Functional conservation of *Nematostella* Wnts in canonical and noncanonical Wnt-signaling

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Summary

Cnidarians surprise by the completeness of Wnt gene subfamilies (11) expressed in an overlapping pattern along the anterior-posterior axis. While the functional conservation of canonical Wnt-signaling components in cnidarian gastrulation and organizer formation is evident, a role of *Nematostella* Wnts in noncanonical Wnt-signaling has not been shown so far. In *Xenopus*, noncanonical Wnt-5a/Ror2 and Wnt-11 (PCP) signaling are distinguishable by different morphant phenotypes. They differ in PAPC regulation, cell polarization, cell protrusion formation, and the so far not reported reorientation of the microtubules. Based on these readouts, we investigated the evolutionary conservation of Wnt-11 and Wnt-5a function in rescue experiments with *Nematostella* orthologs and *Xenopus* morphants. Our results revealed that NvWnt-5 and -11 exhibited distinct noncanonical Wnt activities by disturbing convergent extension movements. However, NvWnt-5 rescued XWnt-11 and NvWnt-11 specifically XWnt-5a depleted embryos. This unexpected ‘inverse’ activity suggests that specific structures in Wnt ligands are important for receptor complex recognition in Wnt-signaling. Although we can only speculate on the identity of the underlying recognition motifs, it is likely that these crucial structural features have already been established in the common ancestor of cnidarians and vertebrates and were conserved throughout metazoan evolution.

Key words: axis duplication, convergent extension, microtubule orientation

Introduction

The Cnidaria represents ancient metazoans which developed 650 millions of years ago (Chen et al., 2002; Conway Morris, 2000). These diploblastic animals possess only two germ layers, the outer ectoderm and the inner endoderm lacking the mesoderm. Cnidarians express many ancestral genes also found in vertebrates, which are missing in *Drosophila* and *C. elegans* (Chapman et al., 2010; Kusserow et al., 2005; Putnam et al., 2007). The best-studied example is the Wnt family. In vertebrates 12 Wnt subfamilies are missing in *C. elegans* and *Drosophila* (Chapman et al., 2010; Putnam et al., 2007), while only 6 are present in *Nematostella* (Guder et al., 2006; Kusserow et al., 2005; Lee et al., 2006). However, functional analyses of the noncanonical Wnt-signaling components Flamingo/van Gogh, JNK, CamKII and PKC are found (Guder et al., 2006; Lee et al., 2006). However, functional analyses of the noncanonical Wnt-signaling pathways in Cnidaria have not been performed so far.

The amphibian *Xenopus laevis* offers an experimental model to study evolutionary conserved functions of Wnt-signaling components because a broad set of readouts are available. For example, Guder et al. (2006) demonstrated by heterologous expression of Hydkk1/2/4 in *Xenopus* that Hydkk1/2/4 behaved as Xdkk1 in inhibiting canonical Wnt-signaling. Thus, heterologous expression of noncanonical Wnt orthologs in *Xenopus* may help to enlighten their putative function in Cnidaria.

In *Xenopus*, Wnt-5a and Wnt-11 activities are distinguishable in gain- and loss-of-function studies. Wnt-11 stimulates the Wnt/PCP-signaling pathway mediated by frizzled, disheveled, Daam1, resulting in activation of the small GTPases Rac1 and RhoA and the JNK (Habas et al., 2003; Habas et al., 2001; Tada and Smith, 2000; Wallingford and Habas, 2005; Wallingford et al., 2000). Activation but also inhibition of Wnt/PCP-signaling results in excluded because expression of dominant-negative β-catenin mutants in *Nematostella* completely inhibited gastrulation (Wikramanayake et al., 2003).
complete block of convergent extension movements in *Xenopus* gastrulation because mesodermal cells fail to polarize (Wallingford et al., 2000), reviewed in (Wallingford and Habas, 2005). Furthermore, in absence of Wnt-11 cells are forming blebs instead of lamellipodia or filopodia (Schambony and Wedlich, 2007). Wnt-5a induces in conjunction with Ror2, PI3K, cdc42, JNK and ATF2 the expression of the protocadherin PAPC. Wnt-5a/Ror2-signaling does not influence cell polarity establishment but is necessary for cell alignment and orientation to the dorsal midline. In absence of Wnt-5a mesodermal cells, although of bipolar shape, move randomly and form supernumerous filopodia at expense of lamellipodia. Neither Wnt-11 nor Wnt-5a are able to replace each other in controlling cellular behavior (Schambony and Wedlich, 2007).

In this study we exploit *Xenopus* embryos as a readout for canonical and noncanonical Wnt-signaling to characterize *Nematostella* (Nv) Wnt ligand properties. By heterologous expression we learn that NvWnt-1 but neither NvWnt-11 nor NvWnt-5 induces secondary axis in *Xenopus* embryos. Instead, only NvWnt-11 and NvWnt-5 exhibit distinct noncanonical functions. Surprisingly, however our various experimental assays revealed the concurrent picture that NvWnt-11 replaces XWnt-5a while NvWnt-5 takes over XWnt-11 function in *Xenopus* gastrulation. These data indicate that specific structural features in the X-Wnt and Nv-Wnt ligands are evolutionary conserved and responsible for the distinct cellular behavior required for the complex cellular machinery of gastrulation.

**Material and Methods**

**Constructs**

The previously described NvWnt-1, -2, -5, -7b and -11 (Kusserow et al., 2005) and NvWnt-3 (Lee et al., 2006) were subcloned via PCR amplification into the EcoRI-XbaI site of pCS2+ (Rupp et al., 1994). Full-length clones for NvWnt-2 and -3 were isolated from 12-120 hours N. vectensis embryos (5'-GGCAATTC-CCGGTCATAGTCCGC-3' as forward and 5'-GCCTTCATAGAAGCCGC-CTAAATACCGGGAT-3' as reverse primer for NvWnt-2; 5'-GGATCCGGA-AAXCAGGCACATCTGAGAG-3' as forward and 5'-TCTAGAGCTGGAG-TATTTTAAAGTGAATGTTAAC-3' as reverse primer for NvWnt-3). The missing 3'end of NvWnt-3 was cloned by the oligonucleotide-capping RACE method using GeneRacer Kit (Invitrogen Ltd, Pailey, UK) (RACEprimer1 5'-GCCTTCATAGAAGCCGC-CTAAATACCGGGAT-3' as forward and 5'-TCTAGAGCTGGAG-TATTTTAAAGTGAATGTTAAC-3' as reverse primer for NvWnt-3).

pCS2+ XWnt-11 (Tada and Smith, 2000), pCS2+ XWnt-5a (Moon et al., 1993) and pCS2+ Mwnt-1 (Doubravskova et al., 2011) were described previously. pCS2+ EBi-GFP was a kind gift of Diane Sepich (Washington University School of Medicine, St Louis, USA). GAP43-mCherry was generated by insertion of mCherry into the SacI-XhoI site of pCS2+ GAP43-GFP (Kim et al., 1998) replacing GFP coding sequence.

*Xenopus* embryo treatment, microinjections and *in situ* hybridization

Embryos were obtained by *in vitro* fertilization, cultured and injected as described previously (Geis et al., 1998), and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Capped mRNAs were synthesized from linearized plasmids using the Ambion mMessage mMachine Kit (Applied Biosystems, Darmstadt, Germany). The injection amount varied depending on construct and is mentioned in the figure legends. To account for less efficient translation of NvWnts than used for the corresponding vertebrate homologs but not more than 500 pg to avoid artefacts. Successful protein synthesis of the NvWnt constructs was detected by the *in vitro* TNT Tetrahyamine Lysate System (Promega, Mannheim, Germany) with radioactive [35S]methionine (GE Healthcare, Little Chalfont, UK) carried out in accordance with manufacturer's instruction. All morpholinos were ordered by GeneTools (Philomath, OR, USA) and injected as followed: 1 pmol XWnt-11 MO (Pandur et al., 2002), 1.6 pmol XWnt-5a MO (Schambony and Wedlich, 2007). For Keller open-face (DMZ) explants mRNA/MOs were microinjected into the dorsal marginal zone of 4-cell or 8-cell stage embryos, whereas for double axis formation assay mRNAs were injected into the ventral side of 4-cell stage embryos. Embryos destined for *in situ* hybridization were single side injected into the animal hemisphere of 2-cell stage embryos. As lineage tracer dextran-FITC (4 pg; Molecular Probes, Eugene, OR, USA) or GAP43-mCherry RNA (250 pg) was co-injected. *In situ* hybridization was performed as described previously (Schneider et al., 2010) using template cDNA XPAPC (Kim et al., 1998).

For the double axis assay the injected embryos were scored at the neurula stage (20, DMZ explants for the analysis of convergent extension movements and time-lapse experiments were prepared at stage 10.25 and cultured, imaged and scored as described previously (Unterscher et al., 2004). Time-lapse movies of growing microtubules were recorded by capturing one image per one second using the Z1 Cell Observer Spinning Disc inverse microscope (Zeiss, Jena, Germany). Tracking of microtubules and measurement of angle were carried out using ImageJ software (open source: http://rsbweb.nih.gov/ij/). Rose diagrams were prepared by the free open-source software OpenRose 0.01.

**Sequence comparison**

Sequence comparison and consensus tree were generated by using the Muscle algorithm and the Geneious software (Geneious v5.3, available from http://www.geneious.com).

**Results and Discussion**

**Conserved function of canonical Wnt-signaling in cnidarians**

*Nematostella* has 11 Wnt gene subfamilies (Kusserow et al., 2005). With the exception of Wnt-8 and Wnt-A, we analyzed all major clusters of these Wnt gene subfamilies on their axis induction capacity in *Xenopus*, i.e. NvWnt-1, NvWnt-2, NvWnt-3, NvWnt-5, NvWnt-7b, and NvWnt-11. NvWnt-1 clusters with NvWnt-6 and NvWnt-10, NvWnt-11 with NvWnt-4. NvWnt-8 was excluded because it has been shown to exhibit a function in PCP signaling (Philipp et al., 2009) and Wnt-A does not exist in vertebrates. In *vitro* transcribed RNA of the Wnts of interest was injected into the ventral blastomeres of 4-cell stage embryos. As shown in Fig. 1 both NvWnt-1 and murine Wnt-1 RNA induced double axes in 70% of the embryos (Fig. 1B,C). In comparison to MWnt-1, however, a 10 times higher concentration of NvWnt-1 was required for the same ratio in phenotype. NvWnt-2, NvWnt-3, NvWnt-5, NvWnt-7b and NvWnt-11 did not induce a secondary axis even when up to 500 pg was injected (Fig. 1D–I).

Appropriate protein synthesis from all NvWnt constructs was confirmed using an *in vitro* reticulocyte lysate system (Fig. 1J). Since Wnts lose activity when they are tagged, expression was detected by radioactive labelling. The observed differences in the synthesis efficiency should not count in our axis induction assay because much higher amounts were injected of NvWnt-2, NvWnt-3, NvWnt-5, NvWnt-7b and NvWnt-11 in comparison to the corresponding vertebrate othologs and also NvWnt-1. We therefore conclude that among the so far tested cnidarian Wnts only NvWnt-1 possesses an axis induction capacity like vertebrate canonical Wnt ligands.

**Conserved function of noncanonical Wnt-signaling in cnidarians**

We next focused on the characterization of the noncanonical function of the selected Wnt subfamilies members. To assay noncanonical function we used a gain-of-function approach in Keller open-face explants. RNA samples of choice were injected into the dorsal blastomeres of 4-cell stage Xenopus embryos. With the onset of gastrulation (stage 10.25) dorsal marginal zone (DMZ) explants were prepared and cultivated over 6–8h. XWnt-5a overexpression resulted in elongated explants lacking the typical constriction (compare Fig. 2A with Fig. 2B,F) while
XWnt-11 overexpression completely inhibited the elongation (Fig. 2D,G).

Among all tested members of the *Nematostella* Wnt clusters only NvWnt-5 and -11 showed effects on convergent extension movements (see Fig. 2). NvWnt-5 expression showed an effect on the elongation and constriction in a dose-dependent manner (Fig. 2C,F) thereby phenocopying XWnt-11 expression. In contrast, NvWnt-11 expressing explants elongated without constriction (Fig. 2E,G) resembling the XWnt-5a gain-of-function phenotype. Thus, NvWnt-5 and NvWnt-11 activate noncanonical Wnt-signaling pathways, however with an ‘inverse’ activity in *Xenopus*.

To further confirm this surprising observation we investigated the rescue capacity of the NvWnt orthologs in XWnt-5a and XWnt-11 morphants. Depletion of XWnt-5a resulted in explants with failures in constriction but normal elongation (Fig. 3B,H). This phenotype was rescued by NvWnt-11 dose-dependently but not by NvWnt-5 (Fig. 3C,D,H). High doses of NvWnt-5 led to elongation defects in XWnt-5a morphants, which corresponds to its activity when expressed in wildtype (compare Figs 3H and 2F). XWnt-11 antisense morpholino (XWnt-11 MO) injections inhibited elongation of the explants, which was rescued by NvWnt-5 in a dose-dependent manner (Fig. 3E,G,I). NvWnt-11 coinjections, however, had no effect (Fig. 3F,I). Coinjections of
Fig. 2. NvWnt-5 and NvWnt-11 act in noncanonical Wnt-signaling by disturbing convergent extension movements. (A; H) Wildtype DMZ explants with normal elongation and constriction. (B) XWnt-5a (100 pg) overexpression impairs constriction of DMZ explants, while (D) XWnt-11 (20 pg) overexpression completely inhibits their elongation. (C) NvWnt-5 expression has both an effect on constriction and elongation. (E) NvWnt-11 expressing DMZ explants elongate but the constriction fails. (I) NvWnt-1 (200 pg), (J) NvWnt-2 (400 pg) and (K) NvWnt-3 (400 pg) expressing DMZ explants show normal elongation and constriction. (F, G, L) Quantification of elongated DMZ explants (black) and of elongated DMZ explants showing normal constriction (gray). # number, *p<0.005 to wildtype DMZ explants, **p<0.05 to wildtype DMZ explants. Error bars indicate standard error; scale bars 750 μm.
either NvWnt-11 or NvWnt-5 led to a weak constriction phenotype in XWnt-11 morphants (Fig. 3I). This is explained by the individual activities of these *Nematostella* Wnts when expressed in wildtype embryos (compare Figs 3I and 2F,G).

To prove the inverse activities of NvWnt-11 and NvWnt-5 in *Xenopus*, we performed an additional rescue experiment, which is based on the specific control of the protocadherin *PAPC* expression by XWnt-5a (Schambony and Wedlich, 2007). *In situ* hybridizations revealed that single-side injections of XWnt-5a MO led to the loss of *PAPC* in late gastrula (Fig. 4A). Coinjection of NvWnt-11 but not NvWnt-5 rescued *PAPC* expression (Fig. 4B–D). We therefore conclude that XWnt-5a and NvWnt-11 are functionally related.

XWnt-11 but not XWnt-5a controls microtubule orientation

Microtubules (MT) play an essential role at the onset of *Xenopus* gastrulation when cells become bipolar (Kwan and Kirschner, 2005; Lane and Keller, 1997). Since *in vivo* imaging of MT...
growth in DMZ explants also revealed MT polarization along the mediolateral cell axis (Kwan and Kirschner, 2005; Shindo et al., 2008) we investigated the role of XWnt-5a and XWnt-11 in such a MT growth orientation assay.

For tracking the growing ends of microtubules we expressed EB1-eGFP together with GAP43-mCherry, a cell membrane marker, in Xenopus embryos and performed high-resolution time-lapse imaging of DMZ explants after 30 minutes and 4 hours using a spinning disc microscope. Within the first 30 minutes (explants isolated at stage 10.25) most cells were of isodiametric shape forming cell protrusions in all directions (multipolar). In these cells microtubules grew in all directions from the centre to the cell periphery (Fig. 5A,A; see movie SM1 in supplementary material), only 20% of the cells were of elongated bipolar shape exhibiting a polarized growth of microtubules towards the cell ends (Fig. 5F). After further cultivation (4 hours at 22°C) when the siblings reached stage 12.5, 70% of the cells in the explants showed polarized growing microtubules from the cell centre towards the mediolateral cell poles (Fig. 5A,A; see also supplementary material movie SM2). In XWnt-5a morphants time-dependent oriented growth remained unaltered (Fig. 5B,B;F; see also supplementary material movie SM3). In XWnt-11 morphants, however, less bipolar cells were detected after 30 minutes. After 4 hours explant cultivation time-lapse imaging revealed that the microtubules failed to polymerize in mediolateral orientation (Fig. 5C,C;F; see also supplementary material movie SM4). Cell shapes remained unpolarized as reported previously when Wnt/PCP-signaling is disturbed (Schambony and Wedlich, 2007; Wallingford et al., 2000).

NvWnt-5 controls microtubule orientation

Using this new cellular readout dissecting Wnt-5 and Wnt-11 function we tested the rescue ability of the Nematostella orthologs in the Xenopus morphants. As shown in Fig. 5D–F (see also supplementary material movies SM5 and SM6) NvWnt-5 but not NvWnt-11 abolished the XWnt-11 morphant phenotype. This indicates that the unique function of specific noncanonical Wnt ligands can be mimicked by Nematostella orthologs. Despite an overall sequence similarity of a given Wnt gene subfamily, hidden sequence motifs might explain the specificity of NvWnt-5 and NvWnt-11 in Xenopus assays.

Sequence comparison

To unravel such motifs, we performed a sequence analysis for the noncanonical Wnts NvWnt-5 and -11 from Nematostella and selected vertebrate Wnt-5 and -11 sequences (XWnt-5a and -11;
Fig. 5. See next page for legend.
HWnt-5a and -11) by using the canonical NvWnt-1 as an outgroup. The sequence comparison revealed a proper allocation according to the overall alignment (Fig. 6) of NvWnt-5 and -11 as members of the Wnt-5 and Wnt-11 subfamilies. Besides conserved Wnt-specific motifs, cysteine (71, 85), glycine (252), methionine (92, 225), proline (176) and tyrosine (277) residues are specific for the noncanonical Wnt-5 and -11 subfamilies.

An analysis for motifs shared by vertebrate Wnt-11 and NvWnt-11 or vertebrate Wnt-5 and NvWnt-5 revealed that 26 residues match in *Nematostella* and vertebrate Wnt-5 and 18 in *Nematostella* and vertebrate Wnt-11. We also identified sites for vertebrate Wnts where the homology of Wnt-5 and Wnt-11 was closer to NvWnt-11 and NvWnt-5, respectively. In NvWnt-11 12 vertebrate specific Wnt-5 residues, and in NvWnt-5 five vertebrate specific Wnt-11 residues were found. We presume that these sites are candidates for the uniqueness at the putative ligand/receptor interface. They are considered to be responsible for the observed specificity of NvWnt-5 and NvWnt-11 in *Xenopus* morphant rescue experiments (Figs 3–5). The number of *Nematostella* residues with ‘crosshomology’ was larger for Wnt-5 than for Wnt-11. Interestingly, also in the canonical NvWnt-1 sequence specific sites from vertebrate and *Nematostella* Wnt-5 and Wnt-11 were found. This might suggest the evolutionary ancestry of the noncanonical Wnt subfamilies 5 and 11 from a canonical Wnt ancestor.

Wnt ligand specificity is conserved in evolution?

Heterologous expression of NvWnt-5 and NvWnt-11 in *Xenopus* revealed that canonical and noncanonical Wnt activities have already been established in ancient metazoan. Thus, regulation of body axis formation and cell shape symmetry break (change in polarity) by Wnts was separated very early in the metazoan evolution. We presume that the secession of noncanonical Wnt functions was related to the implementation of sophisticated gastrulation movements in a cnidarian-like metazoan ancestor. In line with this hypothesis is the inverse switch in the functional relationship between Wnt-5 and -11 and the conservation of corresponding residues to the canonical NvWnt-1. Thus canonical Wnt-signaling might have been the ancestral form of Wnt-signaling in metazoan evolution.

**Fig. 5.** NvWnt-5 rescues microtubule orientation in XWnt-11 morphants. (A–E) In vivo imaging of microtubule growth and cell polarization in DMZ explants expressing EB1-eGFP (500 pg) and GAP43-mCherry (200 pg). Scale bar 20 μm. (A’–E’) Microtubule growth directions of the indicated cell statistically rendered in rose diagrams. (A–E, A’–E’) 30 minutes after explant isolation (stage 10.25) microtubules grow from the cell centre in all directions. After 4 hours (stage 12.5), microtubule growth gets bipolar (A, A’) in control and (B, B’) in XWnt-5a depleted explants, while (C, C’) in absence of XWnt-11 microtubules keep on growing in all directions. (E, E’) NvWnt-5 (400 pg) but not (D, D’) NvWnt-11 (200 pg) expression in XWnt-11 morphants rescues microtubule orientation. (F) Quantification of cells with bipolar microtubule orientation. # number, **p<0.005 to wildtype, *p<0.005 to MO. Error bars indicate standard error.

**Fig. 6.** Alignment of vertebrate and *Nematostella* noncanonical and canonical sequences. The evolutionary conserved backbone of Wnt residues is indicated by black bars. Wnt-5 specific residues are drawn in red, Wnt-11 specific in blue. ‘Crosshomology’ is indicated by red bars for the Wnt-5 subfamily and blue bars for the Wnt-11 subfamily. Note that some of the Wnt-5 or Wnt-11 specific residues are also shared by the canonical NvWnt-1 (outgroup). (For further details see text).
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