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ORIGINAL ARTICLE

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Choice of either β-catenin or Groucho/TLE as a co-factor for Xtcf-3 determines dorsal-ventral cell fate of diencephalon during *Xenopus* development

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Abstract Co-repressor Groucho/Transducin-Like Enhancer of split (TLE) interacts with transcription factors that are expressed in the central nervous system (CNS), and regulates transcriptional activities. In this study, we examined the contribution of Groucho/TLE to CNS development in *Xenopus*. The functional inhibition of Groucho/TLE using the WRPW motif as a competitor resulted in the conversion of the ventral cell into the dorsal fate in the prospective diencephalon. We also found that the neural plate was expanded laterally without inhibiting neural crest development. In tailbud, the disturbance of trigeminal ganglion development was observed. These observations allow us to conclude that Groucho/TLE plays important roles in the induction and patterning of distinct CNS territories. We found that Xtcf-3 is involved in some of the patterning in these territories. We generated the variant of *Xtcf-3*, *Xtcf-3BDN-*, which is suspected to interfere with the interaction between endogenous Groucho/TLE and Xtcf-3. The transcriptional activation of the *Xtcf-3*-target genes in response to endogenous Wnt/β-catenin signaling by the overexpression of *Xtcf-3BDN-* led to a reduction of the ventral diencephalon. This result indicates that transcriptional repression by the Groucho/TLE-Xtcf-3 complex is important for ventral diencephalon patterning. This idea is supported by the finding that the overexpression of the dominant-negative form of *Xtcf-3* or *axil* causes the expansion of the ventral diencephalon. Based on these data, we propose that the localized activation of Wnt/β-catenin signaling, which converts Tcf from a repressor to an activator, is required for the establishment of dorsal-ventral patterning in the prospective diencephalon.

Keywords Dorsal-ventral · Diencephalon · Groucho · Tcf-3 · *Xenopus*

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Introduction

The central nervous system (CNS) is composed of a variety of discrete tissues, such as telencephalon, diencephalon, mesencephalon, metencephalon, myelencephalon and spinal cord, along the anterior-posterior (A-P) axis. These territories are divided along the dorsal-ventral (D-V) axis, which corresponds to the lateral-medial axis at the open neural stage (reviewed by Lumsden and Krumlauf 1996). The functional variation in these territories depends on structural variation in the CNS. In addition, these divisions of the brain reflect the initial regionalization of the neuroectoderm in the developing CNS. Therefore, analyses of the molecular mechanisms controlling CNS development are important for understanding higher brain function.

The CNS primordium arises from ectodermal cells and is specified along the A-P and D-V axes in response to inductive signals originating from the underlying dorsal endomesoderm. The mechanisms of A-P patterning in the CNS have been well-investigated in vertebrate embryos. The neuroectoderm is initially specified as an anterior neural tissue, and subsequently converted into the posterior tissue by such posteriorizing signals as FGF, RA and Wnts (Cox and Hemmati-Brivanlou 1995; Papalopulu and Kintner 1996; McGrew et al. 1995). Transplant studies and molecular marker analyses have shown that the initial A-P division of the neuroectoderm is established during gastrulation (reviewed by Gamse and Sive 2000). The mechanism regulating D-V patterning has been investigated mainly in the prospective spinal cord territory because of its sim-

ple structure. The cell fate along the D-V axis is known to be controlled by the interplay between dorsally derived factors, such as BMPs and ventrally derived factors, such as Shh (Liem et al. 1995; Echelard et al. 1993). Recent studies have indicated that D-V patterning of the forebrain at later stages is also regulated by a mechanism similar to that of the spinal cord (Furuta et al. 1997; Ericson et al. 1995; Shimamura and Rubenstein 1997) despite its complex structure and the difference in underlying tissues. However, the mechanism controlling the initial division of the prospective forebrain territory along the D-V axis is not fully understood.

Groucho was initially identified in *Drosophila* (Hartley et al. 1988). The ortholog of Groucho, Groucho/Transducin-Like Enhancer of split (Groucho/TLE), is found in a wide variety of species and expressed throughout development. Groucho/TLE does not have a DNA binding domain and functions as a co-repressor for specific subsets of transcription factors in several developmental processes such as segmentation, neurogenesis and sex determination (reviewed by Fisher and Caudy 1998). Because a large number of transcription factors, including Bf1, En, Nkx-class factors, Pax factors, Six factors and Tcf (Yao et al. 2001; Jiménez et al. 1997; Choi et al. 1999; Eberhard et al. 2000; Kobayashi et al. 2001; Cavallo et al. 1998), which are known to regulate forebrain development, have a conserved Groucho/TLE binding motif (Smith and Jaynes 1996), Groucho/TLE seems to be important for correct forebrain patterning as well. In addition, the expression domains of these transcription factors show partial overlaps in the prospective forebrain territory. Therefore, within the neuroectoderm, Groucho/TLE seems to act as a co-repressor in the same cells or territories where the transcription factors mentioned are at work. It is thus possible to elucidate the mechanism underlying forebrain development by analyzing Groucho/TLE function.

T-cell factor (Tcf) family proteins are downstream components of the Wnt/β-catenin signaling pathway (Behrens et al. 1996; Molenaar et al. 1996). Tcf alone has no transcriptional activity. In the absence of Wnt/β-catenin signaling, Tcf associates with Groucho/TLE and can act as a repressor (Cavallo et al. 1998; Roose et al. 1998). Upon signaling, Tcf, via its interaction with β-catenin, activates the transcription of the target genes (Behrens et al. 1996; Huber et al. 1996). Wnt/β-catenin signaling plays important roles at two distinct stages of the developing embryo. Whereas Wnt/β-catenin signaling before midblastula transition (MBT) is involved in body axis formation, after MBT, a gradient of Wnt/β-catenin signaling activity is required for pattern formation and regionalization of the neuroectoderm along the A-P axis (reviewed by Niehrs 1999; Yamaguchi 2001). For instance, the artificial activation of Wnt/β-catenin signaling by the overexpression of *Wnt-3*, *Wnt-8* or *β-catenin*, or by lithium exposure of the neuroectoderm after MBT, promotes the induction of the posterior markers and blocks the formation of the anterior CNS (McGrew et al. 1995, 1997; Bang et al. 1999; Fredieu et al. 1997). In addition, the analyses of several Wnt antagonists that are expressed in the anterior ectoderm and the underlying endomesoderm (Glinka et al. 1998; Leyns et al. 1997; Bouwmeester et al. 1996; Bradley et al. 2000; Pera and De Robertis 2000) have indicated that the inhibition of Wnt/β-catenin signaling in the anterior neuroectoderm is important for CNS patterning along the A-P axis. Interestingly, Wnt/β-catenin signaling has also been implicated in the D-V patterning of the neural tube. For example, the activation of Wnt/β-catenin signaling is necessary for the expression of dorsal-specific genes and cell proliferation in the dorsal region of the neural tube (Saint-Jeannet et al. 1997; Chang and Hemmati-Brivanlou 1998). In mouse, perturbation of Wnts gene expression results in loss or reduction of the dorsal neural structure in several CNS territories (Takada et al. 1994; Perry et al. 1995; Ikeya et al. 1997; Lee et al. 2000; Pinson et al. 2000; Muroyama et al. 2002). These studies suggest that Wnt/β-catenin signaling is also involved in the specification of dorsal cell fate in the CNS.

Here, we show that inhibition of the Groucho/TLE function by the tetrapeptide motif WRPW (Trp-Arg-Pro-Trp; Paroush et al. 1994; Fisher et al. 1996; Jiménez et al.1997) leads to expansion of the prospective diencephalon territory. We also show that this alteration of diencephalon patterning along the D-V axis is caused by the inhibition of the formation of the Groucho/TLE-Xtcf-3 complex. Based on these results, we propose a model where the subdivision within the diencephalon territory requires a gradient of Wnt/β-catenin signaling activity along the D-V axis that is established through Groucho/TLE-mediated repression.

Materials and methods

Embryonic manipulations

In vitro fertilization and embryo culture were carried out as described previously (Hawley et al. 1995). Embryos were staged according to Nieuwkoop and Faber (1956) and fixed with MEMFA.

Plasmid construction

To generate Myc-WRPW construct, oligonucleotides were designed (5′-AATTCTGGAGGTGAGTCCGTGTGGCGACCATGGTGAACTACCCTCC-3′ and 5′-TCGAGAGGGAGTTCACCATGGTCGCCACACGGACTCACCTCCAG-3′). The annealed oligonucleotides were subcloned into the *Eco*RI-*Xho*I site of pCS2+NLSMT. Plasmids encoding *Xgrg4*, *Xtcf-3BDN-*, *Xsix3BD-*, *axil* and *dnXtcf-3* were made by using PCR with the following primers: *Xgrg4* (F: 5′-CCCATGCATATGTACCCCCAGACCAGGCACCCG-3′, R: 5′-CCCGTCTAGACTAATAAATAACTTCATAGACTGTGGC-3′), *Xtcf-3BDN-* (F: 5′-CCCGAATTCCACCATGCTTGGAGGGCATT-3′, R: 5′-CCCCGGCGCGCCTCACATATTGGACTTCGT-3′), *Xsix3BD-* (F: 5′-CCCGGAATTCCACCATGGTGTTCAGGTCCCCTCTAG-3′, R: 5′-CCCCGGCGCGCCTCACCAGATGGTTCGGGGCAGA-3′), *axil* (F: 5′-CCCGAATTCCACCATGAGTAGCGCTGTGTTAGTGAC-3′, R: 5′-CCCGGCGCGCCTCAGTGGATCC

TCTCCACTTTGCCC-3′), *dnXtcf-3* (F: 5′-CCCGAATTC CACCATGCCCGGGGAGGGATCCGCC-3′, R: 5′-CCC GGCGCGCCTCAGTCACTGGATTTGGTCACC-3′). The PCR product was inserted into the *Cla*I-*Xba*I site of pCS2+ or *Eco*RI-*Asc*I site of pCS2AT+, which was constructed by insertion of annealed oligonucleotides (5′-TCGAGGGGCG CGCCGATATCTCTAGACGCCCTATAGTGAGTCGTAT TAC-3′ and 5′-GTAATACGACTCACTATAGGGCGTCTA GAGATATCGGCGCGCCC-3′) into the *Xho*I-*Sna*BI sites of pCS2+.

Microinjection

Capped RNA was transcribed using the mMessage mMachine (Ambion). To confirm the injected side, yellow fluorescent protein (YFP) mRNA was co-injected. The plasmids were linearized with *Not*I and transcribed with SP6 polymerase. Injected embryos were cultured to the appropriate stage in 0.1× MBS, fixed for in situ hybridization and sections.

Whole-mount in situ hybridization analysis and sectioning

In situ hybridization was performed following the methods of Harland (1991). Probes were labeled with digoxigenin (Roche) and stained with NBT/BCIP substrates (Roche). The markers used in this experiment were *Xbf-1* (Bourguignon et al. 1998), *Xen-2* (Hemmati-Brivanlou et al. 1991), *Xhairy2b* (Tsuji et al. 2003), *Keratin* (Jonas et al. 1985), *Xkrox20* (Bradley et al. 1993), *Xnkx2.4* (Small et al. 2000), *N-tubulin* (Chitnis et al. 1995), *Xotx2* (Blitz and Cho 1995; Pannese et al. 1995), *Xpax6* (Hirsch and Harris 1997), *Xrx1* (Casarosa et al. 1997), *Xslug* (Mayor et al. 1995), *Xsox2* (Mizuseki et al. 1998) and *Xvax2* (Barbieri et al. 1999). cDNAs of these genes were obtained by RT-PCR from total RNA of the neurula stage using primers based on sequence from GenBank. For sections, embryos were fixed, dehydrated, embedded in paraffin and sectioned in 10-μm slices using a microtome.

Results

Inhibition of Groucho/TLE induces expansion of neural plate size

To investigate whether the Groucho/TLE-mediated repression is involved in CNS development, we attempted to inhibit the Groucho/TLE function in *Xenopus*. It is known that the WRPW motif is necessary for mediating the interaction between Groucho/TLE and Hes- (Hairy and Enhancer of split-) related proteins (Paroush et al. 1994; Fisher et al. 1996). To confirm whether this motif acts as a competitive inhibitor of Groucho/TLE in vivo, we first co-overexpressed Myc-epitope-tagged WRPW (Myc-WRPW; Fig. 1A) with *Xgrg4* or *ESG1* (Choudhury et al. 1997; Molenaar et al. 2000). Myc-WRPW overcame the head defect caused by the overexpression of *Xgrg4* or *ESG1* (Fig. 1B, C and data not shown). These results suggest that Myc-WRPW is sufficient for inhibiting the Groucho/TLE function.

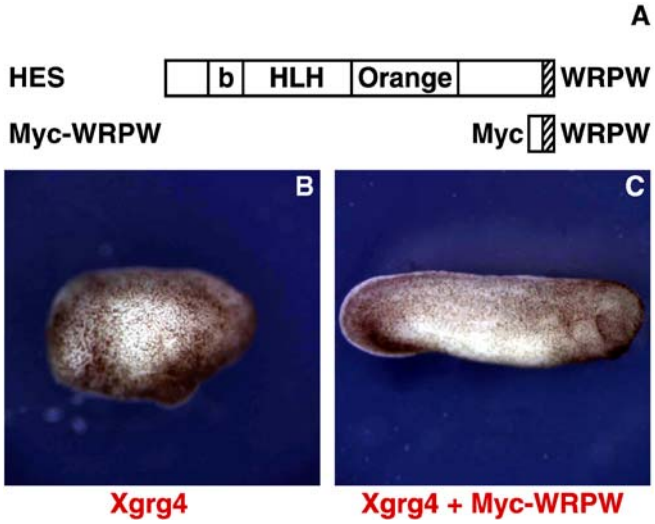

Fig. 1 Overexpression of Myc-WRPW can rescue head defect caused by Groucho/Transducin-Like Enhancer of split (Groucho/TLE). **A** A schematic diagram of the structure of the Myc-WRPW construct. The Hairy and Enhancer of split-related protein (*Hes*) consists of the bHLH domain, the Orange domain and the WRPW domain. *Filled boxes* represent the WRPW domains. **B**–**C** Lateral views. Anterior to the right. Two dorsal blastomeres at the 4-cell embryo stage were each injected equatorially with 1.6 ng *Xgrg4* mRNA alone (**B**; head defect in 66%, n =53) or with 1.6 ng *Xgrg4* and 800 pg Myc-WRPW mRNA (**C**; head defect in 3.3%, n =59)

In order to confirm that Groucho/TLE really functions in the neural patterning processes, embryos were injected with Myc-WRPW mRNA into the prospective neuroectoderm. Overexpression of Myc-WRPW caused an abnormal brain development that is associated with eye defects (91%, n =46), and this abnormality was rescued by *Xgrg4* (data not shown), suggesting that Groucho/TLE play important roles in *Xenopus* brain patterning. To further characterize the changes in neuroectoderm patterning observed in Myc-WRPW-injected embryos, the expression of neural markers was analyzed by using in situ hybridization. At the mid-gastrula stage (stage 11), the expression domain of *Xotx2*, an anterior neural plate marker, was expanded laterally (Fig. 2A and data not shown; n =12), while mesodermal expression of *Xotx2* and *chordin* was unaffected (Fig. 2A and data not shown). These observations indicate that the overexpression of Myc-WRPW leads to changes in ectodermal patterning without affecting that of the endomesoderm.

At the early neulura stage (stage 15) of the Myc-WRPW-overexpressed embryos, lateral expansion of the neural plate, marked by *Xsox2* (Fig. 2B, bracket; n =36), and reduction of the epidermis, marked by *Keratin* (Fig. 2C; n = 12), was observed. In the neural crest, the expression domains of *Xhairy2b* and *Xslug* were also shifted laterally from their original locations (Fig. 3G, H; n =48 and n =14, respectively). These results indicate that the inhibition of Groucho/TLE function results in the expansion of the neural plate without affecting neural crest development. There-

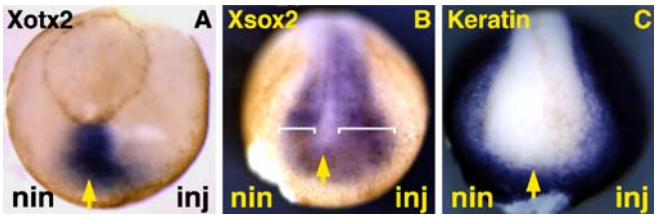

Fig. 2 Myc-WRPW overexpression leads to expansion of neural plate but not neural crest. **A**–**C** 2 ng Myc-WRPW mRNA was injected into one side of the dorsal animal blastomere of 8-cell-stage embryos. The injected embryos were analyzed by whole-mount in situ hybridization at the mid-gastrula stage (**A**, stage 11) and the early neurula stage (**B**–**C**, stage 15) with the following probes: *Xotx2* (**A**), *Xsox2* (**B**) and *Keratin* (**C**). Dorsal view with the anterior side *down* (**A**); anterior views with the dorsal side *up* (**B**–**C**). *Yellow arrows* delineate the midline of the neural plate. *White brackets* indicate the width of the neural plate

fore, Groucho/TLE may regulate initial neural plate border formation, which occurs before the cell-fate decision within the neuroectoderm.

Groucho/TLE influences D-V patterning in the prospective diencephalon territory

In order to examine the effects of Groucho/TLE inhibition on CNS patterning, we investigated the expression patterns of several neural marker genes in detail. At the early neurula stage (stage 15), the prospective mesencephalon region, the prospective isthmic region (*Xen-2*; Fig. 3E, red arrowheads; n =15) and the prospective rhombencephalon region (*Xkrox20*; Fig. 3E, pink arrowheads; n =21) were not significantly affected except for the expansion along the lateral-medial (dorsal-ventral after neural tube closure, D-V) axis. These results are consistent with our observation that the neural plate was expanded laterally (Fig. 2B, C). On the other hand, several alterations to forebrain regionalization were observed. The prospective telencephalon region (*Xbf-1*) was reduced but did not disappear completely (Fig. 3D; n =14). The prospective dorsal diencephalon territory (*Xotx2*; Eagleson and Dempewolf 2002; Fig. 3A; n =45) was expanded into the prospective ventral diencephalon territory (*Xrx1* and *Xvax2*; Fig. 3B, C; n =36, n =12, respectively). However, the anterior floor plate patterning (*Xnkx2.4* and *Xhairy2b*) was relatively unaffected (Fig. 3F, G, yellow arrowhead; n =17 and n =48, respectively). These results, which are summarized in Fig. 3J, show that the overexpression of Myc-WRPW strongly influences diencephalon patterning along the D-V axis. In accordance with these observations, the expression domain of *Xhairy2b* in the prospective forebrain-midbrain boundary (Fig. 3G, white arrowheads) and the expression domain of *Xpax6* in the prospective dorsal diencephalon seemed to be expanded medially (Fig. 3I; n =30). Taken together, these results indicate that the Groucho/TLE function is essential for the patterning of the prospective diencephalon along the D-V axis and the prospective telencephalon.

The alteration of the prospective diencephalon patterning seen at the neurula stage (Fig. 3A) appears to reflect an unusual *Xotx2* expression at the mid-gastrula stage (Fig. 2A). Animal cap and regional marker analyses have provided evidence that the prospective D-V boundaries in the telencephalon and spinal cord territory are already demarcated at the mid-gastrula stage (Zimmerman et al. 1993; Knecht et al. 1995; Knecht and Harland 1997; Chitnis et al. 1995; Ma et al. 1996; Kolm and Sive 1995; Lupo et al. 2002). Taken together, the D-V subdivision of the diencephalon may also occur at the mid-gastrula stage.

Alteration of neural plate patterning leads to eye defects

To further analyze the effect of the Groucho/TLE functional inhibition at later developmental stages, in situ hybridization was performed on a tadpole brain (stage 45). The *Xpax6* expression domain in the forebrain was significantly enlarged (Fig. 4A, brackets; n =20), whereas the expression

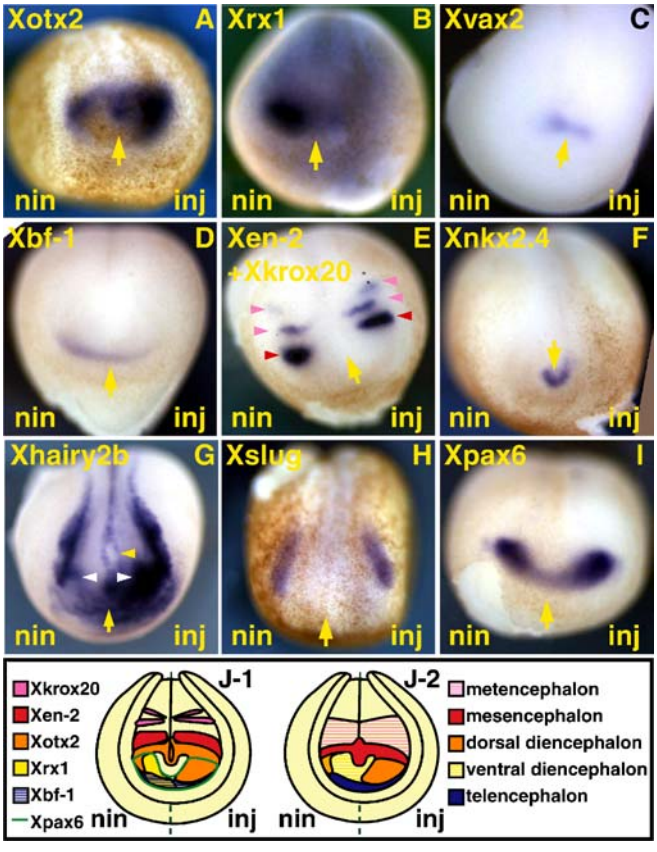

Fig. 3 Overexpression of Myc-WRPW induces expansion of the prospective dorsal diencephalon territory. **A**–**I** Myc-WRPW-injected embryos were analyzed by whole-mount in situ hybridization at the early neurula stage (stage 15) for the expression of *Xotx2* (**A**), *Xrx1* (**B**), *Xvax2* (**C**), *Xbf-1* (**D**), *Xen-2* (**E**, *red arrowheads*), *Xkrox20* (**E**, *pink arrowheads*), *Xnkx2.4* (**F**), *Xhairy2b* (**G**), *Xslug* (**H**) and *Xpax6* (**I**). *Yellow arrows* delineate the midline of the neural plate. **A**–**I** Anterior views with the dorsal side *up*. **G***Yellow arrowhead* indicates the floor plate; *white arrowheads* indicate the prospective forebrain-midbrain boundary. **J** A summary of the results shown in **A**–**E**. The color code of the gene expression domain is on the *left* (**J-1**). The color code of the prospective brain territory in the neural plate is on the *right* (**J-2**)

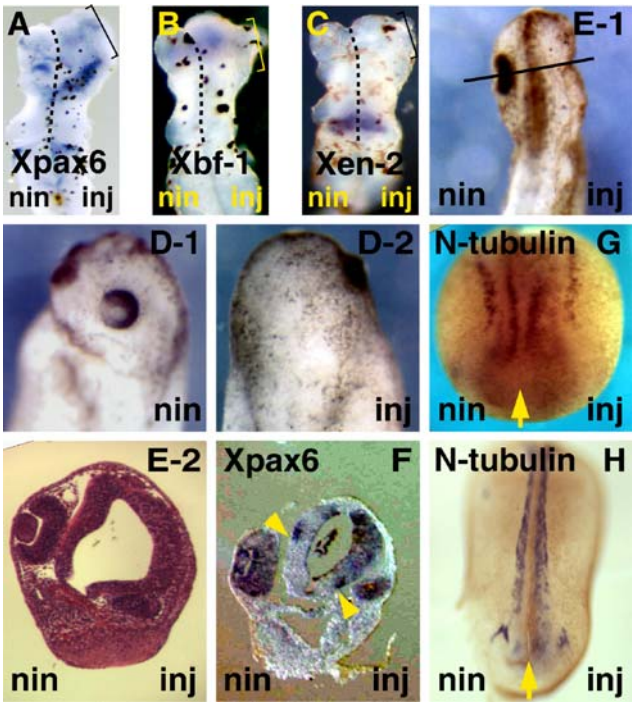

Fig. 4 Overexpression of WRPW causes diencephalon enlargement and an eye defect. **A**–**E** Embryos were injected with Myc-WRPW mRNA and allowed to develop to stage 45 (**A**–**C**), stage 36 (**D**–**F**), stage 15 (**G**) and stage 21 (**H**). In situ hybridization was performed with the following probes: *Xpax6* (**A**, **F**), *Xbf-1* (**B**), *Xen-2* (**C**) and *N-tubulin* (**G**, **H**). **A**–**C** Dorsal views of a dissected brain free of surrounding tissue. Anterior is *up*. *Brackets* indicate width of the expanding diencephalon tissue. *Broken lines* delineate the midline of the brain. **D** Lateral views with the anterior side *up*. Control side (**D-1**) and injected side (**D-2**) of embryos. Dorsal view with the anterior side *up* (**E-1**). The *line* in **E-1** indicates the plane of transverse section shown in **E-2** and **F**. The histological section is at the level of the diencephalon (**E-2**, **F**). Dorsal is *up*. *Yellow arrowheads* indicate the ventral limit of *Xpax6* expression in the dorsal diencephalon territory (**F**). Dorsal views with the anterior side *down* (**G**, **H**)

domains of other markers, such as *Xbf-1* and *Xen-2*, were unaffected (Fig. 4B, C; n =14 and n =11, respectively), indicating that only the diencephalon region is expanded in the tadpole. These results are consistent with the expansion of the prospective dorsal diencephalon territory observed at the early neurula stage (Fig. 3A). In contrast, although the reduction of the prospective telencephalon territory (*Xbf-1*) was observed at the early neurula stage (Fig. 3D), the same territory in the tadpole appeared normal. It has been reported that the stability of the regionalization in the neural plate depends on the axial mesoderm (Camus et al. 2000; Hallonet et al. 2002; Kiecker and Niehrs 2001). Therefore, the underlying mesoderm may influence the reduced prospective telencephalon territory after the neurula stage, resulting in the development of a normal telencephalon in the tadpole. However, the molecular mechanism underlying this recovery remains unknown.

Furthermore, a loss of eye formation (i.e., absence of optic cup and lens) was observed in the tailbud (stage 36; Fig. 4D). Histological analyses revealed that the dorsal diencephalon tissue, marked by *Xpax6*, was significantly enlarged, and the optic vesicle was deformed and displaced proximally to the neural tube (Fig. 4E, F). The enlarged dorsal diencephalon tissue might have pushed out the optic vesicle from its normal position, so that the optic vesicle could not contact the lens placode (reviewed in Ogino and Yasuda 2000). In addition, disturbance of the trigeminal ganglion development, marked by *N-tubulin*, was observed at the tailbud stage (stage 21; Fig. 4H; n =21), although the expression pattern of *N-tubulin* in the early neurula stage embryos was unaffected (Fig. 4G; n =18). Similar to the case of the eye defect, it is suggested that the deformation of the trigeminal ganglion may also be caused by changes in the composition of the head structure that affect the migration, rather than by the depletion, of the lineage cells. These observations indicate that the alteration of diencephalon patterning at the early gastrula stage is subsequently reflected in the eye defects and the deformation of the trigeminal ganglion at the later neurula stage.

Interaction between Groucho/TLE and Xtcf-3 is important for prospective diencephalon patterning

Our studies have indicated that Groucho/TLE-mediated transcriptional repression functions in the patterning of several territories in the neuroectoderm, such as the neural plate border, the telencephalon and the diencephalon. However, the transcription factor that is alone sufficient for regulating the patterning remains unknown. Therefore, various classes of transcription factors may be coordinately involved in the pattern formation by interacting with Groucho/TLE. In order to examine how the pattern formation of these territories is established, we analyzed several transcription factors (*Xtcf-3*, *Xsix3* and *Xoptx2*) that act as repressors when bound to Groucho/TLE (Roose et al. 1998; Kobayashi et al. 2001; Zhu et al. 2002). Briefly, we constructed variants of these factors (*Xtcf-3BD-*, *Xsix3BD-* and *Xoptx2BD-*) which lack the C-terminal domain including the DNA binding domain. It was expected that the overexpression of these variants would activate a specific transcriptional pathway by interfering with the interaction between the corresponding transcription factor and Groucho/TLE. This is a well-known strategy for making a specific competitor and has been adopted for analyses with Sox2 and FoxD3 (Kishi et al. 2000; Sasai et al. 2001). However, because *Xtcf-3BD-* retains the β-catenin binding domain, this variant may also inhibit the interaction between Xtcf-3 and β-catenin. Therefore we also constructed a variant lacking both the DNA binding domain and the β-catenin binding domain. Since this Xtcf-3 variant (Xtcf-3BDN-) was found to induce the expression of -357*Xtwin*/Luc (fourfold and data not shown), a construct containing the Wnt responsive element (Laurent et al. 1997), and to overcome the head defect caused by *Xgrg4* overexpression (data not shown), it is confirmed that *Xtcf-3BDN-* can be used as an inhibitor of Groucho/TLE functions.

The overexpression of *Xsix3BD-* or *Xoptx2BD-* in the neuroectoderm caused eye defects (54%, n =94; Fig. 5B and data not shown) without affecting the D-V patterning

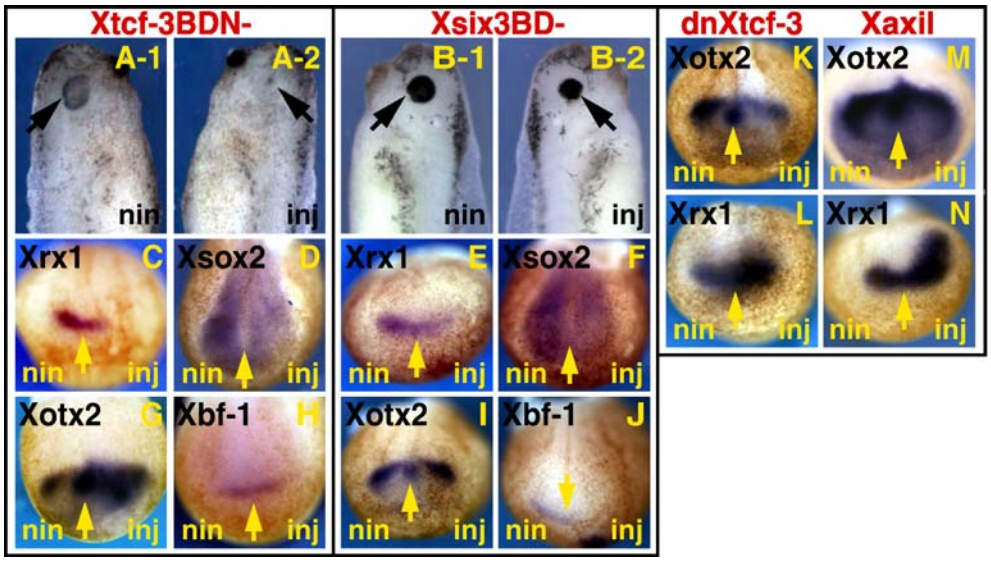

Fig. 5 Inhibition of *Xtcf-3* affects diencephalon patterning along the D-V axis. Two ng of *Xtcf-3BDN*- mRNA (**A**, **C**, **D**, **G**, **H**), 2 ng *Xsix3BD*- mRNA (**B**, **E**, **F**, **I**, **J**), 200 pg *dnXtcf-3* plasmid DNA (**K**, **L**) or 40 pg *axil* plasmid DNA (**M**, **N**) was injected into one side of the dorsal animal blastomere of an embryo at the 8-cell stage. *Yellow arrows* delineate the midline. **A**, **B** Lateral views with the anterior side *up*; control side (**A-1**, **B-1**) and injected side (**A-2**, **B-2**) of embryos. *Black arrows* indicate the eye. Note that the eye was reduced in the *Xtcf-3BDN*-injected embryos (**A-2**) and the *Xsix3BD*-injected embryos (**B-2**). **C**–**N** Anterior views with the dorsal side *up*. In situ hybridization was performed with the following probes at the early neurula stage (stage 15): *Xrx1* (**C**, **E**, **L**, **N**), *Xsox2* (**D**, **F**), *Xotx2* (**G**, **I**, **K**, **M**) and *Xbf-1* (**H**, **J**)

of the diencephalon. Similar results have been reported in experiments in which the constitutively active form of *Xsix3* or *Xoptx2* was overexpressed in *Xenopus* and zebrafish embryos (Zuber et al. 1999; Kobayashi et al. 2001), suggesting that *Xsix3BD*- and *Xoptx2BD*- act as inhibitors of *Xsix3*- and *Xoptx2*-mediated repression. *Xsix3BD*- or *Xoptx2BD*- also caused the reduction of the neural plate (*Xsox2*; Fig. 5F and data not shown; n =15) and the prospective forebrain territories (*Xrx1*, *Xotx2* and *Xbf-1*; Fig. 5E, I, J, and data not shown; n =12, n =12 and n =16, respectively). The analyses of mutant mouse demonstrated that Six3 is required for repressing Wnt1 expression in the anterior neuroectoderm (Lagutin et al. 2003). This mutant embryo lacked telencephalon and an eye field. The phenotypical similarities between mutant mouse and *Xenopus* embryos injected with *Xsix3BD*- or *Xoptx2BD*- suggest that these two variants inhibited Six-family-mediated Wnt repression, resulting in the posteriorization in the anterior neuroectoderm.

The overexpression of *Xtcf-3BDN*- caused eye defects, as well (80%, n =49; Fig. 5A). Unlike *Xsix3BD*- or *Xoptx2BD*-, however, *Xtcf-3BDN*- induced the expansion of the prospective dorsal diencephalon territory into the ventral diencephalon territory (compare *Xrx1*, *Xotx2* in Fig. 5C, G with those in Fig. 3A, B; n =26 and n =20, respectively), and the expansion of the neural plate (*Xsox2*; Fig. 5D; n =19). These features in the expression patterns are similar to those obtained by the overexpression of Myc-WRPW. In addition, as *Xgrg4* could rescue the eye defects caused by the Myc-WRPW, the eye defect caused by *Xtcf-3BDN*- was also rescued by co-injection of *Xgrg4* (data not shown). Taken together, these observations indicate that Xtcf-3 is a candidate for the essential regulator of patterning in the neural plate and D-V diencephalon. For telencephalon patterning, however, Xtcf-3 is unlikely to be involved in its regulation, because a reduction of the prospective telencephalon territory was not observed with overexpression of *Xtcf-3BDN*- (*Xbf-1*; Fig. 5H, compare with Fig. 3D; n =13).

We therefore focused on the function of Xtcf-3 in diencephalon patterning along the D-V axis. It is known that Tcf proteins are converted into transcriptional activators in the presence of β-catenin (Molenaar et al. 1996). Because *Xtcf-3BDN*- is likely to promote the interaction between endogenous Tcf3 and β-catenin by depleting Groucho/TLE that is specifically bound to Xtcf-3, we hypothesized that the Wnt/β-catenin signaling pathway is activated in the prospective diencephalon territory. To prove this hypothesis, we used the dominant-negative form of *Xtcf-3*, *dnXtcf-3* (Molenaar et al. 1996). To avoid disturbance of early neural formation, we injected the pCS2AT-dnXtcf-3 plasmid into the prospective neuroectoderm to inactivate Wnt/β-catenin signaling after MBT (Baker et al. 1999). The overexpression of *dnXtcf-3* led to a reduction of the prospective dorsal diencephalon territory (*Xotx2*; Fig. 5K, n =34) and an expansion of the prospective ventral diencephalon territory (*Xrx1*; Fig. 5L; n =33). These observations suggest that the repression of Xtcf-3-mediated transcription results in opposite features of D-V diencephalon patterning (31%, n =229) obtained through the overexpression of *Xtcf-3BDN*-. In addition, the overexpression of *axil*, which is a negative regulator of the Wnt/β-catenin signaling pathway, also causes alteration of the diencephalon patterning (37%, n =180), similar to those observed with the overexpression of *dnXtcf-3* (Fig. 5M, N; n =19 and n =12, respectively). These observations support the

importance of the relationship between Tcf-dependent Wnt/β-catenin signaling and Groucho/TLE in prospective diencephalon patterning along the D-V axis.

Discussion

Role of Wnt/β-catenin signaling in anterior neural plate

Recent studies have shown that the Wnt-signaling-free zone is maintained by Wnt antagonists within the anterior neuroectoderm (Glinka et al. 1998; Leyns et al. 1997; Bouwmeester et al. 1996; Bradley et al. 2000; Pera and De Robertis 2000; Kiecker and Niehrs 2001; Michiue et al. 2004). Furthermore, the activation of Wnt/β-catenin signaling after MBT causes a loss of the anterior head structure (Bang et al. 1999; McGrew et al. 1995, 1997; Fredieu et al. 1997). On the other hand, our data suggest that the alteration of the diencephalon D-V patterning is due to the transcriptional activation of the Xtcf-3-regulated genes in response to the endogenous Wnt/β-catenin signaling in the anterior neuroectoderm. However, the prospective telencephalon territory was not significantly reduced by overexpression of *Xtcf-3BDN-* (Fig. 5H) suggesting that the anterior neuroectoderm was not posteriorized. Thus, a weak Wnt/β-catenin signal, which is not sufficient to cause posteriorization, may exist in the anterior neuroectoderm. This idea is supported by the observation of low activation of Wnt-responsive reporter constructs such as Top-Flash within the anterior neuroectoderm (Kiecker and Niehrs 2001). Various roles of Wnt/β-catenin signaling in vertebrate development are known. However, why is its role in diencephalon D-V patterning unknown to date? We think it is because most roles of Wnt/β-catenin signaling known to date were determined by inhibition or forced activation of its pathway, and therefore, the mechanisms that are regulated by a slight change of the signaling activity might have been overlooked. In this study, we propose a new mechanism of diencephalon D-V patterning by depleting the inhibitory factor of endogenous Wnt/β-catenin signaling. In fact, overexpression of *axil* or *dnXtcf-3* after MBT resulted in a reduction of the more posterior brain territory in addition to the alteration of the diencephalon territory (Fig. 5K–N and data not shown), because they also repressed the transcription of Xtcf-3-regulated genes regardless of whether the Wnt/β-catenin signaling activity existed or not.

Prospective diencephalon patterning model

At the neural tube level, recent studies have indicated the importance of Wnt/β-catenin signaling that originates from the adjacent ectoderm and mesoderm for the dorsalization of neural tissue (Saint-Jeannet et al. 1997; Chang and Hemmati-Brivanlou 1998). In this study, we showed for the first time that the regulation of Xtcf-3-mediated transcription by Groucho/TLE and Wnt/β-catenin signaling is also important for D-V patterning in the diencephalon territory. Because *Groucho/TLE* and *Xtcf-3* are ubiquitously expressed in the anterior neuroectoderm (Choudhury et al. 1997; Molenaar et al. 1998, 2000), this transcriptional regulation in the prospective diencephalon territory may depend on Wnt/β-catenin signaling specific for the dorsal region. It is therefore possible that similar molecular mechanisms regulate the diencephalon D-V patterning. However, it remains unknown how the dorsal-specific activation of Wnt/β-catenin signaling is established in this territory. The gradient of Wnt/β-catenin signaling along the A-P axis in the neuroectoderm occurs in a direct and long-range fashion from the mesoderm and posterior ectoderm (McGrew et al. 1997; Kiecker and Niehrs 2001; Dorsky et al. 2003; Nordstrom et al. 2002). Although several type-1 Wnts, including *Xwnt-1*, *Xwnt-3A* and *Xwnt-8*, are expressed in the anterior neuroectoderm, no dorsal-specific expression is detected in the prospective diencephalon territory. Accordingly, the D-V gradient of Wnt/β-catenin signaling may be established indirectly through the interaction with the underlying mesoderm and the adjacent non-neural ectoderm (Fig. 6). Furthermore, several recent studies have shown that the interplay between Wnts and Wnt antagonists influences the neural plate patterning. Because some Wnt antagonists (such as *frzb*) are expressed in the mesoderm underlying the prospective ventral diencephalon territory (Leyns et al. 1997; Pera and De Robertis 2000), the D-V patterning may also be regulated by the interplay with these factors (Fig. 6). However, the overexpression of *Xtcf-3BDN-* caused moderate expansion of the dorsal diencephalon territory compared with that of Myc-WRPW. Because it is known that Groucho/TLE can interact with multiple transcription factors, several factors including *Xtcf-3* may participate in these mechanisms of neural regionalization. Thus, the inhibition specific for the Xtcf-3-mediated transcription may not completely mimic the inhibitory effects of Groucho/TLE caused by WRPW. Several alterations in the patterning of other forebrain territories were also caused by the functional inhibition of Groucho/

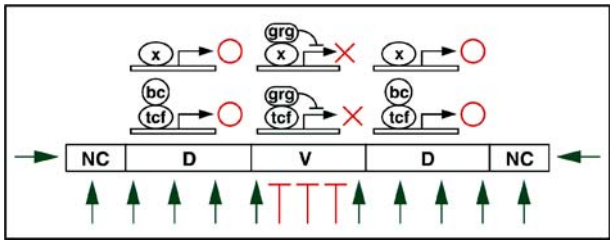

Fig. 6 Model for roles of Groucho/TLE and Wnt/β-catenin signaling in the patterning of prospective diencephalon. The dorsal activity of Wnt/β-catenin signaling is established in the neuroectoderm. In the prospective ventral diencephalon territory (*V*), mesoderm-derived Wnt antagonists suppressed Wnt/β-catenin signaling activity (*red antagonistic arrows*). Therefore, Xtcf-3 (*tcf*) and other Groucho/TLE-binding transcription factors (*X*) interact with Groucho/TLE (*grg*), and repress the transcriptional activation of the target genes. On the dorsal side of the prospective diencephalon (*D*), high activity of Wnt/β-catenin signaling is maintained by diffusing Wnts from the non-neuroectoderm and the mesoderm (*green arrows*). The expression of the dorsal-specific genes may be induced by the interaction between β-catenin (*bc*) and Tcf or other transcription factors. *NC* prospective neural crest

TLE. In order to investigate how the forebrain patterning is regulated at the gastrula stage, it will be necessary to identify other Groucho/TLE binding factors that the overexpression of WRPW inhibits.

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