The involvement of Frodo in TCF-dependent signaling and neural tissue development

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Summary

Frodo is a novel conserved regulator of Wnt signaling that has been identified by its association with Dishevelled, an intracellular component of Wnt signal transduction. To understand further how Frodo functions, we have analyzed its role in neural development using specific morpholino antisense oligonucleotides. We show that Frodo and the closely related Dapper synergistically regulate head development and morphogenesis. Both genes were cell-autonomously required for neural tissue formation, as defined by the pan-neural markers *sox2* and *nrp1*. By contrast, β-catenin was not required for pan-neural marker expression, but was involved in the control of the anteroposterior patterning. In the mesoderm, Frodo and Dapper were essential for the expression of the organizer genes *chordin*, *cerberus* and *Xnr3*, but they were not necessary for the expression of *siamois* and *goosecoid*, established targets of β-catenin signaling. Embryos depleted of either gene showed a decreased transcriptional response to TCF3-VP16, a β-catenin-independent transcriptional activator. Whereas the C terminus of Frodo binds Dishevelled, we demonstrate that the conserved N-terminal domain associates with TCF3. Based on these observations, we propose that Frodo and Dapper link Dsh and TCF to regulate Wnt target genes in a pathway parallel to that of β-catenin.

Key words: Frodo, Wnt, TCF, β-catenin, Neural, *Xenopus*

Introduction

The current understanding of how embryonic cells respond to a small number of extracellular signals to generate patterned tissues and organs has been hampered by the limited knowledge of the intracellular machinery that mediates these responses. One of the signaling pathways that functions to establish cell fates and cell polarity in many developmental processes is the Wnt signaling pathway, which is initiated by secreted factors of the Wnt family (Cadigan and Nusse, 1997). Although Wnt signaling has been implicated in the formation of the vertebrate organizer (Harland and Gerhart, 1997; Sokol, 1999), neural induction (Baker et al., 1999; Sokol et al., 1995), and morphogenetic movements of gastrulation and neurulation (Sokol, 1996; Tada and Smith, 2000; Wallingford et al., 2000), the underlying molecular mechanisms remain unclear.

Recent studies have led to the realization that the Wnt pathway involves multiple branches, including signaling through β-catenin, activation of Jun N-terminal kinases, Rho GTPases and Ca^{2+} ion signaling (Boutros et al., 1998; Habas et al., 2001; Kinoshita et al., 2003; Yamanaka et al., 2002). Despite the existence of many protein targets, it is commonly accepted that the activation of Wnt target genes involves β-catenin and transcriptional factors of the T cell factor (TCF) family. The TCF proteins in complex with Groucho family members repress the transcription of their targets (Cavallo et al., 1998; Roose et al., 1998), but may be activated or de-repressed by an associated co-factor such as β-catenin. TCFs have been reported to function as transcriptional repressors for anteroposterior axis specification in *C. elegans* embryos (Meneghini et al., 1999) and during vertebrate head development (Houston et al., 2002; Kim et al., 2000). The predominant model of canonical Wnt signaling assumes that the upstream components of the pathway, such as Dishevelled (Dsh), stabilize β-catenin and promote its association with TCF, thereby converting TCFs into transcriptional activators (Nusse, 1999).

Dishevelled appears to be essential for all branches of the Wnt pathway (Boutros and Mlodzik, 1999; Sheldahl et al., 2003; Sokol, 2000). In the canonical Wnt pathway, Dsh is proposed to inhibit the activity of GSK3, a serine-threonine protein kinase that targets β-catenin for degradation (Cook et al., 1996; Itoh et al., 1998; Siegfried et al., 1992; Yost et al., 1998). In addition, Dsh associates with and inhibit the function of Axin, a component of the β-catenin destruction complex (Cliffe et al., 2003; Itoh et al., 2000; Kishida et al., 1999; Li et al., 1999; Smalley et al., 1999; Zeng et al., 1997). In an attempt to gain insight into Dsh function, many Dsh-associated proteins have been identified (Wharton, 2003). Among those are Frodo (Gloy et al., 2002; Gillhouse et al., 2004) and Dapper (Cheyette et al., 2002), two closely related proteins that contain a highly conserved N-terminal leucine zipper domain and a C-terminal PDZ-binding domain. Whereas Frodo and Dapper are

90% similar in primary amino acid sequence, they are expressed in different patterns and reveal different activities in functional assays (Cheyette et al., 2002; Gloy et al., 2002). Both Frodo and Dapper have been implicated in mesoderm and neural tissue development, but their specific roles and molecular mechanism of action remain to be elucidated. This study investigates the function of these proteins using the morpholino-mediated loss-of-function approach. Our data suggest that Frodo and Dapper are involved in more than one step of the signaling cascade and may function in a pathway that is parallel to β-catenin.

Materials and methods

Plasmids

The pCTX vector for RNA synthesis was constructed from CMV promoter-containing pCS2 (Turner and Weintraub, 1994), in which *Sal*I and *Kpn*I sites were eliminated by blunting and re-ligation. A multiple cloning site flanked by *Xenopus* β-globin 5′- and 3′-UTRs, and T7 and SP6 promoter sequences was inserted by PCR (S.Y.S., unpublished). pCTX-HA-Frodo was constructed by inserting the *Hin*dIII-*Nsi*I and *Nsi*I-*Xba*I fragments from pXT7-HA-Frodo (Gloy et al., 2002) into pCTX. pCTX-HA-Frd337 and pCTX-HA-Frd186 were obtained by self-ligation of pCTX-HA-Frodo digested with *Spe*I, and *Pst*I and *Spe*I, respectively. pEBG-TCF3 was generated by subcloning the *Sac*I-*Kpn*I fragment of pT7TS-TCF3 (Molenaar et al., 1996) into pEBG (Sanchez et al., 1994). The deletion of the β-catenin-binding domain in pEBG-ΔNTCF3 was constructed by site-directed mutagenesis as described (Makarova et al., 2000) using the primer 5′-GAGCTCGGGGCTAACGACCTCGAGTCGGAGAATCACAGC-3′. pCS2-Flag-Frd337 was generated by PCR (forward primer, 5′-GG-AATTCTAAGCCCATTCCCTCCCC-3′; reverse primer, 5′-GCTC-TAGATCAACTAGTCCTTGGTTTGT-3′) into pCS2-Flag, which encodes the N-terminal FLAG epitope (H.H., unpublished).

Xenopus embryos and microinjections

In vitro fertilization and embryo culture in 0.1×Marc's modified Ringer's solution (MMR) were carried out as described (Peng, 1991). Staging was according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For microinjection, embryos were transferred to 3% Ficoll 400 (Pharmacia) in 0.5×MMR and injected at the 4- to 16-cell stages with 5 nl of mRNA or morpholino solution. For rescue experiments and luciferase reporter assays, the same blastomere was injected with a morpholino, followed by mRNA or DNA injection 15-20 minutes later. For lineage tracing, RNA encoding nuclear β-galactosidase (nβgal) was injected together with morpholinos at 20 pg/embryo, and β-galactosidase activity was visualized with the Red-Gal substrate (Research Organics). FrdMO and DprMO have been characterized previously (Cheyette et al., 2002; Gloy et al., 2002). Control morpholino (CoMO) had the following sequence: 5′-AGAGACTTGATACAGATTCGAGAAT-3′. For mRNA synthesis, pCTX-HA-Frodo, pCTX-HA-Frd337, pCTX-HA-Frd186, pCS2-Flag-Frd337, pCS2-β-catenin (S>A) (Liu et al., 1999), pCS2-TCF3VP16 (Vonica et al., 2000), pCS2-nβgal (Turner and Weintraub, 1994) and pT7TS-HA-TCF3 (Molenaar et al., 1996) were linearized with *Not*I or *Xba*I. Capped synthetic RNAs were generated by in vitro transcription with SP6 or T7 RNA polymerase using the mMessage mMachine kit (Ambion).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out according to Harland (Harland, 1991) with slight modifications as described previously (Hikasa et al., 2002). For Frodo in situ hybridization, embryos were rehydrated in 1×PBS, 0.1% Tween 20 and bisected with a razor blade before hybridization. Digoxigenin-labeled antisense RNA probes were synthesized from pBS59-chd (*chordin*) (Sasai et al., 1994), pDor3 (*Xnr3*) (Smith et al., 1995), pGsc (Cho et al., 1991), pBSSK-Xsox2 (Green et al., 1997), pBSKS-nrp1 (Richter et al., 1990), pBSSK-Frodo (Gloy et al., 2002) and pBSSK-myoD (Hopwood et al., 1989) using the DIG labeling mixture (Boehringer Mannheim). 5-Bromo-4-chloro-3-indolyl phosphate (Sigma) and Nitro blue tetrazolium (Sigma) were used for chromogenic reactions.

RNA isolation and RT-PCR

Total embryo RNA for RT-PCR was extracted from stage 10 or stage 11 embryos injected with morpholinos by proteinase K-phenol extraction as described (Itoh and Sokol, 1997). cDNA was made from DNase-treated RNA using Superscript first strand synthesis system (Invitrogen). RT-PCR was carried out as previously described (Itoh and Sokol, 1997). Primers for RT-PCR were: *cerberus*, 5′-GCTTG-CAAAACCTTGCCCTT-3′ and 5′-CTGATGGAACAGAGATCTTG-3′; *chordin*, 5′-AACTGCCAGGACTGGATGGT-3′ and 5′-GGCAG-GATTTAGAGTTGCTTC-3′; *Xnr3*, 5′-CGAGTGCAAGAAGGTG-GACA-3′ and 5′-ATCTTCATGGGGACACAGGA-3′; *siamois*, 5′-CTCCAGCCACCAGTACCAGATC-3′ and 5′-GGGGAGAGTG-GAAAGTGGTTG-3′; *gsc*, 5′-TTCACCGATGAACAACTGGA-3′ and 5′-TTCCACTTTTGGGCATTTC-3′; *vent1*, 5′-GCATCTC-CTTGGCATATTTGG-3′ and 5′-TTCCCTTCAGCATGGTTCACC-3′; *EF1a*, 5′-CAGATTGGTGCTGGATATGC-3′ and 5′-ACTGC-CTTGATGACTCCTAG-3′.

Transfections, GST pull-down assays, immunoprecipitation and western analysis

COS7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum and 50 μg/ml of gentamicin (Sigma). For GST pull-down assays, cells were transiently transfected using the Fugene 6 transfection reagent (Roche) with the following plasmids: pEBG (0.1 μg), pEBG-XTCF3 (10 μg), pEBG-ΔNTCF (12 μg), pCTX-HA-Frodo (10 μg), pCTX-HA-Frd337 (1 μg) and pCTX-HA-Frd186 (1 μg). After 30 hours in culture, transfected cells were lysed in 500 μl of lysis buffer [1% Triton X-100, 50 mM Tris-HCl (pH. 7.5), 50 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride, 10 mM NaF, 1 mM Na_3VO_4]. Supernatants were cleared at 12,000 ***g*** for 5 minutes and incubated with glutathione-agarose beads (Sigma) for 2 hours at room temperature. The beads were washed three times with lysis buffer and boiled in the SDS-PAGE sample buffer. Immunoprecipitation and western analysis were performed as described (Gloy et al., 2002). Monoclonal 12CA5 and M2 antibodies (Sigma) were used for detection of HA- and FLAG-tagged proteins, respectively. Antibodies to non-phosphorylated β-catenin were from Upstate Biotechnology.

Luciferase reporter assays

Four-cell stage embryos were injected into a single ventral animal blastomere with 30 pg of pSia-Luc reporter DNA (Fan et al., 1998) together with the indicated RNAs. At early gastrula stage (stage 10+), embryos were homogenized in 50 mM Tris-HCl (pH 7.5). Supernatants were cleared by centrifugation at 12,000 ***g*** for 3 minutes and assayed for luciferase activity as previously described (Fan et al., 1998). Every experimental group included four samples, each comprising five embryos. All transcriptional assays were repeated at least three times.

Results

Frodo and Dapper act synergistically during head development

Phenotypes of embryos depleted of Frodo and Dapper during *Xenopus* early embryogenesis were compared after dorsal injection of specific morpholino antisense oligonucleotides (Cheyette et al., 2002; Gloy et al., 2002) into four-cell embryos.

Injections of either FrdMO or DprMO resulted in shortened embryos with mild anterior abnormalities, whereas a control morpholino with a similar base composition did not significantly alter normal development (Fig. 1A-C). The simultaneous injection of FrdMO and DprMO resulted in dorsally bent embryos lacking head structures even at half the dose (Fig. 1D; Table 1), suggesting that Frodo and Dapper synergize during early development. These developmental abnormalities were suppressed by full length Frodo mRNA lacking the morpholino target sequence, indicating that Frodo can functionally substitute for Dapper in this assay (Fig. 1E; Table 1). These findings confirm the specificity of morpholino effects. Interestingly, mRNA for stabilized β-catenin (Liu et al., 1999) also rescued head structures, including cement gland and eyes, but failed to restore proper morphogenetic movements (Fig. 1F; Table 1). These observations indicate that Frodo and Dapper are necessary for both head development and morphogenetic movements accompanying body axis elongation.

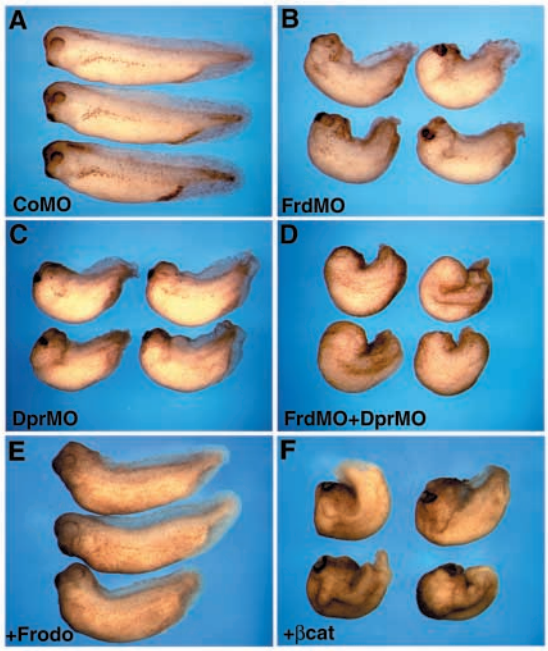

Fig. 1. Frodo and Dapper are required for head development. Two blastomeres of four-cell stage embryos were injected in the dorsal equatorial region with morpholinos and mRNAs as indicated in Table 1. (A) Control morpholino (CoMO)-injected embryos. (B,C) Embryos injected with FrdMO or DprMO show shortened axes with head defects. (D) Co-injection of FrdMO and DprMO leads to severe head defects. (E) The effects of FrdMO and DprMO are reversed by Frodo RNA lacking the target sequences. (F) Head development is rescued in FrdMO and DprMO-injected embryos by stabilized β-catenin (βcat) RNA. Shortened axes are rescued by Frodo RNA, but not by βcat RNA.

Frodo and Dapper, but not β-catenin, are required for neural development

The head and morphogenetic abnormalities in Frodo and Dapper morpholino-injected embryos suggest a likely defect in the formation of neural tissue. To address this possibility, we analyzed the specification of cell fates and cell movements that accompany neurulation. When a single blastomere of 8- to 16-cell embryos was injected with either morpholino, no neural fold formed on the injected side at stages 18-20, leading to an open neural tube (Fig. 2, left panels; Table 2). The neural folds in embryos that were injected with the control morpholino or the neural fold on the uninjected side formed normally. Lineage tracing demonstrated that this morphogenetic defect of FrdMO- and DprMO-injected embryos was observed only in cells that contained morpholinos (Fig. 2, right panels). This neural tube closure defect was rescued by the full-length Frodo mRNA, but not by β-catenin RNA (Table 2). These findings suggest that β-catenin cannot substitute for Frodo or Dapper in the control of morphogenetic movements during neurulation, which is similar to our data on tissue involution during gastrulation (Fig. 1F; Table 1).

At the beginning of gastrulation, Frodo is expressed throughout the animal pole hemisphere, including neuroectoderm (Gloy et al., 2002), suggesting a role in neural tissue development. We therefore examined whether the loss-of-function of Frodo and Dapper influences the pan-neural molecular markers *sox2* and *nrp1* (Fig. 3; Table 3). The muscle-lineage marker *myoD* was also used to evaluate the

Table 1. The effects of FrdMO and DprMO on head formation and morphogenesis

			Phenotypes (%)			
				Morphogenetic defects with		
Experimental groups	Dose/embryo	*n**	Normal	Normal head	Microcephaly	Acephaly
Control MO	16 ng	70	90	4	6	0
FrdMO	16 ng	38	5	26	61	8
DprMO	8 ng	40	15	32	50	3
FrdMO+DprMO	4 ng+2 ng	55	29	18	29	24
FrdMO+DprMO+Frodo	4 ng+2 ng+1 ng	56	59	20	16	5
FrdMO+DprMO	8 ng+4 ng	97	2	10	25	63
FrdMO+DprMO+Frodo	8 ng+4 ng+1 ng	65	22	35	38	5
FrdMO+DprMO+βcat	8 ng+4 ng+10 pg	61	5	67 (18)†	23	5

Morpholinos (MOs) and mRNAs as indicated above were injected into the equatorial region of two dorsal blastomeres at four-cell stage. Morphological defects were scored at stages 33-35 and expressed as percentage of total number of injected embryos.

Incomplete blastomeres closure (n=44, 98%) was observed in embryos injected with FrdMO and DprMO (8 ng+4 ng), and to a lesser degree in embryos injected with FrdMO (16 ng) or DprMO (8 ng) separately (n=40, 20% and n=38, 32%, respectively).

Data were obtained from three separate experiments.

*n, number of scored embryos.

†Percentage of embryos with enlarged head.

effect of FrdMO and DprMO on mesoderm development. In situ hybridization analysis revealed a dramatic effect of the morpholinos on *sox2*, an early neural tissue marker (Green et al., 1997; Mizuseki et al., 1998), in both early gastrula (Fig. 3B,C) and neural plate stage embryos (Fig. 3D-F), suggesting that abnormal neural plate closure may be due to the early defect in neural specification. Lineage tracing confirmed that lack of *sox2* expression was observed only in morpholino injected cells that were β-galactosidase positive. As animal-dorsal blastomeres mainly contribute to ectoderm derivatives, rather than organizer-derived mesoderm (Moody, 1987; Vodicka and Gerhart, 1995), we expect that the lack of *sox2* expression in the neural plate is due to the direct morpholino effect on the responding tissue, rather than to decreased neural inducing properties of the organizer. This conclusion is further supported by our finding that *myoD* was not significantly affected by FrdMO and DprMO in these embryos (Fig. 3G,H; Table 3). Thus, Frodo and Dapper appear to function cell-autonomously during the early phase of neural induction.

We also examined the expression of *nrp1*, a late pan-neural marker (Knecht et al., 1995; Richter et al., 1990), in embryos injected with FrdMO or DprMO into a single dorsal animal blastomere at the 8- to 16-cell stage. Embryos injected with a control morpholino show symmetrical expression of *nrp1* in the brain and the posterior neural tube (Fig. 3I). In FrdMO and DprMO-injected embryos, *nrp1* expression was severely reduced on the injected side in both anterior and posterior neural tube (Fig. 3J,K). The effect of FrdMO on *nrp1* was significantly suppressed by the full-length Frodo mRNA (Fig. 3M). These results indicate that Frodo and Dapper are essential for neural fold formation and pan-neural marker expression.

To test whether *nrp1* is a target of β-catenin signaling, we analyzed *nrp1* levels in embryos injected with β-catenin morpholino (βcatMO). In these embryos, overall levels of *nrp1* were not significantly affected. Whereas the *nrp1* expression

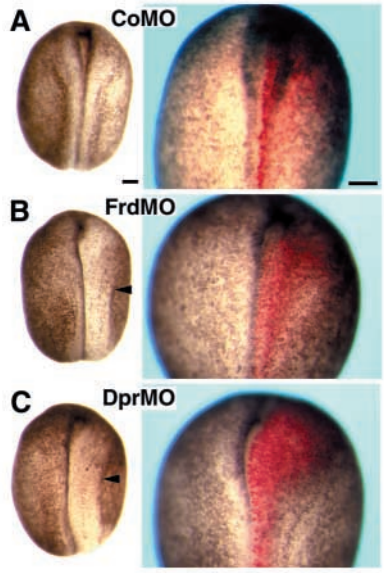

Fig. 2. The effect of FrdMO and DprMO on neural plate closure. (A-C) CoMO (A), FrdMO (B) or DprMO (C) were injected into the right animal dorsal blastomere of 8- to 16-cell stage embryos without (left panels) or with (right panels) nuclear β-galactosidase (nβgal) RNA, a lineage tracer. Doses of MOs and mRNAs are as indicated in Table 2. (B,C) Neural plate closure and neural fold formation is severely disturbed on the injected side as evidenced by RedGal staining. Arrowheads indicate the neural plate border. Scale bars: 150 μm.

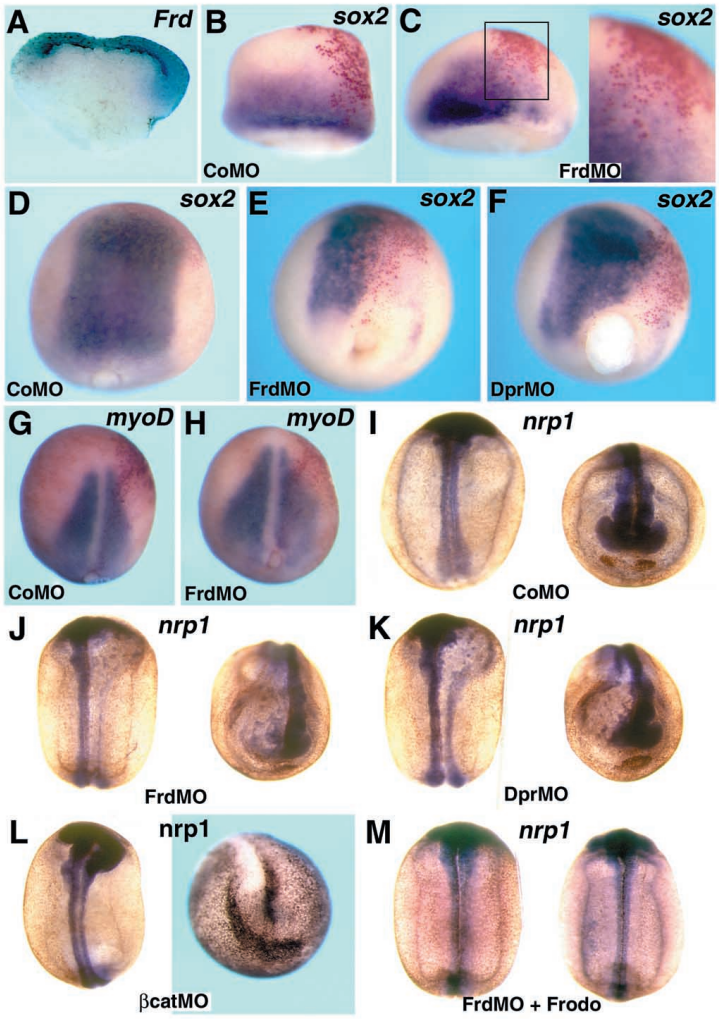

Fig. 3. Frodo and Dapper are required for neural development. (A) Localization of Frodo RNA visualized by whole-mount in situ hybridization on a half-embryo at stage 10, sagittal view. (B-M) Morpholinos and RNAs were injected as indicated in Table 3 into a single right animal-dorsal blastomere of 8- to 16-cell stage embryos with (B-H) or without (I-M) nβgal mRNA. Whole-mount in situ hybridization has been carried out with antisense probes for *sox2* (B-F), *myoD* (G,H) and *nrp1* (I-M). Suppression of *sox2* was observed in cells injected with FrdMO (or DprMO) and nβgal RNA at stage 10.5 (C) or 13 (E,F). (C) The inset is shown on the right at higher magnification. CoMO-injected embryos at stage 10.5 (B), 13 (D) and 20 (I). (G,H) Lack of effect of FrdMO on *myoD* expression at stage 14. (I-K) *Nrp1* expression on the injected side is severely reduced in both anterior and posterior neural tube in FrdMO- and DprMO-injected embryos at stage 20. (L) The *nrp1* expression domain becomes narrow posteriorly, but expands anteriorly in the embryos injected with β-catenin morpholino (βcatMO). Morphology of an embryo injected with βcatMO is shown on the right. (M) The effect of FrdMO on *nrp1* is restored by Frodo RNA (see also Table 3). (B-H,M) Dorsal view. (I-L) Dorsal view (left), anterior view (right).

Table 2. The effect of FrdMO and DprMO on neural plate closure

Experimental groups	Dose/ embryo	*n**	Phenotypes (%) Normal	Open neural plate
Control MO	8 ng	45	93	7
FrdMO	8 ng	42	21	79
DprMO	4 ng	53	28	72
FrdMO+Frodo	8 ng+1 ng	40	62	38
FrdMO+βcat	8 ng+10 pg	42	7	93

Eight- to sixteen-cell stage embryos were injected with MOs and RNAs as indicated above into the right animal-dorsal blastomere. Phenotypes were scored morphologically at stages 18-20.

*Number of scored embryos.

The data were obtained from two separate experiments.

domain narrowed down at the posterior neural tube, it expanded anteriorly (Fig. 3J, left), indicating the anterior shift of cell fates. These embryos had enlarged forebrain, midbrain and the cement gland (Fig. 3L, right), consistent with previously published data (Heasman et al., 2000). Thus, the effect of βcatMO on *nrp1* is clearly different from the effects of FrdMO and DprMO, suggesting that the zygotic β-catenin function is not required for *nrp1* expression.

Selective downregulation of organizer markers in embryos depleted of Frodo and Dapper

The expression of Frodo and Dapper in the dorsal mesoderm (Cheyette et al., 2002; Gloy et al., 2002) suggests that they play a role in the formation and function of the Spemann organizer, a dorsal signaling center conserved in all vertebrates (Harland and Gerhart, 1997). As the neuroectoderm is adjacent to the organizer in the early embryo according to fate maps (Moody, 1987; Vodicka and Gerhart, 1995), deficient head development in FrdMO and DprMO-injected (FDM) embryos (Fig. 1) may be caused by either abnormal neuroectoderm development or impaired organizer. To discriminate between the two possibilities, we studied the organizer markers *chordin*, *Xnr3* and *goosecoid* (*gsc*) by in situ hybridization in embryos dorsally injected with FrdMO or/and DprMO (Fig. 4A; Table 4). Either morpholino significantly reduced *chordin* and *Xnr3* expression, whereas co-injection of both MOs resulted in a much stronger effect on marker expression, suggesting that head defect in FDM embryos is caused by impaired organizer. We note that *chordin* appears to be the marker that is most sensitive to the loss of Frodo and Dapper. Moreover both Frodo and β-catenin RNAs recovered *chordin* and *Xnr3* expression (Fig. 4) and suppressed head defects (Fig. 1) in FDM embryos, supporting the conclusion that Frodo and Dapper are essential activators of *chordin* and *Xnr3*. Surprisingly, *gsc* which is another organizer-specific gene and a target of the β-catenin pathway (Watabe et al., 1995), was not affected by FrdMO and DprMO (Fig. 4A). In fact, we occasionally observed a slight expansion of *gsc* in FDM embryos. By contrast, βcatMO strongly reduced the expression of all three organizer markers (Fig. 4A). These results show that the effect of FrdMO and DprMO on organizer genes is gene specific.

To extend these observations, we used RT-PCR to analyze several molecular markers, including *chordin*, *cerberus*, *Xnr3*, *siamois*, *gsc* and *vent1*, in embryos dorsally and ventrally injected with FrdMO and DprMO (Fig. 4B). These morpholinos synergistically reduced the expression of *chordin*, *Xnr3* and *cerberus* at both early and late gastrula stages. As further evidence of specificity, this effect was reversed in FDM embryos by Frodo RNA. By contrast, the dorsal Wnt target genes *gsc* and *siamois*, and the ventrolateral mesodermal marker *vent1*, were not affected by FrdMO and DprMO.. These results are consistent with the in situ hybridization data in Fig. 4A and further support the notion that Frodo and Dapper function in a gene-specific manner during organizer formation.

Table 3. The effect of FrdMO and DprMO on pan-neural and muscle markers

Gene	Experimental groups	Dose/embryo	Stage	*n**	Gene expression (%) Unchanged	Reduced Weakly	Strongly
sox2	Control MO	8 ng	10.5	30†	90	7	
sox2	FrdMO	8 ng	10.5	31	29	71	
sox2	DprMO	4 ng	10.5	34	44	56	
sox2	Control MO	8 ng	13	25	92	8	0
sox2	FrdMO	8 ng	13	31	13	32	55
sox2	DprMO	4 ng	13	29	38	24	38
sox2	Control MO	8 ng	19	14	100	0	0
sox2	FrdMO	8 ng	19	18	44	17	39
sox2	DprMO	4 ng	19	17	35	18	47
nrp1	Control MO	8 ng	20-21	175	95	5	0
nrp1	FrdMO	8 ng	20-21	265	6	37	57
nrp1	DprMO	4 ng	20-21	28	32	25	43
nrp1	FrdMO+Frodo	8 ng+0.5 ng	20-21	113	44	41	15
nrp1	FrdMO+Frodo	8 ng+1 ng	20-21	100	39	48	13
myoD	Control MO	8 ng	14	25	92	8	0
myoD	FrdMO	8 ng	14	37	78	14	8
myoD	DprMO	4 ng	14	34	79	6	15

MOs and RNAs were injected into a single right animal-dorsal blastomere of 8- to 16-cell stage embryos. Whole-mount in situ hybridization was carried out with the pan-neural markers *sox2* and *nrp1*, and the muscle marker *myoD*. The data obtained from two (*myoD*) or four (*sox2* and *nrp1*) separate experiments are shown.

*Number of scored embryos.

†3% of embryos injected with control MO showed expanded expression of *nrp1*.

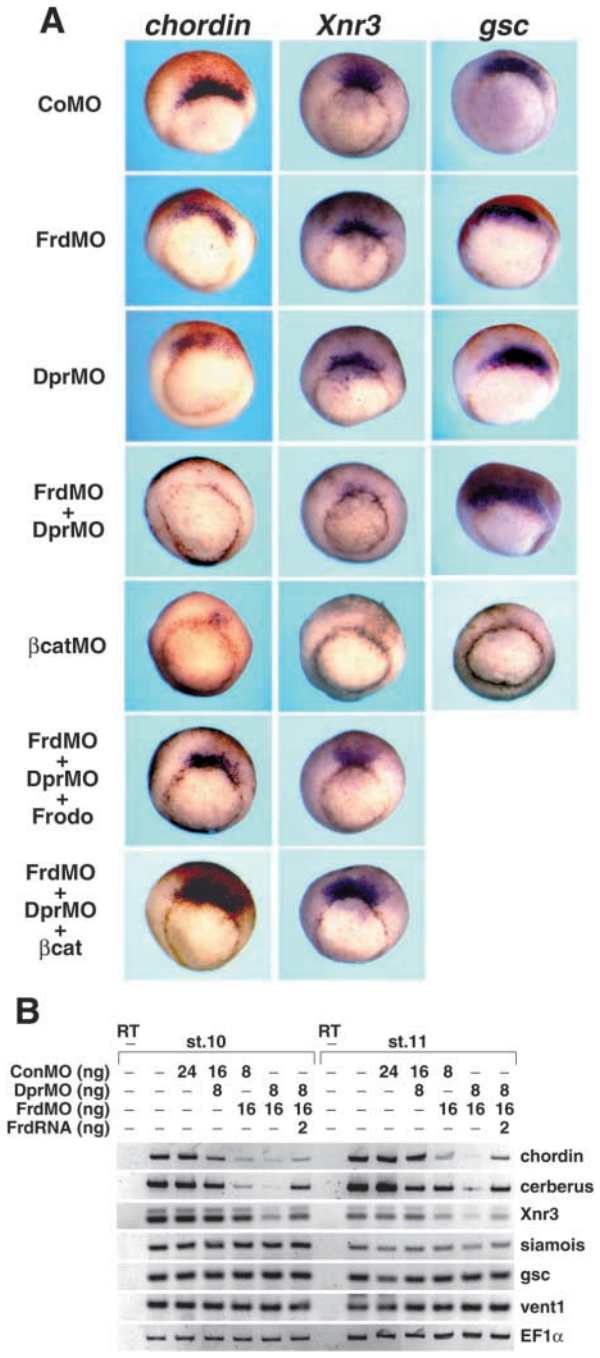

Fig. 4. The effect of FrdMO and DprMO on organizer markers. (A) Four-cell stage embryos were injected in the dorsal equatorial region of two blastomeres with morpholinos and mRNAs as indicated in Table 4, were cultured until stage 10.5 and were subjected to whole-mount in situ hybridization with organizer markers, *chordin* (left panels), *Xnr3* (middle panels) and *gsc* (right panels) as probes. Co-injection of FrdMO and DprMO can reduce *chordin* and *Xnr3* but not *gsc*, whereas βcatMO strongly inhibited expression of all three genes, implying that Frodo and Dapper function in a gene-specific manner. The reduction of *chordin* and *Xnr3* by co-injection of FrdMO and DprMO was reversed not only by Frodo RNA, but also β-catenin RNA. (B) Four-cell stage embryos were injected in the equatorial region of each blastomere with morpholinos and mRNAs as indicated and were analyzed at stage 10 and 11 by RT-PCR, using primers specific for *chordin*, *cerberus*, *Xnr3*, *siamois*, *gsc* and *vent1*. *EF1α* primers were used to control loading.

Frodo associates with TCF3 and is required for TCF-dependent, but not β-catenin-dependent, reporter activation

As organizer formation is thought to depend on early β-catenin function, we next examined the requirement of Frodo and Dapper in canonical Wnt/β-catenin signaling. Despite high sequence similarity, Frodo and Dapper have been shown to oppositely modulate Wnt signal transduction (Cheyette et al., 2002; Gloy et al., 2002). Thus, we directly compared the effects of Frodo and Dapper on a Wnt-dependent luciferase reporter (Fan et al., 1998). Consistent with our previous finding (Gloy et al., 2002), we observed that injections of either Frodo or Dapper RNA enhance Dsh-dependent activation of the reporter at 0.5 ng and 2 ng, whereas reporter activity is suppressed at 6 ng of both RNAs (Fig. 5A). Thus, both Frodo and Dapper synergize with Dsh at low and medium levels, but can act as inhibitors of Dsh signaling at high levels.

We next examined the effect of FrdMO and DprMO on β-catenin levels using an antibody that recognizes unphosphorylated β-catenin (Upstate Biotech). We found that β-catenin levels were decreased by injection of βcatMO, but not by injections of FrdMO and DprMO (Fig. 5B). This finding indicates that Frodo and Dapper influence target gene expression either downstream or parallel to β-catenin. Thus, the downregulation of *chordin*, *cerberus* and *Xnr3* in FDM embryos is unlikely to be caused by altered β-catenin levels.

Whereas Frodo associates with Dsh through its C-terminal region (Gloy et al., 2002), we noticed that both the C-terminal and the N-terminal domains of Frodo can act as Dsh antagonists. This suggests that these two domains associate with different components of Wnt signaling machinery. We then evaluated the possible association of HA-tagged Frodo and TCF3 fused with glutathione-S-transferase (GST-TCF3) in transfected mammalian COS7 cells. This GST pull-down assay demonstrated that Frodo specifically binds GST-TCF3, but not GST (Fig. 5C,D). Further analysis using Frodo deletion constructs revealed that GST-TCF3 binds the large N-terminal fragment of Frodo (Frd337), but not the smaller fragment Frd186 (Fig. 5C,D), implying that the conserved region of Frodo located between amino acids 186 and 337 is necessary for the association of Frodo and TCF3. The binding of the N-terminal region of Frodo and TCF3 has been also confirmed in *Xenopus* embryos using immunoprecipitation analysis (Fig. 5E). These observations indicate that Frodo interacts with both Dsh and TCF and implicate Frodo in Wnt signal transduction downstream of Dsh.

Based on the properties of the C-terminal Dsh-binding domain that acted in a dominant-negative manner, we previously concluded that Frodo acts at the level of Dsh (Gloy et al., 2002). As the N-terminal Frd337 fragment binds TCF, we tested whether this region of Frodo would interfere with signaling by TCF3-VP16, a construct in which TCF3 lacking the β-catenin binding region is fused to the transcriptional activator VP16 (Vonica et al., 2000). This construct is predicted to activate Wnt target genes independently of β-catenin. The N-terminal Frd337 fragment, but not Frd186, inhibited the ability of TCF3-VP16 to stimulate the pSia-luc reporter in a dose-dependent manner (Fig. 5F), suggesting that Frodo acts in the Wnt pathway at the level of TCF.

To determine the role for endogenous Frodo/Dapper in TCF signaling, we evaluated the effect of FrdMO and DprMO on

Table 4. The effect of FrdMO and DprMO on organizer markers

Gene	Experimental groups	Dose/embryo	*n**	Gene expression (%)		
				Unchanged	Reduced	
					Weakly	Strongly
chordin	Control MO	16 ng	67	91	9	0
chordin	FrdMO	16 ng	34	26	68	6
chordin	DprMO	8 ng	30	33	57	10
chordin	βcatMO	16 ng	40	5	30	65
chordin	FrdMO+DprMO	8 ng+4 ng	73	3	36	61
chordin	FrdMO+DprMO+Frodo	8 ng+4 ng+1 ng	55	29	36	35
chordin	FrdMO+DprMO+βcat	8 ng+4 ng+10 pg	65*	46	17	17
Xnr3	Control MO	16 ng	37	92	8	0
Xnr3	FrdMO	16 ng	38	71	26	3
Xnr3	DprMO	8 ng	37	81	16	3
Xnr3	βcatMO	16 ng	40	5	25	70
Xnr3	FrdMO+DprMO	8 ng+4 ng	48	27	42	31
Xnr3	FrdMO+DprMO+Frodo	8 ng+4 ng+1 ng	53	47	38	15
Xnr3	FrdMO+DprMO+βcat	8 ng+4 ng+10 pg	65†	60	9	8
gsc	Control MO	16 ng	49	88	12	0
gsc	FrdMO	16 ng	22	77	23	0
gsc	DprMO	8 ng	20	85	10	5
gsc	βcatMO	16 ng	30	10	20	70
gsc	FrdMO+DprMO	8 ng+4 ng	56‡	62	13	7

Four-cell stage embryos were injected in the dorsal equatorial region of two blastomeres with the indicated morpholinos and mRNAs, cultured until stage 10.5 and subjected to whole-mount in situ hybridization with antisense probes for the organizer markers *chordin*, *Xnr3* and *gsc*.

*Number of embryos scored.

†Expanded expression of *chordin* and *Xnr3* has been observed in 20% and 23% of injected embryos, respectively.

‡Slightly expanded *gsc* expression has been observed in 18% of injected embryos.

Data were obtained from three experiments.

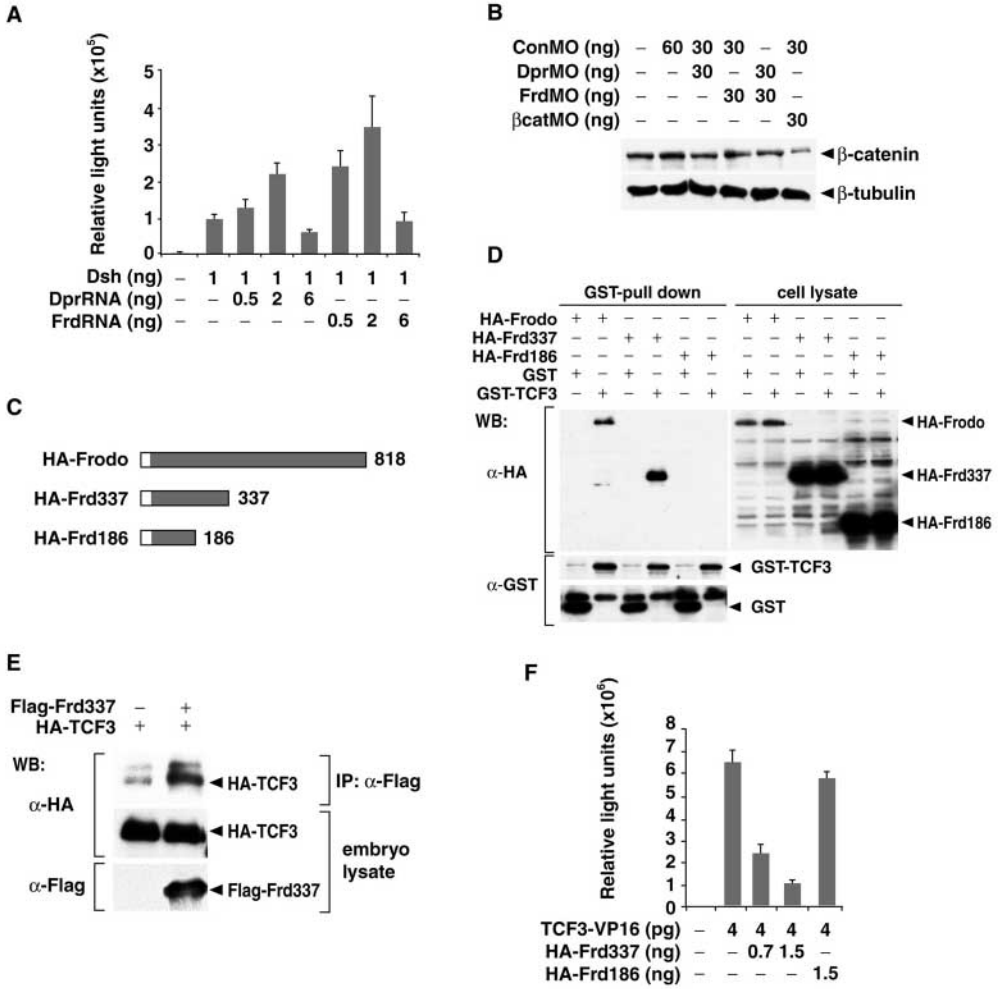

Fig. 5. Physical and functional interactions of Frodo and TCF3. (A) Dose-dependent effects of Frodo and Dapper RNA on the activation of pSiaLuc reporter by Dsh. (B) Levels of non-phosphorylated β-catenin are not changed in stage 10 embryos injected with FrdMO and DprMO in all blastomeres at the eight-cell stage. (C) Frodo constructs used for transfections and microinjections. (D) The association of GST-TCF3 with HA-Frodo and HA-Frd337, but not HA-Frd186, in COS7 cells, revealed in a GST pull-down assay. (E) TCF3 associates with Frodo in *Xenopus* gastrulae. Four- to eight-cell stage embryos were injected with 200 pg of HA-TCF3 RNA and 1 ng of Flag-Frd337 RNA. Protein complexes were immunoprecipitated with anti-Flag antibodies from gastrula stage lysates. (F) The activation of the pSiaLuc reporter by TCF3-VP16 is suppressed by HA-Frd337, but not by HA-Frd186.

the activity of TCF3-VP16 in the pSiaLuc reporter assay. Our results show that the activity of TCF3-VP16 was dramatically downregulated in FDM embryos, but it was not significantly affected by the control morpholino (Fig. 6A). This finding suggests that Frodo and Dapper are required for TCF-mediated transcription. We next tested if overexpression of Frodo influences the binding of β-catenin to TCF3. The amount of β-catenin precipitated with GST-TCF3 in GST pull-down assays was not significantly affected by Frodo (Fig. 6B), indicating that the TCF3-β-catenin and TCF3-Frodo complexes may be independently regulated.

Considering that Frodo/Dapper are required for TCF-dependent transcriptional activation, we further studied the effect of the morpholinos on the transcription activation properties of stabilized β-catenin. β-catenin-dependent luciferase activity was not significantly altered in FDM embryos (Fig. 6C). Surprisingly, we have also observed a slight, but consistent upregulation of reporter activity in response to injected Wnt8 RNA (Fig. 6D), although in the absence of Wnt8 stimulation no significant effect of the morpholinos on the reporter was detected (data not shown). Thus, whereas Frodo and Dapper inhibit Wnt8-dependent transcriptional responses, they are required for TCF-dependent reporter activation. Together, these findings suggest that Frodo and Dapper are involved in more than one step of the signaling cascade and perform different functions at different levels of signal transduction.

Discussion

Frodo and Dapper have been identified as the proteins that bind to Dishevelled and modulate Wnt signaling (Cheyette et al., 2002; Gloy et al., 2002). In this study we used the loss-of-function approach to compare the developmental roles of Frodo and Dapper and further define their mechanism of action. We find that the two proteins are required for head development and neural tissue development, and behave similarly with respect to target gene activation. Our data indicate that Frodo and Dapper regulate TCF-dependent transcriptional responses in a pathway parallel to that of β-catenin. Finally, we show that Frodo associates with TCF3, thereby linking Dsh with a downstream component of the Wnt pathway.

The requirement for Frodo and Dapper in neuroectoderm may reflect the early predisposition of dorsal ectoderm to neural and mesodermal fates observed by others (Sharpe et al., 1989; Sokol and Melton, 1991). As Frodo and Dapper morpholinos inhibit the expression of *chordin*, a gene implicated in neural induction (Wessely et al., 2001), it is possible the effect of FrdMO on neural development is due to the early suppression of *chordin*. However, this explanation is not very likely as the depletion of *chordin* results in the reduced *sox2* expression domain according to a previous study (Oelgeschlager et al., 2003), whereas *sox2* is virtually eliminated in embryos depleted of Frodo or Dapper (Fig. 3). Dorsal animal injection of a dominant interfering TCF construct was reported to reduce *nrp1* expression but did not have an effect on *muscle actin* (Baker et al., 1999). Taken together, these observations raise a possibility that Frodo/Dapper and TCF cooperate in neural tissue development.

Several observations argue that the suppression of pan-neural markers by the morpholinos is not a consequence of decreased organizer activity. First, the requirement of Frodo for neural marker expression can be observed already at early/midgastrula stages. Second, to avoid morpholino effects on the organizer the injections have been performed at the 8- to 16-cell stage into a single dorsal animal blastomere that predominantly contributes to ectodermal tissues. Lineage tracing experiments demonstrate that only the cells injected with the specific morpholinos were affected. If the morpholinos inhibited the organizer, non cell-autonomous effects would be expected. Third, despite profound neural marker defects, *myoD* expression did not change, indicating that mesodermal specification remains largely unaffected (Fig. 3G,H; Table 3). In these experiments, injection of βcatMO at the same location did not have a detectable influence on organizer markers (data not shown), further arguing that the injections were restricted to the responding ectoderm, as βcatMO efficiently inhibited organizer genes when supplied to the dorsal margin (Fig. 4). These observations suggest

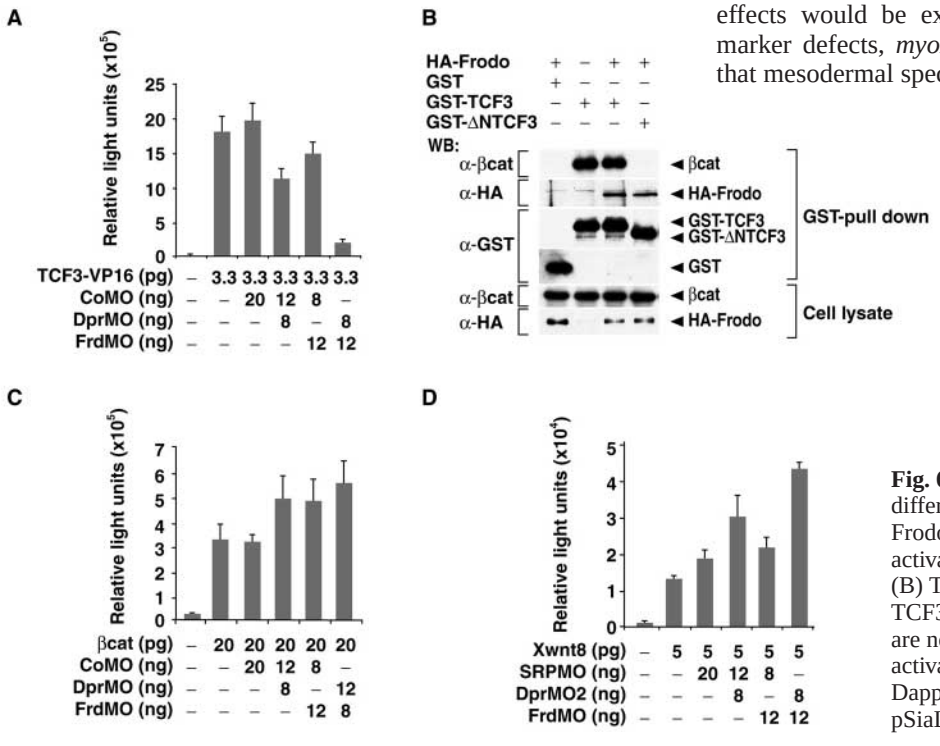

Fig. 6. The involvement of Frodo and Dapper in different steps of Wnt signal transduction. (A) Frodo and Dapper synergize with TCF3-VP16 to activate the pSiaLuc reporter in *Xenopus* embryos. (B) The amount of β-catenin associated with GST-TCF3 is not altered by Frodo. (C) Frodo/Dapper are not required for β-catenin-dependent reporter activation. (D) An inhibitory activity of Frodo and Dapper revealed in Wnt8-dependent stimulation of pSiaLuc.

that Frodo and Dapper are required for early neural development in the responding ectoderm.

Our results also argue that Frodo and Dapper are also needed for the proper function of the organizer, because dorsal marginal injection of Frd/Dpr morpholinos (FDM) significantly reduced organizer markers, such as *chordin* and *cerberus*, Wnt responsive genes (Sasai et al., 1994; Wessely et al., 2001), and *Xnr3*, a direct Wnt target (McKendry et al., 1997; Smith et al., 1995). In contrast to these genes, other targets of Wnt signaling, such as *siamois* and *gsc* (Cho et al., 1991; Watabe et al., 1995), were not affected in FDM embryos, suggesting that Frodo and Dapper are involved only in some aspects of Wnt signaling. The latter observation reinforces the idea of the heterogeneity of the organizer (Zoltewicz and Gerhart, 1997) and the conclusion that different organizer-specific genes are regulated by different molecular mechanisms (Hamilton et al., 2001).

The available evidence is consistent with the view that Frodo and Dapper are structurally and functionally related and play redundant roles during development. The effects of Frodo and Dapper morpholinos are very similar in the assays that we have conducted. Simultaneous injection of both morpholinos revealed significant synergy of Frodo and Dapper. The functional differences between Frodo and Dapper proposed in the early reports (Cheyette et al., 2002; Gloy et al., 2002) may be due to the doses or specific assays used. As Frodo is likely to play a scaffolding role in signal transduction, its effect on signaling is predicted to be dose-sensitive. In fact, we observed that at low doses Frodo and Dapper act synergistically with Dsh in both axis induction and reporter assays, whereas at higher doses they behave as antagonists (Fig. 5A) (Gloy et al., 2002). Moreover, our transcriptional assays (Fig. 6) reveal opposing roles for Frodo/Dapper at different levels of the signaling cascade. Although these proteins appear to be required for TCF-mediated transcriptional activation, they function as negative regulators for Wnt8-dependent responses. Detailed analysis of the molecular mechanisms involved warrants further studies.

Our data reveal significant functional differences between Frodo/Dapper and β-catenin. First, whereas Frodo and Dapper are required for *sox2* and *nrp1* expression, β-catenin does not seem to be necessary for pan-neural marker expression, although our data support its role in anteroposterior patterning of the neural tissue. Second, expression of organizer markers, including *chordin*, *Xnr3* and *gsc*, is reduced in β-catenin-depleted embryos, whereas only *chordin* and *Xnr3*, but not *gsc*, are affected in FDM embryos. Third, Frodo RNA, but not β-catenin RNA, can restore normal morphogenetic movements during gastrulation and neurulation. Finally, Frodo and Dapper morpholinos strongly suppress TCF-dependent stimulation of the pSiaLuc reporter, but not β-catenin-dependent stimulation of the reporter. These results allow us to propose a model in which Frodo transduces Wnt signals to target genes in a pathway parallel to that of β-catenin. Consistent with this model, we find that Frodo associates with both Dsh and TCF. The physical interaction of Frodo and TCF may provide an additional, β-catenin-independent control over TCF function. As Frodo is predominantly found in cell nuclei (data not shown), it may be involved in the direct activation of TCF-dependent transcription or derepression of TCF3. Future studies will be aimed at the elucidation of the molecular mechanism used by Frodo to upregulate TCF3 activity.

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