cell population that gives rise to a variety of derivatives including neurons and glia of the peripheral nervous system, cranial cartilage and bone, and pigment cells. Neural crest cell fate is induced during gastrulation in a dorsolateral region adjacent to the prospective neural plate (Essex et al., 1993; Mayor et al., 1995; O'Donnell et al., 2006; Sasai et al., 2001). Morphogenetic movements of gastrulation and neurulation bring these cells first to the lateral border of the neural plate and later, as the neural folds elevate and fuse, the neural crest cells are brought to the dorsal part of the neural tube. Neural crest cells subsequently undergo an epithelial-to-mesenchymal transition and migrate actively and collectively on defined routes from the dorsal neural tube through the embryonic body to their final destination (Cheung et al., 2005; Theveneau and Mayor, 2011; reviewed in Theveneau and Mayor, 2012).

Neural crest induction in the dorsal ectoderm requires intermediate levels of BMP activity in combination with Wnt/β-catenin and fibroblast growth factor (Fgf)/MAP kinase pathways (Hong et al., 2008; LaBonne and Bronner-Fraser, 1998; Mayor et al., 1995, 1997; Neave et al., 1997; Wilson et al., 1997). The combined activity of these major signaling pathways induces the expression of transcription factors including Gbx2, Dlx, Msx1 and Msx2, Pax3 and Pax7, Ap2alpha (also known as Tfap2a), and Zic1 (Brewer et al., 2004; Khadka et al., 2006; Li et al., 2009; Luo et al., 2003, 2001; Monsoro-Burq et al., 2005; Plouhinec et al., 2014; Woda et al., 2003) in an area between neural and non-neural ectoderm. These factors cooperatively define the neural plate border (NPB) and interact to induce expression of neural crest specific genes. Neural crest genes such as *snail1*, *snail2*, *c-myc*, *foxd3*, *sox8*, *sox9* and *twist* control specification, proliferation and survival, multipotency and migration of the neural crest (Aybar et al., 2003; Bellmeyer et al., 2003; Honoré et al., 2003; Hopwood et al., 1989; LaBonne and Bronner-Fraser, 2000; O'Donnell et al., 2006; Sasai et al., 2001; Spokony et al., 2002).

In addition to the well-defined role of Wnt/β-catenin signaling in neural crest induction, a study by Ossipova and Sokol (2011) has shown that β-catenin independent Wnt signaling is also implicated in neural crest induction. In the later phases of neural crest development, namely during migration, particularly β-catenin independent Wnt signaling plays a crucial role. The Wnt/planar cell polarity (PCP) pathway controls cell polarity, collective cell migration and the recently described contact inhibition of locomotion by local activation of the Rho family small GTPases RhoA and Rac1 (Becker et al., 2013; De Calisto et al., 2005; Matthews et al., 2008; Mayor and Theveneau, 2014; Shnitsar and Borchers, 2008).

Ror2 is a Wnt (co-)receptor that binds Wnt ligands directly via its cysteine-rich domain, which is highly similar to the Wnt-binding CRD domain of frizzled receptors. In addition, Ror2 interacts with frizzled receptors to receive and transduce Wnt signals (Forrester, 2002; Masiakowski and Carroll, 1992; Saldanha et al., 1998). Its role as receptor for Wnt5a and activator of PCP and c-Jun N-terminal kinase (Jnk, also known as Mapk8) signaling is considered one key function of Ror2 that is conserved among vertebrates (Gao et al., 2011; Hikasa et al., 2002; Mikels and Nusse, 2006; Mikels et al., 2009; Nishita et al., 2006; Oishi et al., 2003; Schambony and Wedlich, 2007; Wang et al., 2011; Yamamoto et al., 2008). In addition to activating PCP signaling, in early *Xenopus* embryos Ror2 signals via an RTK-like pathway involving Shc,

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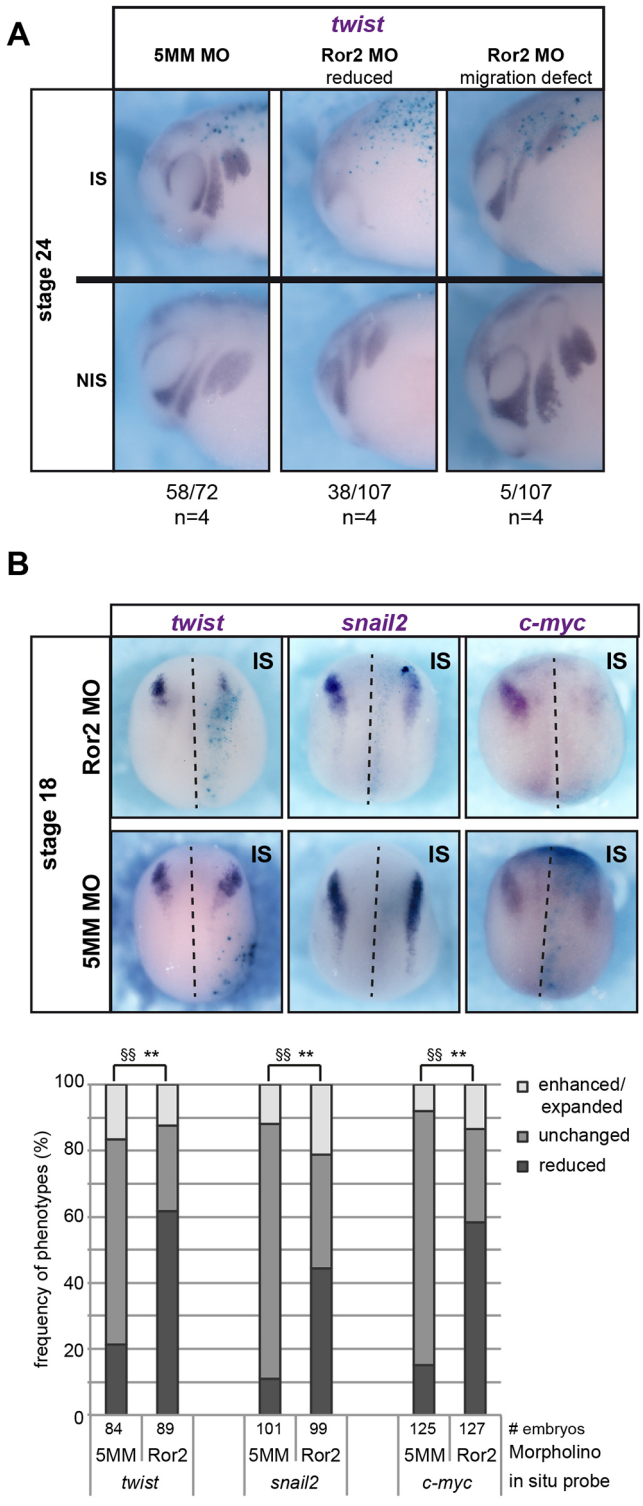

Fig. 1. Ror2 loss-of-function results in decreased expression of neural crest marker genes. Ror2 was knocked down by targeted injection of Ror2 antisense morpholino (Ror2 MO) into one dorso-animal blastomere of 8-cell stage embryos. Controls were injected with the corresponding 5-mismatch morpholino (5MM). A plasmid encoding *lacZ* was co-injected as lineage tracer and the injected side was identified by β-gal staining. (A) *In situ* hybridization with a *twist* probe at stage 24. Images show injected (IS) and non-injected (NIS) side of representative embryos injected with either control 5MM MO or Ror2 MO; two phenotypes were obtained after Ror2 MO injection (reduced expression and migration defects). Numbers below the images indicate the overall frequency of the shown phenotype in four independent experiments. (B) Examples of embryos injected as indicated and probed with the indicated probe at stage 18 are shown; the injected side (IS) is oriented to the right. The graph shows the frequency of observed phenotypes after injection of Ror2 MO or a 5-mismatch control MO (5MM) from at least three independent experiments. The total number of embryos is indicated below each column. Statistically significant differences between Ror2 MO and control 5MM MO according to the χ^2 test are indicated by $^{\S\S}P<0.01$, $^{\S}P<0.05$, and according to the Wilcoxon rank sum test by $^{**}P<0.01$, $^{*}P<0.05$.

phosphatidylinositol-4,5-bisphosphate 3-kinase (Pik3, also known as Pi3k) and Jnk to activate c-Jun and Atf2-dependent transcription of paraxial protocadherin (Papc, also known as Pcdh8) (Feike et al., 2010; Liu et al., 2007, 2008; Schambony and Wedlich, 2007). Ror2 is expressed in the neural plate, at the neural plate border, in the neural crest and the neural crest-derived branchial arches in *Xenopus laevis*, chicken and mouse from late gastrula stages onward (Feike et al., 2010; Hikasa et al., 2002; Matsuda et al., 2001; Stricker et al., 2006). This expression pattern, together with defects in craniofacial development observed in individuals with Robinow syndrome (van Bokhoven et al., 2000) and Ror2 knock-out mice (DeChiara et al., 2000; Nomi et al., 2001), indicates a role of Ror2 in neural crest development. To characterize this role, we have knocked down Ror2 in *Xenopus laevis* embryos and analyzed the development of the neural crest in Ror2 morphant embryos. Here we report that Ror2 is specifically required in the dorsolateral marginal zone to induce the neural plate border. Ror2 acts indirectly via transcriptional regulation of *papc* and the related protocadherin *pcns* (also known as *pcdh8l*) in the dorsolateral marginal zone, to control cell polarity in the lateral neural plate and to induce local Gdf6-mediated BMP signaling at the neural plate border in late gastrulation, which is essential for neural plate border specification and neural crest induction.

RESULTS

Ror2 is required for neural crest induction and specification of the neural plate border

We investigated the role of Ror2 in neural crest development using a targeted morpholino oligonucleotide (MO)-mediated knockdown approach. Ror2 MO was injected into one animal-dorsal blastomere at the 8-cell stage to target predominantly the dorsal ectoderm, and early tadpole stage embryos were analyzed by *in situ* hybridization against the neural crest marker gene *twist*. We expected a role of Ror2 in neural crest migration because of its role in Wnt/PCP signaling and tissue morphogenesis (Gao et al., 2011; Oishi et al., 2003; Schambony and Wedlich, 2007) and the well-known requirement of this pathway in neural crest migration (De Calisto et al., 2005; Mayor and Theveneau, 2014). However, the dominant phenotype observed after Ror2 knockdown was a downregulation of *twist* on the injected side (Fig. 1A). Only in rare cases (<5%) we observed impaired or incomplete migration of the cranial neural crest. Next, we analyzed the expression of neural crest markers prior to the onset of cranial neural crest migration at mid-neurula stages. Knockdown of Ror2 resulted in downregulation of the neural crest markers *twist*, *snail2* and *c-myc* (Fig. 1B,C) in the pre-migratory neural crest, which indicated that Ror2 is required at an early stage of neural crest specification.

Therefore, we analyzed the expression of genes involved in the definition of the neural plate border territory, from which neural crest precursors arise. The neural plate border (NPB) is first specified at early gastrula stages in an area lateral to the prospective neural plate that receives intermediate levels of BMP signaling within the dorsoventral BMP gradient (for review see Milet and Monsoro-Burq, 2012), which functionally interacts with Wnt/β-

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catenin signaling to induce the expression of a specific set of neural plate border-specifying genes (for review see Betancur et al., 2010; Milet and Monsoro-Burq, 2012). We analyzed the expression of *gbx2*, *zic1*, *msx1*, *msx2* and *pax3* at late gastrula/early neurula stages 12/13 and interestingly found that all genes except *pax3* were downregulated in Ror2 morphant embryos (Fig. 2A,B). Notably, in some cases we also observed lateral expansion of *gbx2* expression. *msx1* and *msx2* expression was rescued by co-injection of a morpholino-insensitve *ror2* RNA (Fig. 2C,D; Fig. S1), which confirmed the specificity of the morpholino. We also showed that expression of the pan-neural and mesodermal markers *sox2* and *myoD* was unaffected to exclude ectodermal or mesodermal mispatterning (Fig. S2). These results clearly demonstrated that Ror2 was specifically required in the very first steps of neural crest development, the definition of the neural plate border territory.

In order to further narrow down the level on which Ror2 influences neural crest induction, we analyzed *ap2alpha*, which is required for NPB specification downstream of Wnt and Fgf (de Crozé et al., 2011). In contrast to other NPB genes, we found *ap2alpha* upregulated and downregulated in Ror2 MO-injected embryos with almost equal frequency at stage 12 and at stage 18 (Fig. 3A). This phenotype was specific as confirmed by co-injection of MO-insensitive *ror2* RNA, which fully restored *ap2alpha* expression to levels equal to the uninjected control side. Among the early NPB genes, *ap2alpha* and *gbx2* have been identified as direct targets of the Wnt/β-catenin pathway (de Crozé et al., 2011; Li et al., 2009).

Ror2 has been shown to modulate Wnt/β-catenin signaling and interestingly, both positive and negative regulation of the Wnt/β-catenin pathway has been reported (Billiard et al., 2005; Green et al., 2007; Henry et al., 2015; Li et al., 2008; Mikels and Nusse, 2006; Rasmussen et al., 2013). However, neither inhibition nor activation of Wnt/β-catenin signaling by co-injection of effective doses of dominant-negative (dn)*lef1* RNA or *tcf1* RNA with Ror2 MO, respectively, rescued the downregulation of *msx1* (Fig. 3B; Fig. S3). Moreover, we did not see any synergy between Ror2 loss-of-function and either Wnt/β-catenin gain- or loss-of-function, indicating that Ror2 and Wnt/β-catenin signaling acted independently in NPB specification.

Ror2 signaling is required for local activation of BMP signaling at the neural plate border

Notably, the majority of genes that are expressed at and define the NPB were affected in Ror2 morphant embryos, suggesting a rather

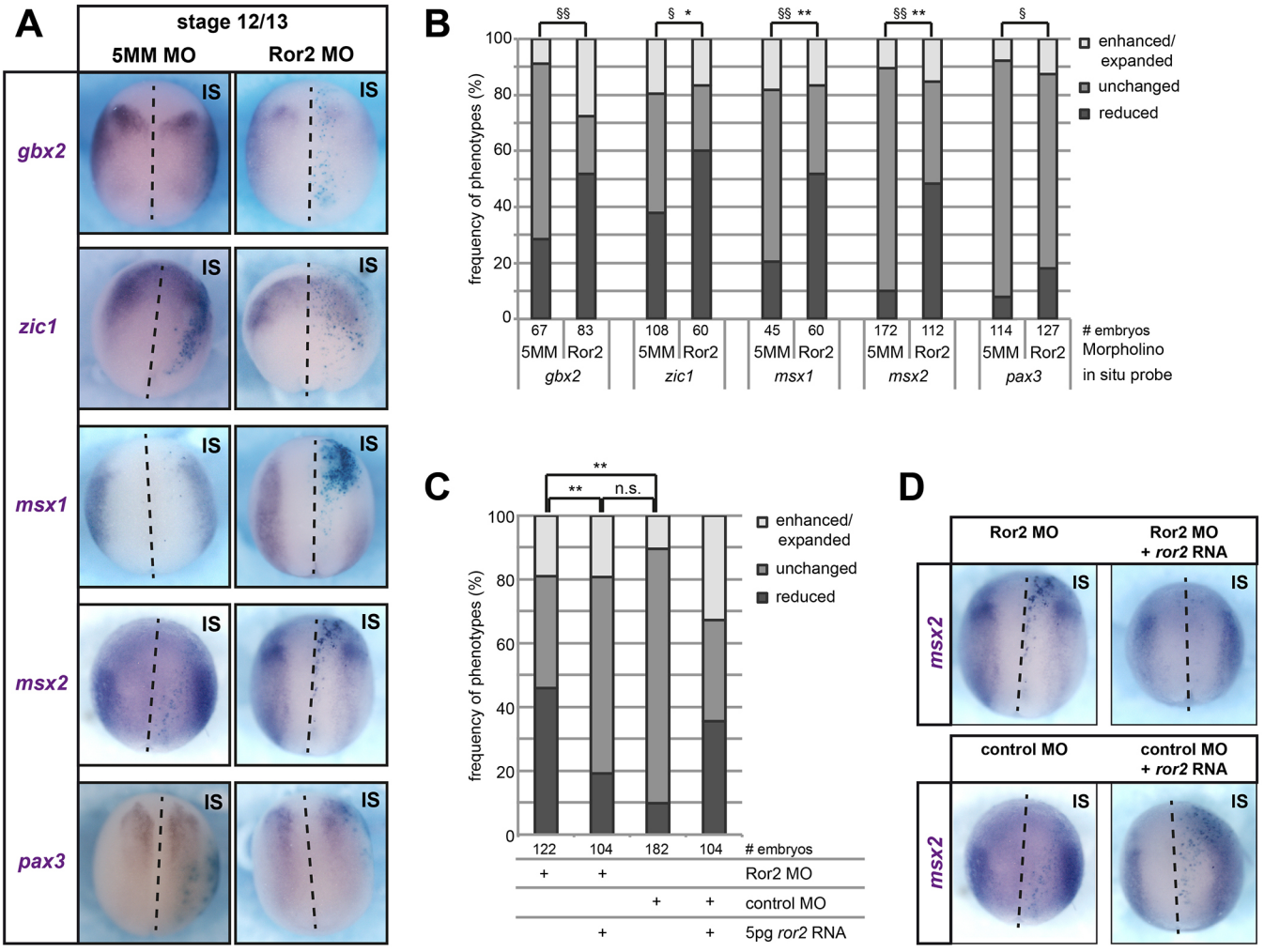

Fig. 2. Ror2 is required for neural plate border specification. Images show representative examples of embryos injected as indicated; the injected side (IS) is oriented to the right. (A) Examples of embryos injected as indicated, fixed at stage 12/13 and probed with the indicated probe. (B) Graph of phenotype frequency corresponding to A. (C) Co-injection of 5 pg MO-insensitive *ror2* RNA rescued *msx2* expression. (D) Representative images corresponding to C. At least three independent experiments are summarized in the graphs. The total number of embryos is indicated below each column. Statistically significant differences according to the χ^2 test are indicated by $^{\S\S}P<0.01$, $^{\S}P<0.05$, according to the Wilcoxon rank sum test by $^{**}P<0.01$, $^{*}P<0.05$.

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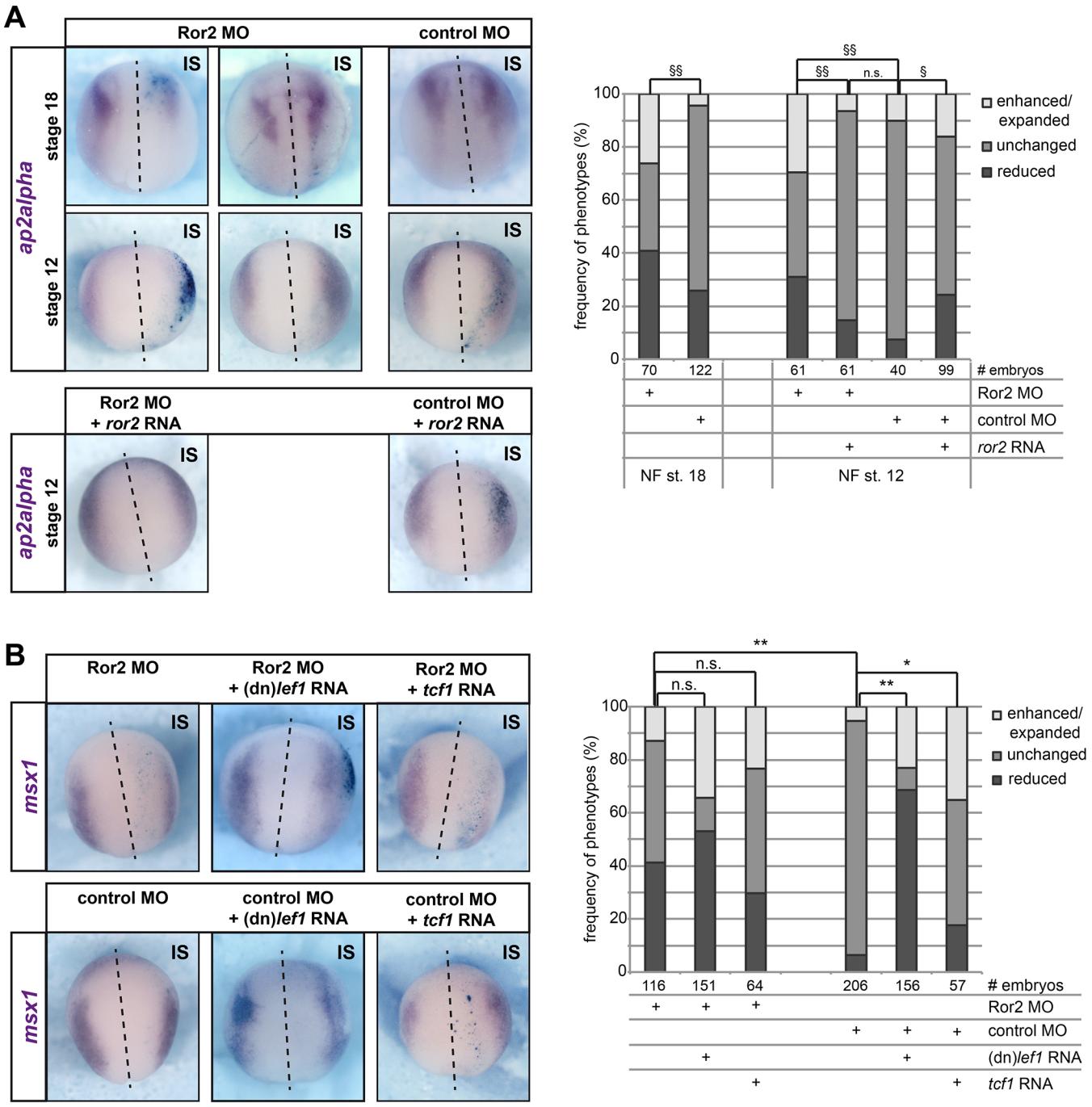

Fig. 3. Ror2 acts independently of Wnt/β-catenin signaling in neural plate border specification. Images show representative examples of embryos injected as indicated; the injected side (IS) is oriented to the right. (A) *ap2alpha* was up- or downregulated at approximately the same frequency by Ror2 MO injections at stage 12/13 or stage 18; *ap2alpha* expression was restored to normal by co-injection of 5 pg *ror2* RNA. (B) Neither dn*lef* RNA nor *tcf1* RNA restored *msx1* expression when overexpressed in Ror2 morphant embryos. Upon co-injection with the control MO, the expected Wnt/β-catenin loss- or gain-of-function phenotype, i.e. down- or upregulation of *msx1*, was observed. At least three independent experiments are summarized in the graphs. The total number of embryos is indicated below each column. Statistically significant differences according to the χ^2 test are indicated by $^{\S\S}P<0.01$, $^{\S}P<0.05$, according to the Wilcoxon rank sum test by $^{**}P<0.01$, $^{*}P<0.05$.

broad and early effect of Ror2. Among those genes, *msx1*, *zic1*, *pax3* and *msx2* are indirectly or directly responsive to BMP (Brugger et al., 2004; Tribulo, 2003; reviewed in Betancur et al., 2010). Neural induction in general was not altered in these embryos (Fig. S1), therefore, we investigated if Ror2 loss-of-function interfered with BMP signaling at the NPB and carried out whole-mount immunostaining against phosphorylated Smad1, Smad5 and Smad8 (pSmad1,5,8). At the end of gastrulation, we observed two distinct stripes of pSmad1,5,8-positive cells along the NPB. Unilateral knockdown of Ror2 resulted in a decrease of pSmad1,5,8-positive cells at the injected side in more than 60% of the embryos and the phenotype was rescued by co-injection of *ror2* RNA (Fig. 4A). Ror2 gain-of-function induced a complex distribution of phenotypes with roughly equal proportions of

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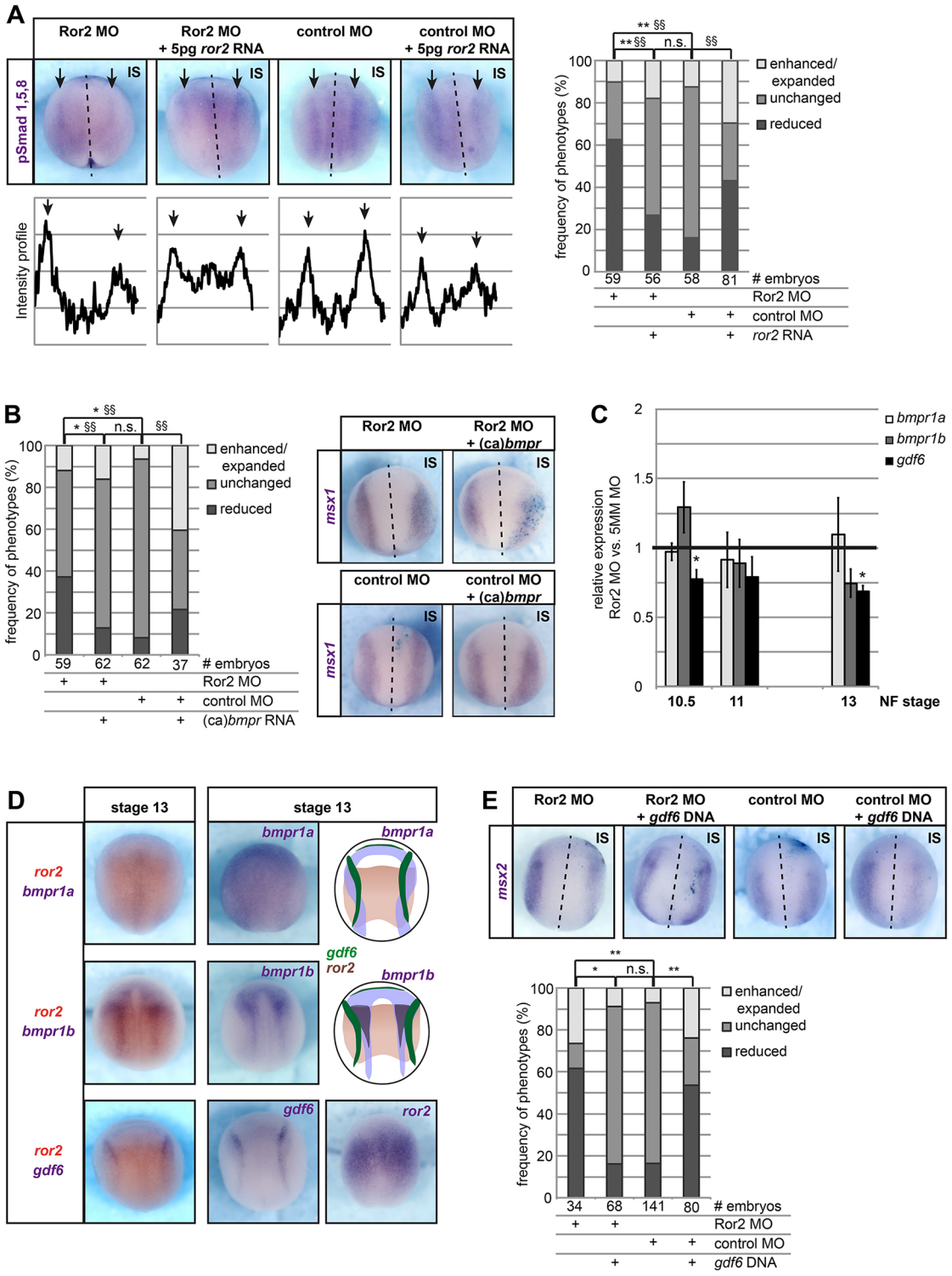

Fig. 4. Ror2 loss-of-function results in decreased BMP signaling at the neural plate border. Images show representative examples of embryos injected as indicated; the injected side (IS) is oriented to the right. Graphs show the frequency of observed phenotypes from three independent experiments. (A) BMP signaling activity was detected in two distinct stripes by whole mount immunostaining against phospho-Smad1,5,8 in embryos injected as indicated. Plots of averaged and smoothed intensity profiles (see Materials and Methods for details) are provided below images of representative embryos. (B) Restoring BMP signaling activity by expression of caBMPR rescued *msx1* expression in Ror2 morphant embryos. (C) The expression of *gdf6*, *bmpr1a* and *bmpr1b* in Ror2 MO-injected embryos relative to controls was determined by real-time RT-PCR at the indicated developmental stages (mean±s.e.m.). $^{*}P<0.05$ significant difference from control-injected embryos (*t*-test for the mean). (D) *Ror2* expression overlaps with expression of the BMP ligand *gdf6* and the type I BMP receptor *bmpr1b* at the neural plate border. Stage 13 embryos were probed for *ror2* (brown) and *gdf6* (purple), *bmpr1a* (purple) or *bmpr1b* (purple) as indicated; single-probe *in situ* hybridizations are provided for comparison. The overlap of *ror2*, *gdf6* and *bmpr1a* or *bmpr1b* expression is illustrated in the schematics. (E) Overexpression of Gdf6 (25 pg plasmid DNA) restored *msx2* expression in Ror2 morphant embryos. At least three independent experiments are summarized in the graphs. The total number of embryos is indicated below each column. Statistically significant differences in A,B,E according to the χ^2 test are indicated by $^{\S\S}P<0.01$, $^{\S}P<0.05$, according to the Wilcoxon rank sum test by $^{**}P<0.01$, $^{*}P<0.05$.

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embryos with increased, unaffected and decreased pSmad1,5,8 staining intensity (Fig. 4A), which was highly reminiscent of the phenotype frequency we observed for *msx1* and *msx2* (Fig. 2C,D). Consistently, *msx1* expression at the NPB was restored by over-expression of a constitutively active BMP receptor (caBMPR) in Ror2 morphant embryos (Fig. 4B). As expected, overexpression of this caBMPR induced *msx1*, which confirmed its activity (Fig. 4B).

We wondered which BMP receptors and ligand might mediate this local activation of Smad1,5,8. In late gastrula *Xenopus* embryos, *bmpr1a* is expressed ubiquitously whereas *bmpr1b* is expressed in the anterior preplacodal region and in two distinct anterior-posterior stripes along the neural plate (Schille et al., 2016). It has been reported that the BMP ligand Gdf6 is expressed specifically in the preplacodal region and along the NPB in *Xenopus* and zebrafish (Chang and Hemmati-Brivanlou, 1999; Reichert et al., 2013), which made Gdf6 a likely candidate for such restricted local activation of the pathway. The relative expression of *gdf6* was moderately reduced from early gastrula stage 10.5 until early neurula stage 13 (Fig. 4C) in Ror2 morphants whereas *bmpr1a* and *bmpr1b* transcript levels were not significantly changed although *bmpr1b* expression also seemed to be sensitive to Ror2 levels. A virtual overlay of expression areas of *ror2*, *bmpr1a*, *bmpr1b* and *gdf6* at Niewkoop and Faber (NF) stage 13 showed that *bmpr1a*, *bmpr1b* and *gdf6* are co-expressed in the anterior preplacodal region that does not express *ror2* and confirmed that all four genes are co-expressed at the posterior neural plate border that gives rise to the neural crest (Fig. 4D). Co-expression of *ror2* and *gdf6* persisted at the neural plate border until late neurula stages (Fig. S4). Indeed, co-injection of a plasmid encoding Gdf6 was sufficient to restore *msx2* expression in Ror2 morphant embryos (Fig. 4E) confirming the functional relationship and placing Ror2 upstream of Gdf6 in NPB specification.

Ror2 acts through β-catenin-independent Wnt signaling to regulate *gdf6* expression and neural cell polarity

We confirmed downregulation of *gdf6* in Ror2 MO-injected embryos by *in situ* hybridization (Fig. 5A). Again, g*df6* expression was rescued by co-injection of *ror2* RNA, confirming the specificity of the phenotype. In addition, we investigated whether the role of Ror2 in NPB specification was Wnt-dependent. A Ror2 mutant that lacks the Wnt-binding extracellular cysteine-rich domain (Ror2ΔCRD) was not able to rescue *gdf6* expression in Ror2 morphant embryos, confirming that Ror2 function at the NPB was indeed Wnt-dependent (Fig. 5A). Next, we co-injected Dvl2ΔDIX, which activates β-catenin independent Wnt pathways (Axelrod et al., 1998) with Ror2 MO and observed a partial rescue of *gdf6* expression (Fig. 5B). This result pointed towards an activation of β-catenin-independent signaling downstream of Ror2 in NPB specification. In our previous work, we have demonstrated that Wnt5a/Ror2 signaling is essential for the upregulation of paraxial protocadherin (Papc) in gastrulating embryos and that Papc acts as a positive modulator of Wnt/PCP signaling (Schambony and Wedlich, 2007; Unterseher et al., 2004). Accordingly, we co-injected *papc* RNA or an RNA encoding the related and functionally redundant ectodermal protocadherin Pcns (Rangarajan et al., 2006; Schneider et al., 2014) to restore Wnt/PCP signaling in Ror2 morphant embryos. Indeed, expressing either Papc or Pcns was sufficient to rescue *gdf6* expression in Ror2 deficient embryos (Fig. 5C). Papc or Pcns gain-of-function by co-injection with the control MO likewise induced a loss of *gdf6* (Fig. 5C). Overexpression of Papc or Pcns in Ror2 morphant embryos also restored *msx2* and *msx1* expression, respectively (Fig. S5A,B). Interestingly, both protocadherins were able to rescue NPB formation in Ror2 morphant embryos, although *papc* is expressed in the organizer and paraxial mesoderm whereas *pcns* is expressed ubiquitously in the ectoderm in gastrula stage embryos (Fig. S5C-F).

The neural crest is induced by the dorsolateral marginal zone (DLMZ), which lies adjacent to the future neural crest at early gastrula stages and is internalized and positioned beneath the NPB by gastrulation movements (Monsoro-Burq et al., 2003; Steventon et al., 2009). Our observation that *gdf6* was downregulated in Ror2 morphants at early gastrula stages indicated that Ror2 is required prior to or during morphogenetic movements. First we asked whether Ror2 depletion affected morphogenetic movements not only in the mesoderm as we have reported in our earlier work (Schambony and Wedlich, 2007), but also in the neuroectoderm and the NPB. Indeed, we observed that the neural folds did not elevate on the injected side after unilateral Ror2 knockdown (Fig. 6A) but shaped normally when control MO was injected (Fig. 6B). Moreover, Ror2 knockdown resulted not only in reduced *gdf6* expression but also in flattening of the *gdf6* expressing NPB (Fig. 6A), indicating that Ror2-dependent morphogenetic movements of the dorsal ectoderm might be functionally related to the local upregulation of *gdf6*. Consistent with defective morphogenetic movements, we observed disrupted cell polarity in the lateral neural plate of Ror2 morphant embryos. Control cells were oriented perpendicular to the embryonic anterior-posterior axis in the lateral neural plate, whereas epidermal cells were oriented in parallel to this axis. The knockdown of Ror2 caused a failure in cell orientation in the injected half of the neural plate (Fig. 6C,E). Ror2-deficient cells were no longer oriented perpendicular to the neural plate border, but showed a randomized orientation (Fig. 6C). By contrast, orientation of epidermal cells was not affected (Fig. S6A,B). In the underlying mesoderm, the notochord has already formed and both axial and paraxial mesodermal cells show a mediolateral orientation (Fig. 6D,F; Fig. S6C,D) that was not significantly affected by Ror2 knockdown. Interestingly, cell orientation in the neural plate was efficiently rescued by co-expression of Papc and also mesodermal cells seemed more uniformly aligned (Fig. 6C-F), indicating that Papc might play a role downstream of Ror2 or act redundant to Pcns in the neuroectoderm.

In order to further elucidate the role of Ror2 in NPB formation, we first dissected stage 10.5 embryos into animal cap ectoderm (AC), dorsal marginal zone (DMZ) and dorsolateral marginal zone (DLMZ) (Fig. 7A). We detected expression of *ror2* in all three tissues, although levels were low. Expression of *pcns* was strongest in the AC but still weakly detectable in the DLMZ whereas *papc* was not detected in any explant. At this stage *papc* expression is limited to the dorsal blastopore lip (Fig. S5), which is removed from DMZ explants. The identity of tissues was confirmed by expression of *brachyury* (*bra*) in DMZ and DLMZ, *ectodermal cytokeratin* (*eck*) in AC and DLMZ and *chordin* (*chd*) in the DMZ and weakly in the DLMZ. Interestingly, the neural plate border gene *msx2* was expressed in all three tissues with the strongest expression in the DLMZ (Fig. 7A). When DLMZ explants were cultured until sibling embryos reached stage 13, we detected a mild increase in *ror2*, but observed a strong induction of *pcns* and *papc* as well as moderate increase of *msx2* (Fig. 7A).

Next, we sought to determine whether Ror2 is required in the dorsal ectoderm or the DLMZ. DLMZ explants were combined with AC-naïve ectoderm to induce NPB fate (Fig. 7B). When sandwiched to DLMZ explants, a robust expression of *sox8*, *msx2* and *gdf6* was induced as compared with uninduced AC explants (Fig. 7B). In addition, and consistent with the above-mentioned

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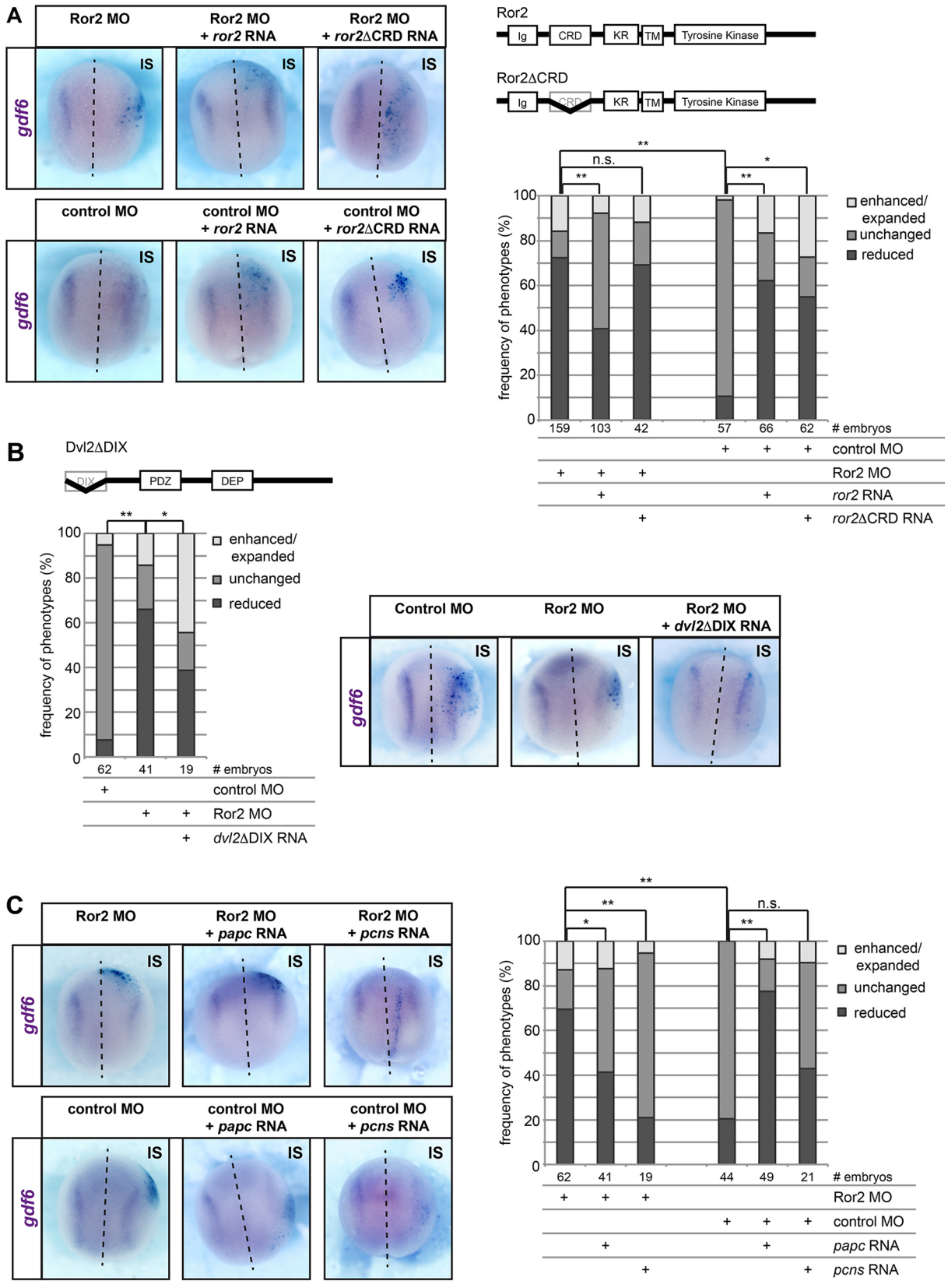

Fig. 5. Ror2 is required for *gdf6* expression at the neural plate border in a Wnt-dependent manner. Embryos were injected into one animal-dorsal blastomere at the 8-cell stage as indicated; the injected side (IS) is oriented to the right. The images show representative embryos injected as indicated. Frequencies of the observed phenotypes in three independent experiments are summarized in the graphs. Decreased *gdf6* expression on the injected side of Ror2 MO-injected embryos was rescued (A) by full-length *ror2* RNA, but not by *ror2*ΔCRD RNA, which lacks the Wnt-binding CRD domain, (B) partially by DvlΔDIX, which activates Wnt/PCP signaling and (C) by the related protocadherins Papc and Pcns. The two-sample Wilcoxon rank sum test was performed to determine differences between experimental groups; statistically significant differences are indicated by $^{**}P<0.01$; $^{*}P<0.05$; n.s., not significant.

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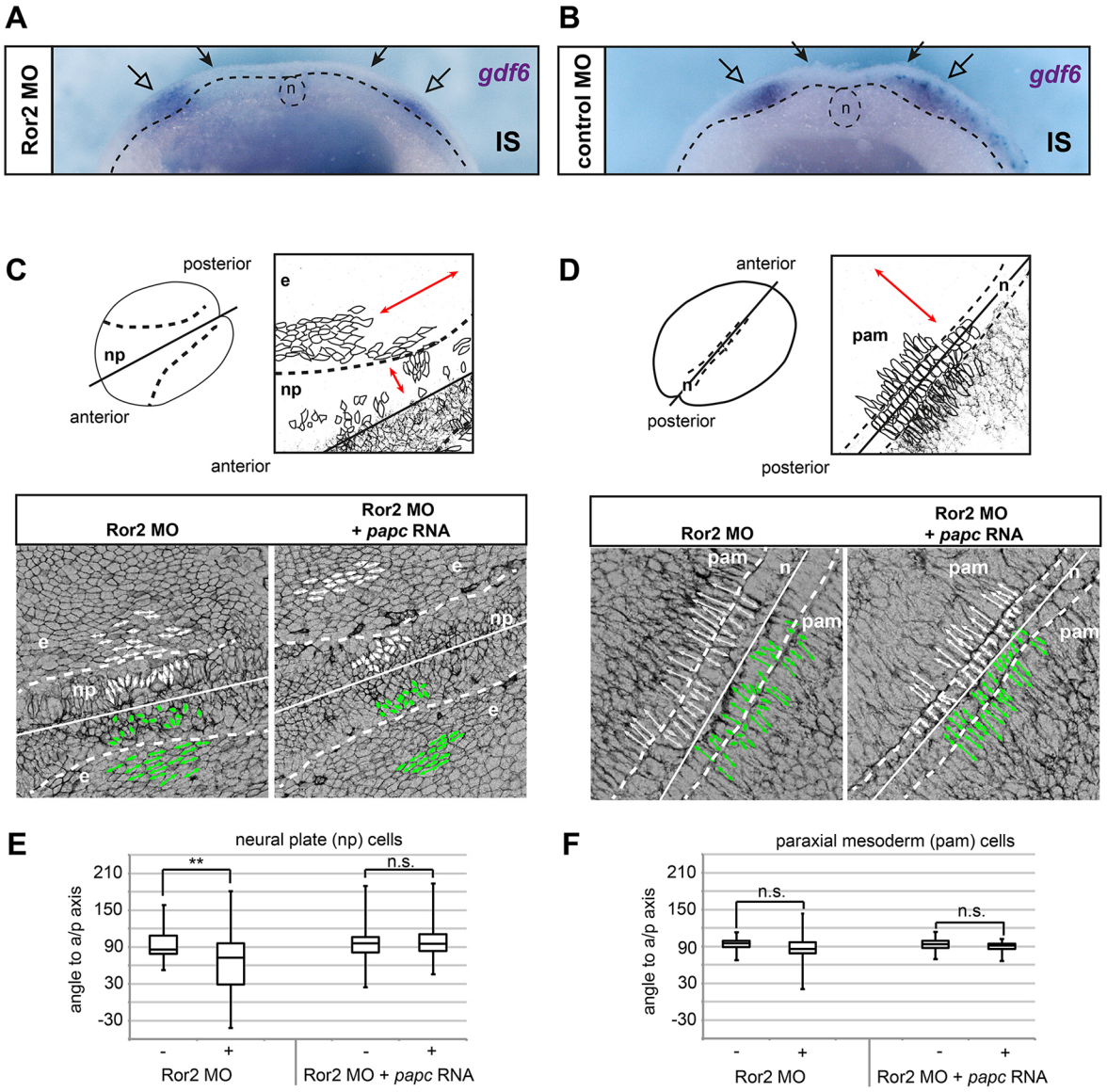

Fig. 6. Ror2 regulates morphogenetic movements and cell orientation in the neural plate. (A,B) Ror2-knockdown (A) disrupts neural fold elevation and thickening of the *gdf6*-expressing NPB area, which was not seen in embryos injected with control MO (B). (C-F) The neural plate of embryos injected as indicated in C,D was explanted at stage 13, fixed and stained with phalloidin and the injected side identified by co-injection of *mGFP* RNA. (C) Upper panels: outline of an explant showing the neural plate and anterior-posterior axis and illustration of cell orientation in the ectoderm. Lower panels: laser scanning microscopy images corresponding to the outlines in upper panels. Cell orientation is indicated by arrows; white, uninjected side; green, injected side; position of the neural plate (np) outlined by dashed lines; anterior-posterior axis indicated by a straight line; e, epidermis. (D) Upper panels: outline of an explant showing the paraxial mesoderm (pam), notochord (n) and anterior-posterior axis and an illustration of cell orientation in the mesoderm. Lower panels: laser scanning microscopy images corresponding to the outlines in upper panels. Cell orientation is indicated by arrows; white, uninjected side; green, injected side; position of the notochord outlined by dashed lines; anterior-posterior axis indicated by a straight line. (E) Box plots plotting the angle between long axes of individual cells and the anterior-posterior (a/p) axis in the neural plate. $^{**}P<0.01$, two-sided separate variance *t*-test. (F) Box plots plotting the angle between long axes of individual cells and the anterior-posterior (a/p) axis in the paraxial mesoderm (pam). Two independent experiments are summarized in the graphs.

findings, *pcns* and *papc* were strongly upregulated. It should be noted that expression levels have been determined relative to uninduced AC explants, which do not express *papc*, but do express *pcns* and *msx2*, which accounts for large differences of relative expression levels. Irrespective of such differences, DLMZ explants from Ror2 morphants were significantly less able to induce NPB markers in ACs from control or Ror2 morphant embryos. We observed a 1.5- to twofold decrease of NPB markers when the DLMZ was explanted from Ror2-deficient embryos as compared with control sandwiches. By contrast, knockdown of Ror2 in the ectoderm did not affect induction of NPB genes. These experiments clearly demonstrated that Ror2 is required in the DLMZ to induce NPB formation.

In summary, we have demonstrated that Ror2 signaling via β-catenin independent pathways in the DLMZ is crucial for NPB specification, particularly for local upregulation of Gdf6 and thus BMP activity. In addition, Ror2 contributes to the regulation of cell polarity and morphogenetic movements in the neuroectoderm, which shapes and positions the NPB in early neurula stages. Interfering with these early functions of Ror2 in the DLMZ and dorsal ectoderm disrupts NPB formation and all subsequent specification and development of the neural crest.

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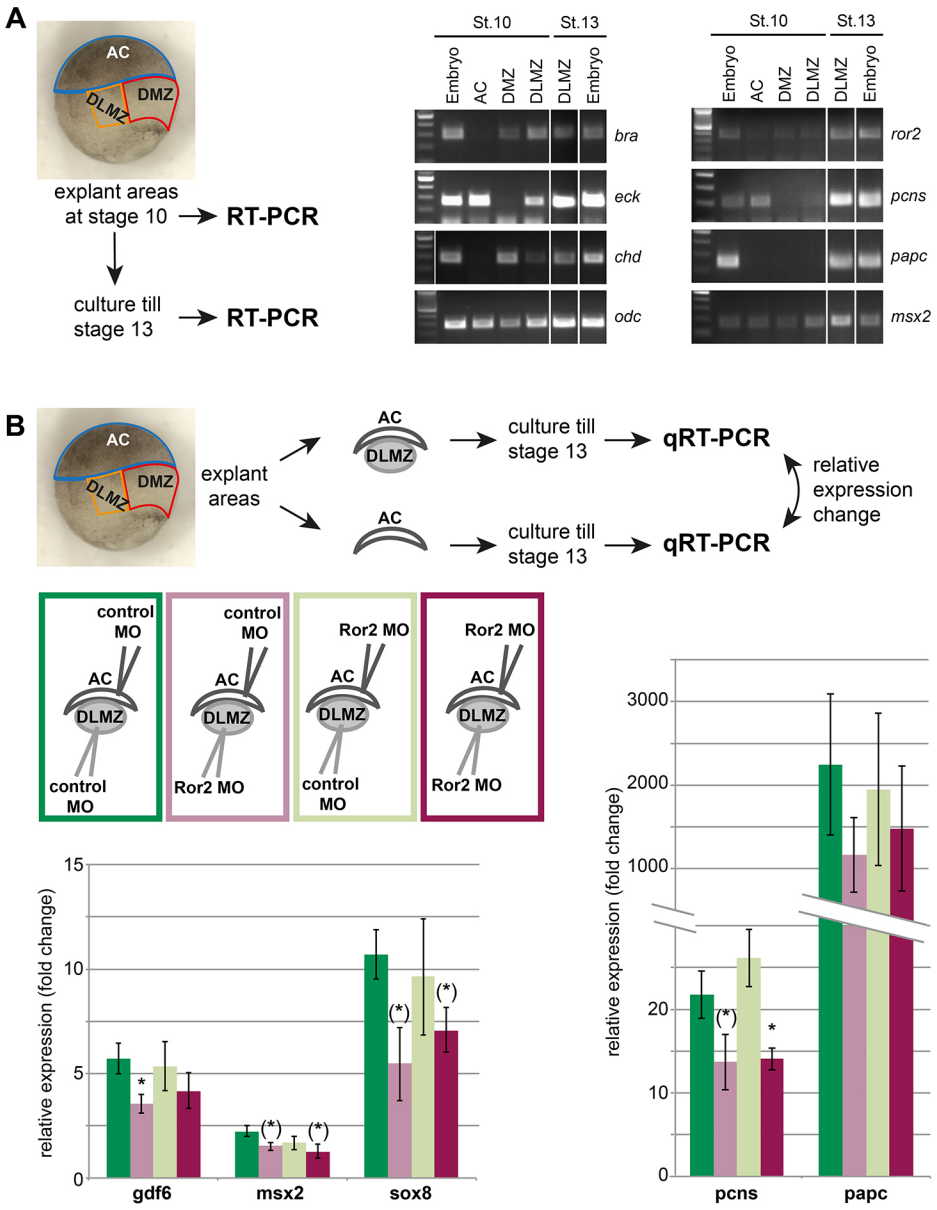

Fig. 7. Ror2 is required in the DLMZ to induced NPB markers. (A) The animal cap (AC), dorsal marginal zone (DMZ) and dorsolateral marginal zone (DLMZ) were explanted from stage 10 embryos and expression of the indicated genes analyzed by RT-PCR. In a second experiment, the same explants were cultured until sibling embryos reached stage 13 and analyzed as before. *bra* and *chd* were analyzed on the same gel and, therefore, the same markers are shown for these gels. (B) DLMZ and AC explants were combined and cultured until sibling embryos reached stage 13. Induction of the indicated genes in combined explants was determined relative to uninduced control AC explants by real-time RT-PCR (qRT-PCR) and plotted as relative changes. Boxed illustrations show the color codes for each combination of DLMZ explants and AC explants. Significant deviations from induction by control MO-injected DLMZ in control MO-injected AC explants (green box/columns) are indicated by asterisks; **P*<0.05, (*)*P*<0.1, two-sided separate variance *t*-test.

DISCUSSION

In this study we have characterized the role of Ror2 in induction of the NPB and neural crest. Our data suggested a broad effect of Ror2 loss-of-function on NPB specification and early neural crest induction. Knockdown of Ror2 resulted in a downregulation of *ap2alpha*, *msx1* and *msx2* as well as the posteriorizing factor *gbx2*, but not *pax3*, at the neural plate border. *pax3* expression depends on *ap2alpha* and *gbx2* (de Crozé et al., 2011; Li et al., 2009), but is additionally positively regulated by Fgf (Hong et al., 2008) and retinoic acid (Pruitt et al., 2004), which might provide partial compensation. Together with the complex effect of Ror2 on *ap2alpha* and *gbx2* the multifactorial regulation of *pax3* might result in the apparent insensitivity of *pax3* to Ror2-mediated signaling at the NPB.

Regulation of even the earliest neural crest-specifying genes *ap2alpha* and *gbx2*, as well as *msx* genes and *zic1*, by Ror2, however, established an essential role of Ror2 in early patterning of the dorsal ectoderm. NPB genes interact to induce bona fide neural crest fates. For example, Pax3 and Zic1 cooperate, but depend on Ap2alpha, to induce *snail2* and *foxd3* (de Crozé et al., 2011; Monsoro-Burq et al., 2005; Sato et al., 2005). Consistently, downstream targets such as *c-myc*, *snail2* and *twist* were downregulated in the pre-migratory neural crest of Ror2 morphant embryos most likely as a consequence of such early specification defects.

NPB specification is positively regulated by BMP and Wnt/β-catenin signaling. *gbx2* is a direct target gene (Li et al., 2009) and *ap2alpha* is one of the immediate early Wnt/β-catenin responsive genes at the neural plate border (de Crozé et al., 2011). Although Ror2 is a major receptor in β-catenin-independent Wnt signaling, it has also been reported that Ror2 can positively or negatively regulate Wnt/β-catenin signaling depending on the cellular context (Billiard et al., 2005; Green et al., 2007; Henry et al., 2015; Li et al., 2008; Mikels et al., 2009; Mikels and Nusse, 2006; Rasmussen et al., 2013). However, we found that neither lowering nor increasing Wnt/β-catenin activity by overexpression of dnLef or Tcf1, respectively, rescued NPB specification in Ror2-depleted embryos. Moreover, we did not observe synergistic effects in

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either condition; therefore, we conclude that Wnt/β-catenin signaling and Ror2 signaling act predominantly independent of each other and any putative antagonistic regulation within the Wnt signaling network represents only a minor effect of Ror2 function in NPB formation.

Conversely, our experiments revealed that Ror2 loss-of-function prevents the local upregulation of BMP-induced pSmad1,5,8 signaling at the NPB in late gastrulation. We have further identified Gdf6 as one BMP ligand that is downregulated in Ror2-depleted embryos and was sufficient to rescue NPB formation in these embryos. *gdf6* expression in the dorsal neural tube and neural crest derivatives is conserved in mouse, frog and zebrafish (Chang and Hemmati-Brivanlou, 1999; Mortlock et al., 2003; Rissi et al., 1995) and overlaps with the expression of *bmpr1a*, *bmpr1b* and *ror2* (this study). Elevated BMP activity is observed at the NPB and in the dorsal neural tube in zebrafish, *Xenopus* and chick embryos (Barth et al., 1999; Faure et al., 2002; Reichert et al., 2013; Sakai et al., 2005; Wu et al., 2011) and a recent study in zebrafish identified Gdf6 as the factor required for this local activation of BMP signaling (Reichert et al., 2013). Our findings are consistent with a specific role of Gdf6 in NPB and neural crest specification and suggest that Ror2 indirectly specifies the NPB by directly or indirectly regulating *gdf6* expression.

Regulation of *gdf6* expression downstream of Ror2 is Wnt-dependent and mediated by β-catenin-independent pathways as confirmed by the rescue of *gdf6* expression by overexpression of Dvl2ΔDIX, Papc or Pcns. The requirement of Ror2-mediated Wnt/PCP signaling in NPB formation was further supported by the observation that Ror2 morphant embryos exhibited morphogenesis defects in the neural plate. Specifically, Ror2 depletion caused disruption of coordinated cell orientation and polarity at the lateral neural plate and flattening of the NPB area. Neural PCP activity and vertical signals from the underlying mesoderm interact to coordinate cell polarity and convergent extension movements in the neural plate (Elul and Keller, 2000; Poznanski et al., 1997; Wallingford and Harland, 2002). Disturbed morphogenesis during gastrulation and early neurulation would result in aberrant positioning of these tissues and thereby expose NPB precursors to different concentrations of morphogens from the dorsoventral gradients and the underlying mesoderm. *ror2* itself as well as *pcns* are expressed in the deep layer of the neuroectoderm. However, overexpression of Papc fully restored cell polarity in the neural plate of Ror2 morphants. Consistent with our earlier studies (Schambony and Wedlich, 2007), *papc* but also *pcns* was downregulated in the DLMZ of Ror2-depleted embryos. Papc coordinates convergent extension movements in the mesoderm (Medina et al., 2004; Schambony and Wedlich, 2007; Unterseher et al., 2004) and could thus contribute indirectly to neural morphogenesis. Notably, functional redundancy of Papc and Pcns has been reported in the context of neural crest migration (Schneider et al., 2014). Such redundancy most probably also accounts for the full rescue of neural cell polarity by Papc and the ability of both protocadherins to rescue NPB formation in Ror2-deficient embryos.

Further characterization established that Ror2 is specifically required in the dorsolateral marginal zone (DLMZ) to upregulate NPB and early neural crest genes such as the direct BMP target *msx2* (Brugger et al., 2004), the Pax3- and Zic1-responsive *sox8* (Bae et al., 2014), and also *gdf6* and *pcns* in adjacent ectoderm. Interestingly, the known downstream target of Ror2, *papc* (Schambony and Wedlich, 2007), was also downregulated in Ror2-depleted DLMZ/ectoderm explants, which further supports a role for both protocadherin 8 orthologs downstream of Ror2 in NPB specification. Endogenously, however, additional functions of Ror2 must be assumed, because simultaneous depletion of both Pcns and Papc did not affect neural crest induction (Schneider et al., 2014).

NPB specification depends on signals from neighboring ectodermal and underlying mesodermal tissues including BMP and Fgf (Bang et al., 1999; Bonstein et al., 1998; Liem et al., 1995; Mancilla and Mayor, 1996; Marchant et al., 1998; Monsoro-Burq et al., 2003). The DLMZ mesoderm is located adjacent to the future neural crest territory at early gastrula and becomes positioned beneath the dorsal ectoderm during gastrulation movements. In combined DLMZ/ectoderm explants morphogenetic defects in the DLMZ should not affect relative positioning of these tissues, which rules out an indirect role of Ror2 only by regulating morphogenesis. By contrast, our results clearly indicate that Ror2 signaling is required to induce factors that are most likely secreted, which then act on the adjacent ectoderm. The molecular nature of such factors remains to be confirmed, but one likely candidate might be *gdf6*. *gdf6* is expressed in the DLMZ of stage 10 embryos and was downregulated at stage 9 and stage 10 in Ror2 morphant embryos (Fig. 4C), at a time prior to the internalization of the DLMZ but concurrent with the onset of NPB specification.

As mentioned above, *gdf6* expression was rescued by DvlΔDIX, Papc or Pcns, which indicated that Ror2 acts via β-catenin-independent signaling. A strong dependence of *gdf6* expression on β-catenin-independent Ror2 signaling is further supported by the observation that both Ror2 overexpression and Ror2 depletion, as well as overexpression of the inactive Ror2ΔCRD, resulted in a downregulation of *gdf6*. Indistinguishable phenotypes in gain- and loss-of-function situations are a key feature of Wnt/PCP signaling that coordinates cell polarity and mass cell movements. In either case, polarity is not established or maintained, which results in randomized cell polarity and loss of coordinated behavior (Darken et al., 2002; reviewed in Wu and Mlodzik, 2009).

In contrast to *gdf6*, pSmad1,5,8, *msx1* and *msx2* were more complexly affected by Ror2 overexpression. *ror2* RNA-injected embryos showed partially decreased and partially expanded staining for these factors. However, *msx1* and *msx2* expression was rescued by *gdf6* overexpression in Ror2-depleted embryos, which clearly demonstrated that these genes are indirectly regulated by Ror2 consistent with the well-established regulation of *msx* genes by BMP signaling (Brugger et al., 2004; Tribulo, 2003). In addition, Wnt/β-catenin signaling also contributes to NPB induction (reviewed in Milet and Monsoro-Burq, 2012). Although we were able to rule out that Ror2 endogenously acts via modulation of Wnt/β-catenin signaling in NPB specification, Ror2 overexpression likely affects both β-catenin-dependent and -independent Wnt signaling, which would further add to the complexity of Ror2 gain-of-function phenotypes.

Consistent with the assumption that Ror2 acts through Wnt/PCP signaling to induce *gdf6*, its expression phenotype at the NPB was rescued by both *Xenopus* protocadherin 8 orthologs, Papc and Pcns. Although, based on the expression pattern, it seems likely that Pcns and Ror2 mediate PCP signaling in the neural plate, it remains unresolved which protocadherin, Papc or Pcns, is acting downstream of Ror2 in the DLMZ. PAPC acts as a modulator and co-factor of Wnt/PCP signaling that itself activates RhoA and Jnk signaling (Medina et al., 2004; Unterseher et al., 2004). Signaling activity of Pcns has not be characterized in detail, but a recent study showed that Papc can functionally substitute for Pcns in neural crest migration, indicating that both exhibit redundant signaling function (Schneider et al., 2014). When overexpressed, both proteins likely restore PCP

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signaling in the DLMZ and dorsal ectoderm irrespective of their endogenous expression, which impedes discrimination between functional compensation and true epistatic relationships.

Nevertheless, these findings establish the unprecedented regulation of the BMP ligand Gdf6 by Wnt/PCP signaling in early *Xenopus* embryos and its requirement downstream of Ror2 in NPB formation.

Interestingly, modulation of BMP/Smad signaling in mouse models of brachydactyly type B and recessive Robinow syndrome has been reported (Wang et al., 2011; Witte et al., 2010). Both syndromes are caused by mutations in the *ROR2* gene in humans (Oldridge et al., 2000; van Bokhoven et al., 2000), which indicates that this regulatory interaction might be conserved between humans, mice and the early *Xenopus* embryo and that NPB induction in *Xenopus* might serve as a model to further elucidate the underlying molecular interactions and mechanisms.

Taken together, our results clearly demonstrate a multifaceted role of Ror2-mediated Wnt/PCP signaling in neural crest induction. In the intact embryo, it is likely that two major aspects contribute to the role of Ror2 in NPB formation; Ror2 is already essential for the definition of the NPB territory by the DLMZ in early gastrulation, upstream of the induction of NPB-specifying genes, and in addition, contributes indirectly by regulating cell polarity and morphogenetic movements to position and shape the NPB.

MATERIALS AND METHODS

Xenopus laevis embryos and microinjection

Xenopus embryos were generated and cultured following general protocols and staged according to the normal table of Niewkoop and Faber (1994). All procedures were performed according to the German animal use and care law (Tierschutzgesetz) and approved by the local authorities (animal care and housing approval: I/39/EE006, Veterinäramt Erlangen; animal experiments approval: 54-2532.2-8/08, German State Administration Bavaria/Regierung von Mittelfranken).

RNA for microinjection was prepared using the mMessage mMachine Kit (ThermoFisher Scientific); plasmid templates are described in supplementary Materials and Methods. Injection amounts of synthetic RNA or plasmid DNA were as indicated, for knockdown experiments 0.2 pmol Ror2 MO or control 5MM MO (Schambony and Wedlich, 2007) were injected if not indicated otherwise. Embryos were injected and cultured until they reached the desired stage. DLMZ explants were prepared at stage 10.5, combined with AC explants from stage 8-9 embryos and cultured until sibling embryos reached stage 13.

For subsequent *in situ* hybridization, single-side injections were performed and traced by co-injection of 100 pg pCS2-*lacZ* DNA. The injected side was visualized by β-galactosidase (β-gal) staining and *in situ* hybridizations were carried out as described by Harland (1991) using the indicated probes (see also Table S2).

Statistical analysis

The χ^2 test was used to determine overall differences between control 5MM MO-injected and Ror2 MO-injected embryos and in addition, a two-sample Wilcoxon rank sum test was performed to compare differences in outcomes of two treatments. A Kruskal–Wallis rank sum test was applied to data from rescue experiments in order to determine overall differences within individual experiments. Further, for pairwise comparisons of particular treatments within the rescue experiments a two-sample Wilcoxon rank sum test was performed. All statistical analyses were performed using free statistical software R (version 3.0.2, https://www.r-project.org/; R Team, 2014).

RT-PCR and real-time RT-PCR

Total RNA was extracted from *Xenopus* embryos or tissue explants of the indicated developmental stages (High Pure RNA Isolation Kit, Roche), reverse-transcribed using MMLV reverse transcriptase (New England Biolabs) and the indicated transcripts were amplified from the resulting cDNA using OneTaq Polymerase (New England Biolabs). For real-time RT-PCR, Brilliant III SYBR Green 2× Master Mix (Agilent Technologies) was used. For primer sequences, see Table S1.

Whole mount immunostaining

Embryos were single-side-injected as indicated, cultured until they reached stage 13 and fixed in 4% buffered formaldehyde for 1 h at room temperature and in methanol overnight at −20°C. After rehydration in a descending methanol series, embryos were washed twice in PBT and blocked for 1 h in 20% horse serum. The primary antibody (anti-pSmad1,5,8; ThermoFisher, PA5-17914) was diluted 1:500 in 20% horse serum, 5% DMSO and incubated overnight at 4°C. Embryos were washed three times in PBT and incubated in anti-mouse-AP (Cell Signaling Technologies, 7054) diluted 1:1000 in 20% horse serum, 5% DMSO for 4 h at room temperature. Embryos were again washed three times in PBT, twice in AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$) and stained using chromogenic alkaline phosphatase substrate NBT/BCIP. Staining was terminated by washing three times in PBT and post-fixation in 4% buffered formaldehyde for at least 1 h at room temperature.

Embryos were scored for pSmad staining intensity at the injected side as compared with the uninjected internal control side. Images of individual embryos were acquired using a Leica S8apo stereomicroscope (Leica Microsystems). Mediolateral intensity profiles of pSmad1,5,8 staining were measured after image conversion to grayscale and linear contrast adjustment using the ImageJ software package (NIH) as described further in supplementary Materials and Methods.

Neural plate explants

Embryos were injected as indicated, co-injected with an RNA encoding membrane-tethered GFP (mGFP), and cultured until stage 12.5. Neural plates including the underlying mesoderm were explanted (Wilson and Keller, 1991), fixed for 1 h in 4% buffered formaldehyde and F-actin was stained using Atto647-conjugated phalloidin (Sigma-Aldrich, 65906), diluted 1:100 in 20% horse serum).

Explants were imaged on a Leica SP5 confocal laser scanning microscope (Leica Microsystems); *z*-stacks spanning the neuroectoderm or the mesoderm were acquired for each explant. The angle between the anterior-posterior axis and the long axis of individual cells was measured for at least 14 cells in two explants per treatment. For further details see supplementary Materials and Methods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.S. carried out experiments, documented and interpreted results. M.B. and A.B. designed and carried out statistical analysis. A.S. conceived of the study, participated in its design, coordination and data analysis and wrote the manuscript.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.135426.supplemental

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