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 Ca2+ 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 (Chyette 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 SacI and KpnI 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 HindIII-NsiI and Xsl-XbaI 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 SpeI, and PstI and SpeI, respectively. pEBG-TCF3 was generated by subcloning the SacI-KpnI fragment of pT7TS-TCF3 (Molenaar et al., 1996) into pEBG (Sanchez et al., 1994). The deletion of the β-catenin-binding domain in pEBG-ANTCF3 was constructed from site-directed mutagenesis as described (Makarova et al., 2000) using the primer 5′-GAGCTCGGGGCTAACGACCTCGAGTCGGAGAA TCACAGC-3′; 3′-AATCTGCGAGCTGGAATTTC-3′; chordin, 5′-AAGCCTGGAAGGACACAGGA-3′; and 5′-GGCCAGATTGTTGCTGGA TA TGC-3′.

 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′-AACTGCCAGGACTGGA TGGT-3′; gsc, 5′-TCACCGATGAAACAACCTGGA-3′; and 5′-TTCCACATTGCCCATTTCCT-3′; vent1, 5′-CAGATCCTGGGATTGCATGC-3′; EF1α, 5′-CAGATTCGCTGATGATGC-3′; and 5′-ACTGC TTGGATGCCTAC-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 with 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 phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM NaN3]. Supernatants were cleared by centrifugation 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 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.

**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

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

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Dose/embryo</th>
<th>n*</th>
<th>Normal</th>
<th>Normal head</th>
<th>Microcephaly</th>
<th>Acephaly</th>
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<td>70</td>
<td>90</td>
<td>4</td>
<td>6</td>
<td>0</td>
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<tr>
<td>Frodo</td>
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<td>38</td>
<td>5</td>
<td>26</td>
<td>61</td>
<td>8</td>
</tr>
<tr>
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<td>50</td>
<td>3</td>
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<td>18</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td>FrdMO+DprMO+Frodo</td>
<td>4 ng+2 ng+1 ng</td>
<td>56</td>
<td>59</td>
<td>20</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>FrdMO+DprMO+10 pg</td>
<td>8 ng+4 ng</td>
<td>97</td>
<td>2</td>
<td>10</td>
<td>25</td>
<td>62</td>
</tr>
<tr>
<td>FrdMO+DprMO+Frodo</td>
<td>8 ng+4 ng+1 ng</td>
<td>65</td>
<td>22</td>
<td>35</td>
<td>38</td>
<td>5</td>
</tr>
<tr>
<td>FrdMO+DprMO+βcat</td>
<td>8 ng+4 ng+10 pg</td>
<td>61</td>
<td>5</td>
<td>67 (18)†</td>
<td>23</td>
<td>5</td>
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</tbody>
</table>

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 (L). (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).
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 b-catenin RNAs recovered 
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 embry 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 b-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 b-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, bcatMO 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

<table>
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<th>Gene</th>
<th>Experimental groups</th>
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<th>Strongly</th>
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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.

13% of embryos injected with control MO showed expanded expression of nrp1.
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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Experimental groups</th>
<th>Dose/embryo</th>
<th>n*</th>
<th>Gene expression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Unchanged</td>
</tr>
<tr>
<td>chordin</td>
<td>Control MO</td>
<td>16 ng</td>
<td>67</td>
<td>91</td>
</tr>
<tr>
<td>chordin</td>
<td>FrdMO</td>
<td>16 ng</td>
<td>34</td>
<td>26</td>
</tr>
<tr>
<td>chordin</td>
<td>DprMO</td>
<td>8 ng</td>
<td>30</td>
<td>33</td>
</tr>
<tr>
<td>chordin</td>
<td>βcatMO</td>
<td>16 ng</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>chordin</td>
<td>FrdMO+DprMO</td>
<td>8 ng+4 ng</td>
<td>73</td>
<td>3</td>
</tr>
<tr>
<td>chordin</td>
<td>FrdMO+DprMO+Frodo</td>
<td>8 ng+4 ng+1 ng</td>
<td>55</td>
<td>29</td>
</tr>
<tr>
<td>Xnr3</td>
<td>Control MO</td>
<td>16 ng</td>
<td>37</td>
<td>92</td>
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<td>FrdMO</td>
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<td>DprMO</td>
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<td>8 ng+4 ng</td>
<td>48</td>
<td>27</td>
</tr>
<tr>
<td>Xnr3</td>
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<td>8 ng+4 ng+1 ng</td>
<td>53</td>
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<tr>
<td>gsc</td>
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<td>49</td>
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<td>FrdMO+DprMO</td>
<td>8 ng+4 ng</td>
<td>30</td>
<td>10</td>
</tr>
</tbody>
</table>

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-neuronal 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...
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 (Zołtewicz 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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