MOLECULAR AND CELLULAR BIOLOGY, Jan. 2007, p. 340–351 Vol. 27, No. 1
0270-7306/07/$08.00+0 doi:10.1128/MCB.00811-06

Gbx2 and Otx2 Interact with the WD40 Domain of Groucho/Tle Corepressors▽‡

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Received 8 May 2006/Returned for modification 18 July 2006/Accepted 27 September 2006

One of the earliest organizational decisions in the development of the vertebrate brain is the division of the neural plate into *Otx2*-positive anterior and *Gbx2*-positive posterior territories. At the junction of these two expression domains, a local signaling center is formed, known as the midbrain-hindbrain boundary (MHB). This tissue coordinates or "organizes" the development of neighboring brain structures, such as the midbrain and cerebellum. Correct positioning of the MHB is thought to depend on mutual repression involving these two homeobox genes. Using a cell culture colocalization assay and coimmunoprecipitation experiments, we show that engrailed homology region 1 (eh1)-like motifs of both transcription factors physically interact with the WD40 domain of Groucho/Tle corepressor proteins. In addition, heat shock-induced expression of wild-type and mutant *Otx2* and *Gbx2* in medaka embryos demonstrates that Groucho is required for the repression of *Otx2* by Gbx2. On the other hand, the repressive functions of Otx2 on *Gbx2* do not appear to be dependent on corepressor interaction. Interestingly, the association of Groucho with Otx2 is also required for the repression of *Fgf8* in the MHB. Therefore Groucho/Tle family members appear to regulate key aspects in the MHB development of the vertebrate brain.

Gbx2 is a member of the homeobox gene family and has been identified in mammalian, avian, amphibian, and fish species (12, 24, 36, 56, 66). *Gbx* genes are related to the *Drosophila unplugged* gene, which is involved in tracheal branching (18). *Gbx2* is a key player in the early patterning of the vertebrate brain and is expressed in the local signaling center known as the midbrain-hindbrain boundary (MHB) or isthmic organizer, which is positioned between the presumptive midbrain and hindbrain (reviewed in references 34, 54, 59, and 70). The *Gbx2* expression domain is located at the region of the hindbrain, while the homeobox gene *Otx2* is expressed in the presumptive forebrain and midbrain and thereby forms a common border with the *Gbx2* domain at the position of the prospective MHB. *Gbx2* mutant mice lack the anterior hindbrain and reveal a posterior expansion of the midbrain (67). In contrast, the anterior brain rostral to rhombomere 3 is deleted in Otx2-null mutant mice (3, 44). Misexpression of *Gbx2* represses *Otx2* expression in the posterior midbrain (46, 66), whereas misexpression of *Otx2* in the anterior hindbrain represses *Gbx2* expression in this region (11, 35). Studies in *Xenopus* suggest that Otx and Gbx proteins needed for the positioning process function primarily as repressors rather than activators (28).

Tle4 is one of the four full-length Groucho proteins in mammals (39, 63). The founding member of this conserved corepressor family is the *Groucho* gene of *Drosophila*. *Groucho* is known to play important roles in various developmental processes, including sex determination, segmentation, neurogenesis and dorsoventral patterning (22, 50). Groucho family members are characterized by a conserved N-terminal glutamine-rich region (Q domain) and a conserved C-terminal WD40 repeat domain (reviewed in references 15 and 26). The WD40 repeat domain binds to a variety of proteins (21, 32, 65). The Q domain is required for the homotetramerization of Groucho (17, 51, 62) but is also involved in several protein-protein interactions (9, 23, 43, 55). The central region of Groucho contains three less-well-conserved domains, the GP domain, the CcN domain, and the SP domain. The GP region is important for interactions with histone deacetylase HDAC1 (9, 16, 19), which results in a repressive chromatin structure, thus explaining the corepressor function of full-length Groucho proteins. In contrast, a truncated member of the family, *Aes* (amino-terminal enhancer of split) contains Q and GP domains but lacks WD40 repeats and does not interact with HDAC1 (9). When expressed in the same cell, inactive heteromultimers involving Aes proteins and full-length Groucho family members form. This dominant-negative function of Aes on Tle-mediated repression has been demonstrated in both in vitro and in vivo systems (6, 43, 57).

A number of developmentally important transcription factors contain a small peptide motif that was first identified in

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‡ Supplemental material for this article may be found at http://mcb.asm.org/.

▽ Published ahead of print on 23 October 2006.

Engrailed proteins. This eh1 (engrailed homology region 1) motif is highly conserved through evolution and was initially characterized as a transcriptional repressor element (61). Groucho corepressor proteins were then determined to interact with eh1 motifs via WD40 domains located at their C termini (32). eh1-mediated interactions control various developmental processes, such as the dorsoventral polarization of the neural tube. Here, repression by Nkx proteins requires the association of Tle proteins with eh1-like motifs of these homeodomain factors (19, 48). Pax proteins also exert transcriptional repression by recruiting Tle family members via their so-called octapeptide, which shows extensive sequence similarity to the eh1 motif (23). On the other hand, Tcf/Lef transcription factors, the nuclear targets of canonical Wnt signaling, were shown to interact with the N-terminally located Q domain of Groucho proteins (57).

Contiguous expression and mutual repressive interactions of *Otx2* and *Gbx2* in the early embryo define the position of the MHB. During further development of this organizing center, the Groucho interaction partners *Engrailed*, *Pax2/5/8*, and *Tcf/Lef* play key roles. In this report, we show that also Otx2 and Gbx2 are associated with Groucho proteins. Using pull-down, coimmunoprecipitation, and colocalization assays, we demonstrate that Gbx2 is able to interact with Tle4 in vitro as well as in vivo. Similar to Otx2, Gbx2 contains an eh1-like motif, and we show here that both elements physically interact with the WD40 repeat domain of Tle4. Using a heat-inducible promoter for misexpression we found that these eh1 motifs function as Groucho-dependent repression domains in the MHB of medaka embryos. Together, these data indicate that Gbx2 as well as Otx2 can function as active repressors by recruiting corepressors of the Groucho/Tle family, thus offering a molecular explanation of how these homeodomain proteins repress the transcription of target genes in an essential organizer during vertebrate brain development.

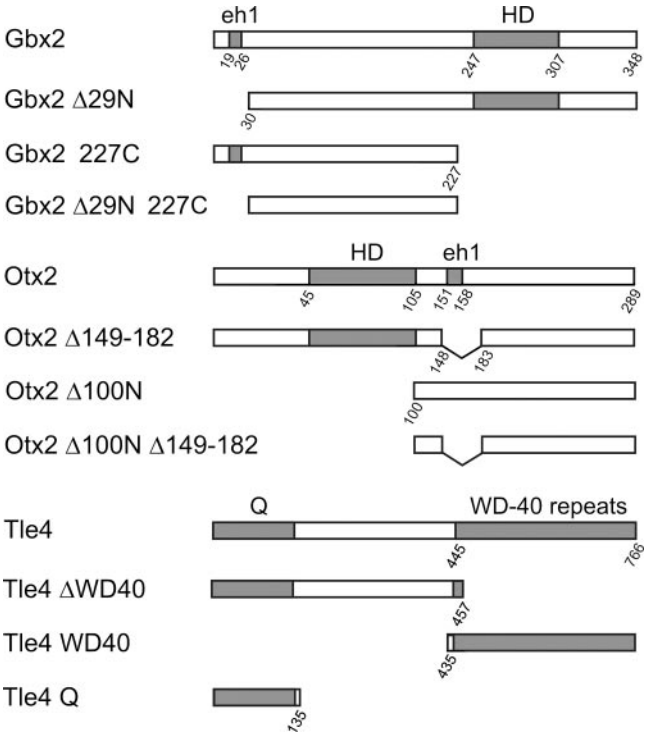

FIG. 1. Schematic diagram of the transcription factors Gbx2, Otx2 and the corepressor Tle4. The structures of wild-type and deletion mutant variants are illustrated. The different domains of each protein are indicated together with the corresponding amino acid positions. Gbx2 and Otx2 contain a DNA binding homeodomain (HD) and an eh1 motif involved in Groucho interaction. Tle4 includes two highly conserved N- and C-terminal domains (Q domain and WD40 repeat domain).

MATERIALS AND METHODS

DNA constructs. For transient reporter assays, the expression vector pMC (25) was used; for other cell culture experiments pKW10 (4) or pKC (polylinker modification of pKW) vectors (all three vectors contain cytomegalovirus promoters) were used. For all experiments, mouse Tle4, mouse Gbx2, and mouse Otx2 cDNAs were used. Deletion mutants were generated by PCR, except for Otx2 with a deletion of residues 149 to 182 (Otx2 Δ149–182) (52), which was subcloned from existing Otx2 constructs. For coimmunoprecipitation experiments, an N-terminal Flag tag and 18 copies of a myc tag were added. Green fluorescent protein (GFP) and DsRed were also fused N terminally. The glutathione *S*-transferase (GST)–Tle4 construct was described previously (23). The F65E mutation (61) in En2 was introduced by PCR.

A medaka Gbx2 fragment was amplified by PCR from cDNA of 24- to 48-h medaka embryos with the primers 5′-GGAATTCGCAAGAAGTACCTGTCC TTA-3′ and 5′-GCGAGAAGCTTCAGAGCTGCAACAGTTTAA-3′. For heat shock-induced expression in the embryos, a heat shock element (HSE)-driven promoter was used (7).

Cell lines and cell transfection assays. Monkey COS-7 kidney cells, human U2-OS osteosarcoma cells, and mouse NIH 3T3 fibroblasts were transfected in 24-well plates using polyethylenimine (8). For luciferase reporter assays, 300 ng of the Gal4-responsive construct *lGC-luc* (10) and 50 ng of the *Renilla* luciferase expression plasmid *phRG-TK* (Promega) were transfected. Luciferase activities were measured 48 h after transfection with a Dual-Luciferase reporter assay (Promega). The activity of firefly luciferase was standardized relative to the reference level of *Renilla* luciferase to normalize for transfection efficiencies. The expression levels of Gal4 fusion constructs were analyzed by Flag tag detection in Western blotting. The cellular extracts were obtained from upscaled transient transfection experiments in six-well plates. For colocalization assays, U2-OS cells were transfected with Lipofectamine 2000 (Invitrogen) according to the supplier's instructions. Cells were analyzed 48 h after transfection.

Coimmunoprecipitation and GST pull-down assays. Transiently transfected COS-7 cells were lysed after 48 h in buffer A (20 mM Tris-HCl, pH 7.9, 120 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.2% NP-40, 1 mM dithiothreitol, and 10% glycerol) and supplemented with a protease inhibitor cocktail (Sigma) and 1 mM phenylmethylsulfonyl fluoride. Lysates were incubated for 30 min on ice, cleared of cellular debris by centrifugation, and subsequently mixed with 20 μl of anti-Flag M2 affinity beads (Sigma) for 2 h at 4°C under constant rotation. Beads were extensively washed with buffer A. For coimmunoprecipitation of Tle4 with Gbx2 and En2, the concentration of KCl was increased to 500 mM. The precipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using monoclonal anti-Myc (9E10) and anti-Flag M2 affinity beads (Sigma). GST pull-down assays were performed as previously described (23).

Fish strains and DNA injection. Medaka embryos and adults of the Cab inbred strain were used for all experiments (42). Embryonic stages were determined according to Iwamatsu (29). DNA was coinjected with the I-SceI meganuclease enzyme (64) into single blastomeres at the one- to two-cell stage. After injection, the embryos were incubated at 28°C. Heat treatments were performed for 2 h at 39°C (7), followed by an incubation at 28°C for 6 to 24 h.

Whole-mount in situ hybridization. Embryos were fixed in 4% paraformaldehyde–2PTW (2× concentrated phosphate-buffered saline at pH 7.5 and 0.1% Tween 20). Whole-mount in situ hybridization was performed at 65°C (53).

RESULTS

Tle4 interacts with Gbx2. We analyzed the transcriptional behavior of *Otx2* and *Gbx2* in cell culture assays. Both genes are known to act as repressors during early brain development

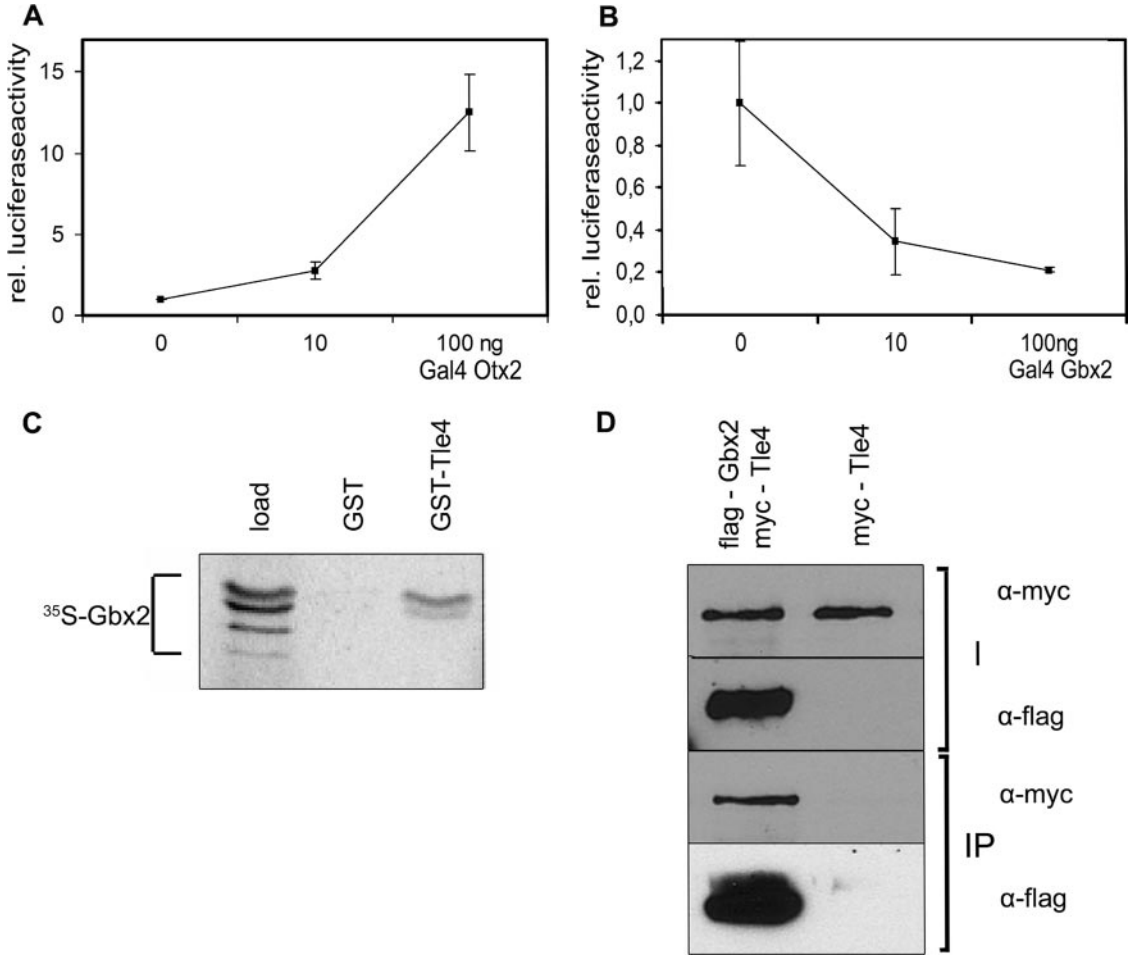

FIG. 2. Gbx2 physically interacts with Tle4 in vitro and in vivo. Otx2 acts as transcriptional activator (A), and Gbx2 exerts transcriptional repression (B) in transient transfection assays. Expression constructs encoding the Gal4 DNA binding domain fused to full-length Otx2 or Gbx2 were cotransfected with the Gal4-responsive reporter into NIH 3T3 cells. Luciferase values are shown relative to the basal level of the reporter. Average values of two experiments are shown, and error bars indicate standard deviations. (C) In vitro binding of Gbx2 to Tle4 by GST pull-down assays. In vitro translated ^{35}S-labeled Gbx2 protein was tested with GST or GST-Tle4 proteins bound to glutathione-Sepharose. The first lane (load) contained 10% of the Tle protein input. (D) In vivo interaction between Tle4 and Gbx2. myc-tagged Tle4 was coexpressed with (lane 1) or without (lane 2) the Flag-tagged Gbx2 component in COS-7 cells. Immunocomplexes obtained with an anti-Flag M2 antibody were analyzed by Western blotting with anti-Myc or anti-Flag M2 antibody. Three percent of total input was used for the load (l). IP, immunoprecipitation.

in vertebrates (28, 66). In order to avoid interference from other homeodomain proteins, we created heterologous fusion constructs consisting of full-length mouse Otx2 and the Gal4 DNA binding domain (illustrations of the cDNAs used are presented in Fig. 1). Cotransfection of increasing amounts of the Gal4-Otx2 fusion construct with a Gal4-responsive reporter into NIH 3T3 fibroblast cells resulted in more than a 10-fold increase in luciferase activity, indicating that Otx2 acts here as a transcriptional activator (Fig. 2A). In contrast, a Gbx2 fusion containing the Gal4 DNA binding domain repressed basal activity of the reporter (Fig. 2B), showing that Gbx2 acts as a repressor in NIH 3T3 cells. Similar results were obtained using U2-OS cells (data not shown).

To test whether the repressing function of Gbx2 is induced by a Groucho corepressor interaction, we applied an in vitro protein-binding assay. As a representative member of the corepressor family, we selected Tle4. Full-length Tle4 fused to GST was synthesized in *Escherichia coli* and subsequently analyzed in GST pull-down assays (Fig. 2C). Indeed, retention of Gbx2 by the GST-Tle4 fusion protein indicated an interaction between the two proteins (Fig. 2C, right lane). In addition to the full-length version of Gbx2, several truncated proteins resulted from the in vitro translation of Gbx2 cDNA (Fig. 2C, left lane). Of these, only the largest fragment bound to Tle4 efficiently, thus confirming the specific nature of the interaction and indicating a terminally located interaction motif.

To investigate this protein complex in vivo, we expressed a myc epitope-tagged Tle4 alone and with Flag-tagged Gbx2 in COS-7 cells. Gbx2 was then precipitated from cell lysates with a monoclonal anti-Flag antibody and analyzed for the presence of Tle4 by Western blotting. A low, but significant amount of myc-tagged Tle4 was detected (Fig. 2D). Therefore, Tle4 interacts with Gbx2 in vitro and in vivo.

The interaction of Gbx2 with full-length Tle4 depends on an eh1-like motif. Gbx proteins display extensive similarity throughout their amino-terminal region including a proline-

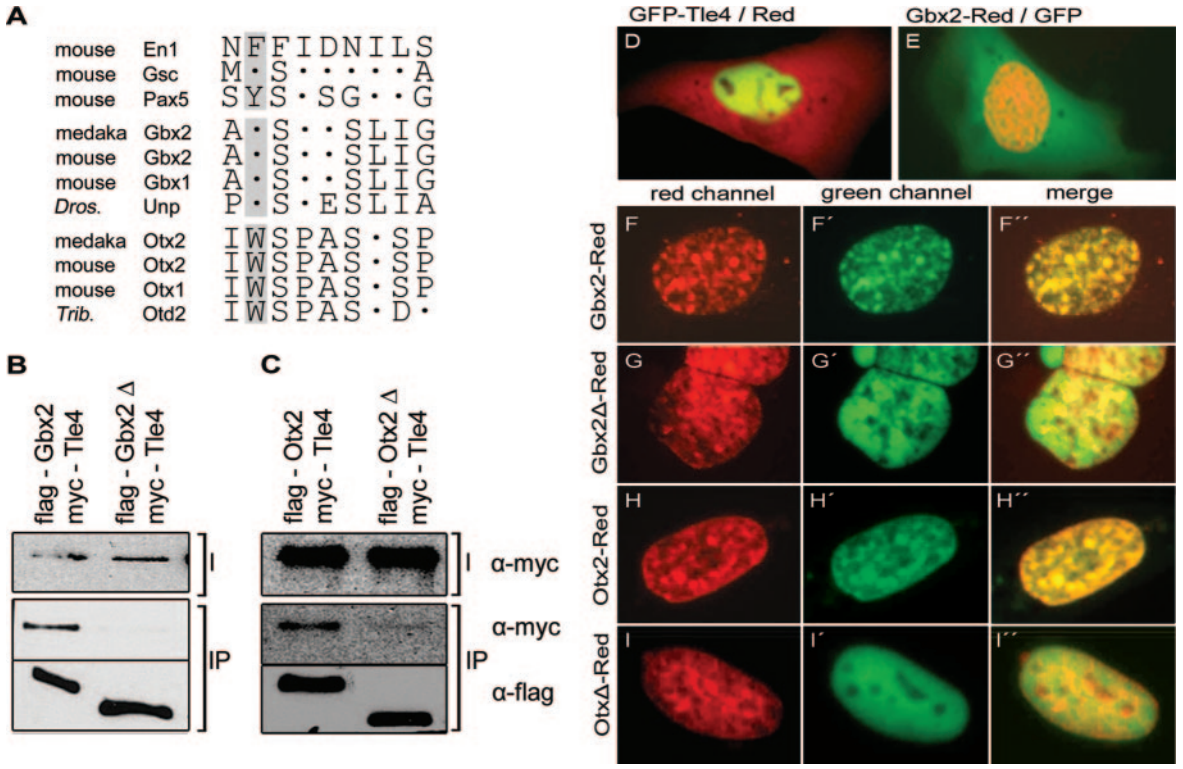

FIG. 3. Conserved function of the eh1 region in mediating physical interaction with Groucho/Tle. (A) Alignment of the eh1-like region of Gbx2 with related motifs from different species (Dros., *Drosophila melanogaster*; Trib., *T. castaneum*). Note the presence of a strongly conserved seven-amino-acid core, which starts with an aromatic residue (position 2 in alignment). (B and C) eh1-dependent interaction of Gbx2 and Otx2 with Tle4, analyzed by coimmunoprecipitation assays. myc-tagged Tle4 was coexpressed with Flag-tagged Gbx2 227C (B, lane 1) or Flag-tagged Otx2 Δ100N (C, lane 1) in transiently transfected COS-7 cells. The corresponding Gbx2 Δ29N 227C and Otx2 Δ100NΔ149–182 deletion mutants lacking the eh1-like motif (lanes 2) showed a dramatically reduced interaction. Immunocomplexes with the anti-Flag M2 antibody were analyzed by Western blotting first with a monoclonal anti-Myc and then with an anti-Flag M2 antibody. Three to five percent of total input was used for the load (l). IP, immunoprecipitation. (D to I) Colocalization assay showing the involvement of the eh1-like motif of Gbx2 and Otx2 for Tle4 interaction by fluorescence microscopy. A GFP-Tle4 fusion is distributed in the nucleus of a U2-OS cell in a "nonspeckled" manner (D). The cell is visualized by coexpression of a Red protein. Gbx2-Red or Otx2-Red localizes in a "speckled pattern" within the nucleus (E; coexpression of GFP). Gbx2- and Otx2-Red fusion proteins colocalize with GFP-Tle4 in speckled patterns within the nucleus (F and H). Otx2 or Gbx2 mutants lacking the eh1 motif fail to colocalize with GFP-Tle4 (G and I). A total of 10 ng of pKC Red Gbx2 or pKC RedGbx2 Δ29N expression vectors was cotransfected with 100 ng of pKW-GFP-Tle4. For pKC Red Otx2 or pKC Red Otx2 Δ149–182, 100 ng was used.

rich domain of unknown function and a highly conserved motif of 10 amino acids located N-terminally to this domain. We identified this region as an eh1-like motif, present also in other classes of homeoproteins such as engrailed, goosecoid, Nkx/Nk, Msx/Msh, and Hesx/Anf (61). The eh1 motif mediates interactions with Groucho corepressors (21, 32, 48). This motif consists of a conserved seven-amino-acid core with divergent flanking residues. The core sequence, starting with a phenylalanine residue (Fig. 3A), is identical in mouse Gbx1 and Gbx2 proteins. The *Drosophila* homologue of Gbx, Unplugged, also displays low divergence. Within the core sequence, a single conservative amino acid exchange has occurred during evolution (position 5 in the alignment). Figure 3A shows a comparison of eh1-like core sequences present in selected proteins of different organisms. The eh1-like motif of Otx proteins is similarly conserved among vertebrates but also occurs in the invertebrate species *Tribolium castaneum*.

To see if the interaction between Gbx2 and Tle4 depends on the eh1-like motif of Gbx2, we generated a Gbx2 deletion mutant lacking the first 29 amino acids (Δ29N). In addition, we deleted the C-terminal region starting from amino acid 227 (227C) that includes the homeodomain (Fig. 1, Gbx2 227C and Gbx2 Δ29N 227C), which we previously found to reduce the cytotoxic effects of overexpression. Coimmunoprecipitation assays resulted in a Tle4 signal for Gbx2 227C but showed a strong reduction for Gbx2 Δ29N 227C which lacks the eh1-like sequence (Fig. 3B). As a control, we performed a coimmunoprecipitation experiment for mouse En2 proteins under the same conditions (data not shown). A strong interaction was detected for the wild-type protein but not for an F65E mutation within the eh1 motif (61). We therefore conclude that the eh1-like motif of Gbx2 is able to interact with Tle4 but at a lower level than that of En2.

Since physical interaction between Otx2 and Tle4 was recently demonstrated (52), we repeated the experiment using a homeodomain-truncated Otx2 construct lacking 100 N-terminal residues, similar to our strategy used for Gbx2 (Fig. 1, Otx2 Δ100N). As expected, we detected Tle4 protein in the coimmunoprecipitate, which was significantly reduced for the Otx2 mutant protein (Otx2 Δ100N Δ149–182) (Fig. 3C). A compar-

ison of the precipitated bands (Fig. 3B and C) indicates that the affinity of Tle4 for Otx2 was below that for Gbx2. Taken together, these results demonstrate the importance of the eh1-like motif of Gbx2 and Otx2 for Tle4 interaction.

Development of a colocalization assay for sensitive detection of Tle4 interactions. To independently verify the results of the coimmunoprecipitation experiments, we established a cell culture colocalization assay. We initially examined the subcellular distribution of a Gfp-Tle4 fusion protein and found it localized to the nucleus of U2-OS cells (Fig. 3D), as previously reported for Groucho proteins (63). Gbx2 and Otx2 were also localized to the nucleus when fused with DsRed protein (Fig. 3E) but, in contrast to Tle4, displayed a "speckled" pattern. When coexpressed with the homeodomain proteins, Tle4 reproduced the nuclear signal pattern of Otx2 and Gbx2, suggesting a physical interaction between each pair of expressed proteins (Fig. 3F to F″ and H to H″). To test for the specificity of these interactions, we performed the same experiments with the eh1 mutant versions. Both Gbx2 Δ29N and Otx2 Δ149–182 failed to alter the GFP-Tle4 pattern (Fig. 3G to G″ and I to I″). As references, we tested En2 and Pax5, which were previously identified as Groucho interaction partners. In both cases we observed a dramatic relocation of Gfp-Tle4 to produce the speckled nuclear patterns characteristic of both En2 and Pax5, alone. Likewise, mutations within the eh1-like motifs of both references failed to do this. Therefore, this assay detected eh1-specific interactions of Otx2 and Gbx2 with Tle4 in the nucleus.

Tle4 represses the transcriptional activity of Gbx2 and Otx2 in an eh1-dependent manner. Gbx2 has been shown to operate both as a transcriptional repressor (28, 66) and activator (40). To investigate the functional consequences of its physical interaction with Tle4, we tested whether the corepressor can modify the repression of Gbx2 in cell culture. The basal activity of the Gal4-responsive reporter was increased after cotransfection with small amounts of a Gal4-VP16 fusion construct. Gbx2 weakly reduced the transcriptional activity of the reporter (partly due to positive effects on the expression of the *Renilla* luciferase reference construct [data not shown]). But when Gal4-Gbx2 was coexpressed with Tle4, the Gal4 promoter activity dropped considerably (sevenfold below basal expression), even though the Gal4-Gbx2 expression level was reduced due to the coexpression of Tle4 (Fig. 4A). The expression of Tle4 alone did not significantly alter reporter activity, showing that the repressive effect on the promoter is exerted specifically by Gal4-Gbx2. Furthermore, the combined effect of Gbx2 and Tle4 was not seen when the eh1 mutant of Gbx2 (Gal4-Gbx2 Δ29N) was used. The effect of Gbx2 and Tle4 together became more pronounced when higher amounts of Gal4-Gbx2/Gal4-Gbx2 Δ29N expression vectors (30 ng) were used (eightfold difference in reporter activity) (data not shown). In the absence of Tle4, both Gbx2 versions repressed Gal4 reporter gene activity equally (Fig. 4A). These data show that in cell culture, Tle4 mediates transcriptional repression of Gbx2 depending on its interaction with the eh1-like motif of Gbx2.

Contrary to Gbx2, Otx2 behaves as a transcriptional activator in cell culture (Fig. 2A). The expression of Gal4-Otx2 and the corresponding eh1 mutant (Gal4-Otx2 Δ149–182) significantly increased the activity of the Gal4-responsive reporter (Fig. 4B). However, coexpression of Tle4 completely abolished

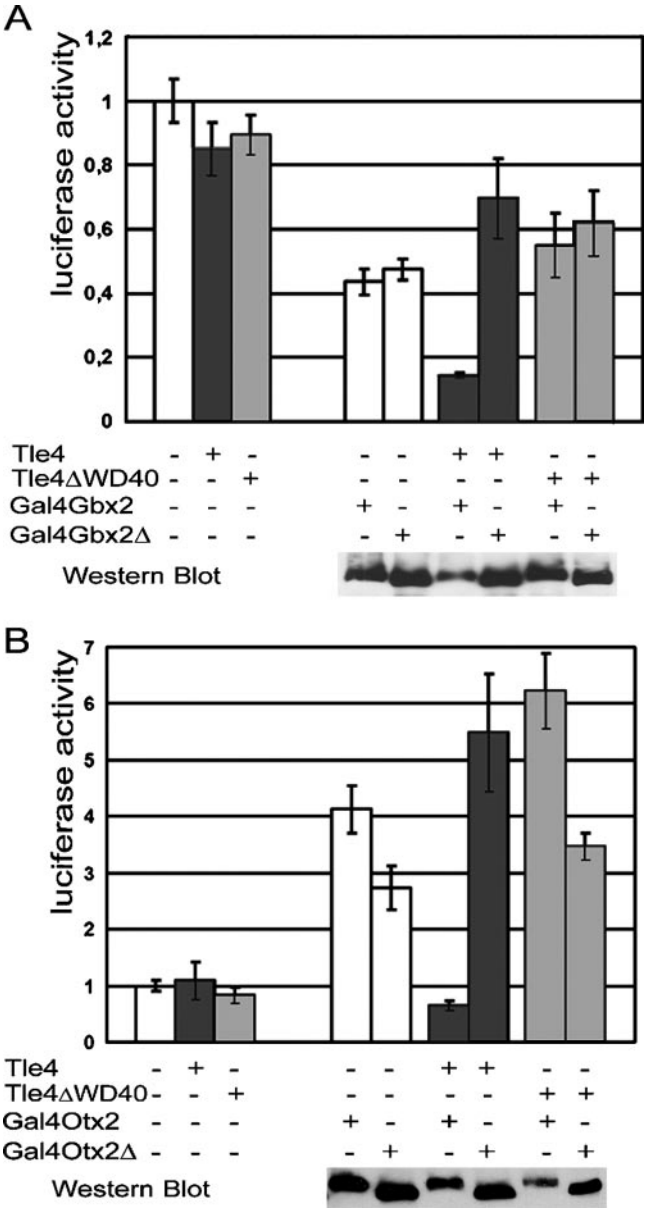

FIG. 4. The eh1 motif of Gbx2 and Otx2 mediates repression by Groucho/Tle corepressors. Transient reporter assays in human U2-OS cells. Ten nanograms of the indicated Gal4 fusion constructs pMCGal4Gbx2 and pMCGal4Gbx2Δ29N (A) or pMCGal4Otx2 and pMCGal4Otx2Δ(149–182) (B) was cotransfected with either pKW10 (empty expression vector), pKW-Tle4 or pKW-Tle4ΔWD40 (each, 30 ng). The basal expression level of the reporter was raised for the Gbx2 experiments (A) by cotransfection of a pKCGal4-VP16 expression vector (10 ng). Normalized luciferase values are shown relative to the reporter gene activity. The means and standard deviations of four independent transfections are shown. Similar results were obtained for at least three separate experiments. Protein levels of the Gal4 fusion constructs were analyzed by Western blotting of cell extracts and are presented below each diagram.

the effect of wild-type Otx2, whereas the Otx2 protein lacking the eh1 motif was unaffected by the corepressor. This reduction may in part be caused by a reduced Gal4-Otx2 level (due to Tle4 coexpression, but note an even lower level upon

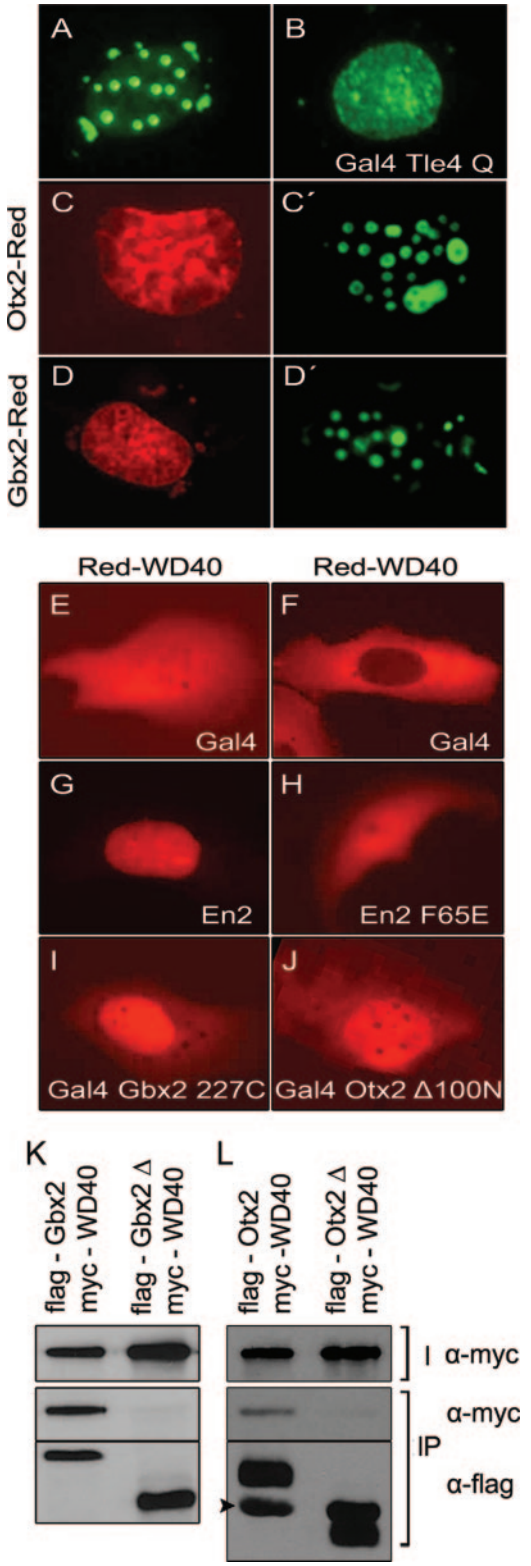

FIG. 5. Colocalization assay using the WD40 domain. (A) Distribution of a GFP-Tle4 mutant protein lacking the WD40 repeats in small aggregates in the nucleus of U2-OS cells. (B) A Gal4-Tle4 Q fusion (400 ng of pMC Gal4-Tle4-Q) was able to relocate the Tle4 ΔWD40 mutant into a speckled pattern. (C and D) Gbx2-Red and Otx2-Red fusion proteins (150 ng of pKC DsRed-Gbx2 or pKC DsRed-Otx2) localize in speckled patterns within the nucleus but do not colocalize with the GFP-Tle4 mutant in the same cells (C′ and D′) (100 ng of pKW GFP-Tle4 ΔWD40 was used). Note that the expression level of the pMC vector in these cells is lower compared to that of pKC/pKW. The distribution of a Red-WD40 fusion (100 ng of pKC DsRed-Tle4-WD40) was found throughout the cells (E) or with a preference to the cytoplasmic compartment of U2-OS (F), when coexpressed with a Gal4-DNA binding domain (200 ng of pKC Gal4). En2 (200 ng of pKCEn2) recruits the Red-WD40 protein into the nuclear compartment (G), whereas an En2 mutant [200 ng of pKC En2(F65E)] was unable to relocate the WD40 fusion protein (H). Coexpression of Gal4-Gbx2 227C (200 ng) or Gal4-Otx2Δ100N (200 ng) with Red-WD40 resulted in a recruitment of the WD40 fusion to the nucleus (I and J) (Table 1). The nuclear distribution of the fusion proteins was analyzed by fluorescence microscopy. (K and L) WD40-mediated interaction of Tle4 with Gbx2 and Otx2, analyzed by coimmunoprecipitation assays. The myc-tagged WD40 domain was coexpressed with Flag-tagged Gbx2 227C or Flag-tagged Otx2 Δ100N in COS-7 cells. The corresponding Gbx2 Δ29N 227C (flag-Gbx2Δ) and Otx2 Δ100N Δ149–182 (flag-Otx2Δ) deletion mutants lacking the eh1-like motif showed a dramatically reduced interaction. Immunocomplexes with the anti-Flag M2 antibody were analyzed by Western blotting first with a monoclonal anti-Myc and then with an anti-Flag M2 antibody. Three to five percent of total input was used for the load (l). Immunoglobulin G light chain signal is indicated by an arrowhead. IP, immunoprecipitation.

coexpression of Tle4ΔWD40, resulting in strong activation). Our cell culture experiments are therefore consistent with a Groucho-mediated repression conferred by the eh1-like domains of these two homeobox proteins.

The WD40 repeat domain of Tle4 is sufficient for the interaction with Gbx2 and Otx2. After identifying the eh1-like sequences as Groucho interaction motifs, we next asked which domain of Tle4 mediates this contact. The eh1 region of Engrailed has been shown to interact with the WD40 repeat domain of Groucho (32). We therefore tested a Tle4 mutant lacking the carboxy-terminal region including the WD40 repeat domain in our reporter assays (Fig. 4A, Tle4 ΔWD40). Whereas full-length Tle4 in the reporter assays reached only low levels of expression, the ΔWD40 version was highly expressed in these experiments (data not shown). Nevertheless, the mutant Tle4 protein was not able to confer repressive activity on Otx2 and Gbx2 (Fig. 4A and B). These results suggest that the WD40 repeat domain is essential for full repression exerted by Gbx2 and Otx2 and the Tle4 corepressor, possibly by a direct interaction involving the eh1 motif.

We next investigated the potential physical interaction with the WD40 repeat domain using colocalization assays. In contrast to results with the full-length protein, overexpression of Gfp-Tle4ΔWD40 resulted in the formation of small aggregates within the nucleus or closely associated with it (Fig. 5A). Such distributions are often observed for truncated proteins fused with GFP following overexpression (27). However, coexpressing Gal4 fused with the Tle4 Q domain (Gal4-Tle4 Q) distributed the GFP fusion protein again into a speckled pattern within the nucleus (Fig. 5B), suggesting that the two proteins interact at the Q domain. On the other hand, neither Otx2 nor Gbx2 coexpression had such an effect (Fig. 5C and D), indicating that the Tle4 protein lacks the corresponding interaction domain.

To demonstrate this interaction directly, we tested the isolated WD40 domain. A Red-WD40 fusion protein lacks the

TABLE 1. Nuclear relocation of Red-WD40 by Otx2 and Gbx2

Construct (amount)	Accumulation (%) of Red-WD40 in:[a]	
	Nucleus	Cytoplasm
Gal4-Otx2 Δ100N (250 ng)	25 (45/183)	2.7 (5/183)
Gal4-Gbx2 227C (100 ng)	47 (58/123)	0 (0/123)
Gal4 (250 ng)	1.8 (5/279)	29 (81/279)

[a] U2-OS cells were transfected with the indicated constructs. Numbers of counted cells are given in parentheses (no. of cells in nucleus or cytoplasm/total no. of cells).

endogenous nuclear localization signal and thus localizes throughout the cell (Fig. 5E), though it primarily occurs in the cytoplasm (Fig. 5F). This fusion protein revealed a highly specific interaction with En2, being recruited to the nucleus in almost 100% of the cells (Fig. 5G). In contrast, mutation of the eh1 motif [En2(F65E)] abolishes this interaction (Fig. 5H). Since initial experiments with full-length Otx2 and Gbx2 were negative, we switched to mutants that lacked their homeodomains. As expected, the elevated expression levels of these constructs resulted in the nuclear localization of the Red component in 47% of the cells expressing Red-WD40 and the Gbx2 protein, whereas only 1.8% of the cells cotransfected with Red-WD40 and Gal4 alone showed any accumulation of the Red fusion in the nucleus (Table 1). In the case of Otx2, around 25% of the cells revealed nuclear relocation. These data indicate that the WD40 repeat domain is sufficient for interactions with Gbx2 and Otx2 truncated proteins. The nuclear accumulation of these proteins, however, is somewhat below that of En2. This may reflect weaker complexes involving Tle corepressors and Gbx2 and Otx2 transcription factors, which is in agreement with our coimmunoprecipitation data.

Finally, we looked for direct interactions between the WD40 domain and Gbx2 and Otx2 by coimmunoprecipitation. This assay revealed a significant interaction with both Gbx2 and Otx2 (Fig. 5K and L, left lanes), whereas the eh1 mutant versions of both proteins showed a clear reduction in precipitated bands (Fig. 5K and L, right lanes). Taken together, our results demonstrate that the WD40 domain of Tle4 is not only essential but also sufficient for its interaction with the eh1-like domains of both Gbx2 and Otx2.

Expression of Gbx2 in medaka embryos. So far, no data are available on *Gbx2* function in medaka; we therefore performed a BLAST search using the zebra fish Gbx2 protein sequence in a medaka draft genome database (UT Genome Browser; http://medaka.utgenome.org). A homologous gene appeared that was consistent with the results of a previous PCR-based screen for homeobox genes in medaka (41). We then isolated a cDNA fragment, including the homeodomain region, by reverse transcription-PCR and analyzed the expression pattern of the *Gbx2* gene during medaka embryogenesis.

The results of whole-mount in situ hybridization experiments with wild-type embryos are shown in Fig. 6. In medaka, *Gbx2* transcripts are first detectable at 70% epiboly as a patchy expression domain of two stripes at the level of the prospective hindbrain (Fig. 6A). At late neurula stages, *Gbx2* is expressed in the neural tube within the hindbrain region (Fig. 6B). The most anterior part of this domain forms a ring within the neural tube (Fig. 6B to G, arrowheads). A stripe with a more posterior positioning also appears transiently during early somitogenesis (Fig. 6B and D, arrows) and is lost around the seven-somite stage (Fig. 6E and F). The anterior *Gbx2*-positive ring initially appears diffuse (Fig. 6C, arrowhead) but sharpens during early somitogenesis at its anterior boundary (Fig. 6D, arrowhead). This domain marks the anterior-most hindbrain in other species. Two-color in situ hybridization experiments show that at four somites, this boundary coincides with the posterior limit of the *Otx2* territory, thus identifying the region as the MHB (Fig. 6C and D). In addition, *Gbx2* is expressed in neural crest cells at the beginning of the neurula stage. Migration of *Gbx2*-positive neural crest cells during later stages generates a rapidly changing, diffuse pattern within the hindbrain (Fig. 6C to F). At the seven-somite stage, *Gbx2* transcripts appear in the dorsal telencephalon and otic primordia (Fig. 6E and F) and are later detected in neurons of the spinal cord and the medial side of otic vesicles (Fig. 6H). The medaka expression domains of *Gbx2* largely coincide with those documented in zebra fish (36, 56).

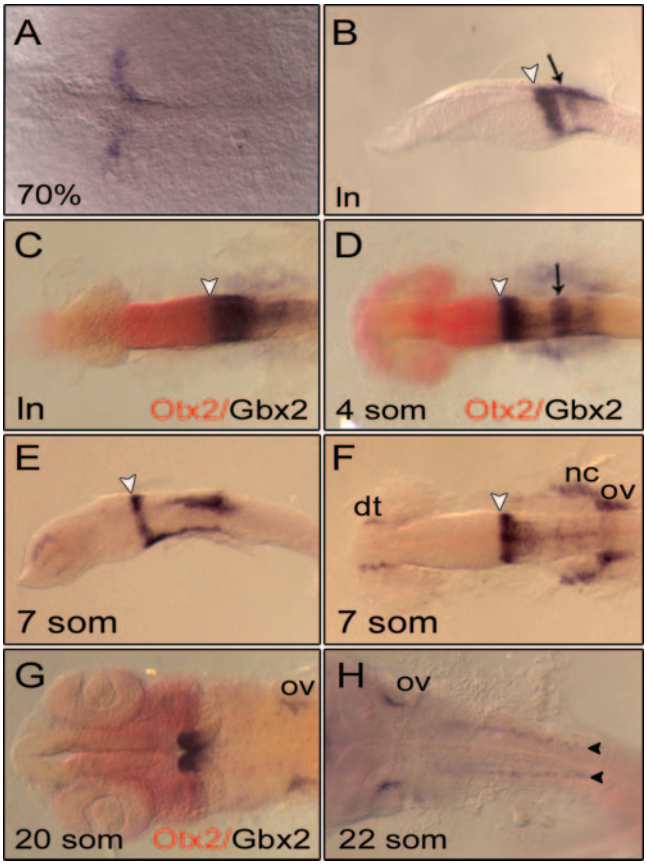

FIG. 6. Expression pattern of Gbx2 in medaka embryos. (A) At 70% epiboly *Gbx2* is first expressed in two stripes in the prospective hindbrain region of the embryo. (B) Late neurula stage embryo showing *Gbx2* expression in the hindbrain. (C, D, and G) Double in situ hybridization was conducted for *Otx2* (red) and *Gbx2* (purple). (E and F) Seven-somite stage embryo (dt, dorsal telencephalon; nc, neural crest cells; ov, otic vesicles). (H) *Gbx2* expression at 22 somites in the spinal chord (black arrowheads). Anterior is to the left. Panels A, C, D, and F to H are dorsal views; panels B and E are lateral views. White arrowheads show the position of the MHB, and arrows indicate a second stripe of Gbx2 expression in the hindbrain.

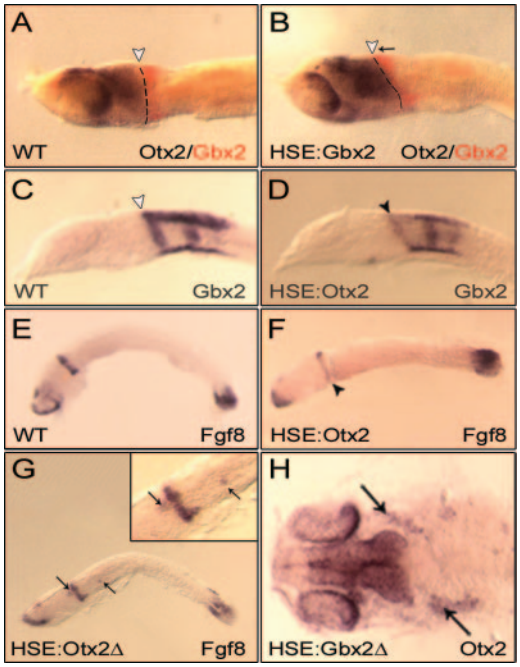

FIG. 7. Groucho mediates repression on Gbx2 and Otx2 in the MHB region of medaka. (A) Wild-type expression of *Otx2* (purple) and *Gbx2* (red) in medaka embryos. (B) Heat-induced misexpression of *Gbx2* results in an anterior shift of the common expression border at the MHB. Wild-type expression of both *Gbx2* (C) and *Fgf8* (E) is repressed by ectopic *Otx2* (D and F, respectively). Ectopic *Fgf8* is induced by misexpression of Otx2Δ (G; inset shows higher magnification of MHB territory) and ectopic *Otx2*, by Gbx2Δ (H). Embryos were heat treated at mid-gastrula (stage 14) and fixed at somitogenesis, 20 to 24 h after induction. Heat treatment for Otx2/Otx2Δ was done at late gastrula, and fixation was 6 h after this. Stage 23 (12 somites) embryos were used for experiments shown in panels A and B; stage 19 (2 somites) for panels C and D; stage 22 (9 somites) for panels E, F, and G; and stage 24 embryos (16 somites) for panel H. For all embryos, anterior is to the left. Panels A to G are lateral views, and panel H is a dorsal view. Black arrowheads depict repression of a gene, arrows point to ectopic expression, and white arrowheads show the position of the MHB.

Interactions between Otx2/Gbx2 and Groucho/Tle affect the developmental regulation of the mid-hindbrain organizer in fish. The embryonic expression pattern of *Tle* genes in medaka (5) along with their physical interaction with Otx2 and Gbx2 described here suggests that Tle proteins participate in the genetic control of MHB development in fish. To examine the functional significance of the Gbx2-Tle and Otx2-Tle interactions in medaka embryos, we overexpressed these transcription factors and their corresponding mutants that lack the eh1-like motif. For misexpression, we chose a heat-inducible HSE system (7) that allows the ectopic activation of a gene of interest in a stage-dependent manner. Heat-inducible constructs were injected at the one- to two-cell stage and then induced during early gastrulation. After 6 to 24 h, the surviving embryos were analyzed for GFP fluorescence and fixed. In addition to the Gbx2 and Otx2 variants, GFP is expressed in a bidirectional manner from the HSE-driven promoter and thereby labels misexpressing cells. Only embryos with strong GFP expression were used for whole-mount in situ hybridizations.

Injection of *Gbx2* mRNA into zebra fish blastomeres was reported to result in reduced forebrains and eyes (36). Activation of the *Gbx2* HSE expression construct at gastrulation strongly reduced the expression of this phenotype. Additionally, we observed an anterior shift of the caudal *Otx2* limit on the dorsal side of the embryos (in 19% of the GFP-positive embryos) (Fig. 7B and Table 2). Endogenous *Gbx2* expression domains followed the new *Otx2* boundaries, indicating a complete shift of the MHB (Fig. 7B). These data are in agreement with reports demonstrating that *Gbx2* represses *Otx2* expression in the posterior midbrain (46, 66). The repressive effect of *Gbx2* on *Otx2* was lost when the *Gbx2* mutant lacking the eh1-like domain (Gbx2 Δ29N) was used for misexpression (Table 2), indicating that Tle is required for this repression. In 50% of the embryos, we observed ectopic expression of *Otx2* in both nonneural (Fig. 7H) and neural tissue (data not shown). *Otx2* ectopic expression became more pronounced when embryos were incubated to late stages. Taken together, these data indicate that *Gbx2* represses *Otx2* in a Tle-dependent manner.

Misexpression of *Otx2* in the anterior hindbrain of transgenic mice was reported to repress *Gbx2* expression in the hindbrain (11, 35). *Otx2* mRNA injections result in severe disruptions during gastrulation (47). Using the HSE-inducible system to misexpress *Gbx2* after the sensitive gastrula stage, we observed the repression of endogenous *Gbx2* in 54% of the embryos, predominantly in the first transverse stripe next to the *Otx2* territory (Fig. 7D and Table 2). Although the endogenous *Gbx2* expression pattern is highly dynamic, the first *Gbx2* stripe at the MHB remains constant (see above). The strongest repressive activity was observed when misexpression was induced at the end of gastrulation followed by a 6-h incubation. However, forced overexpression of the eh1-deleted *Otx2* mutant caused similar repression effects on endogenous *Gbx2* (Table 2), indicating a Tle-independent repression mechanism exerted by *Otx2* on *Gbx2*.

In addition, we analyzed the repressive effect of *Otx2* on fibroblast growth factor 8 (*Fgf8*). Secreted Fgf8 protein plays a pivotal role in isthmic organizer activity at early somite stages. In this region, *Otx* gene products are required to repress *Fgf8* in nonexpressing tissues, thereby restricting the spatial expres-

TABLE 2. Effects of Gbx2/Otx2 misexpression on endogenous Otx2, Fgf8, and Gbx2

Target gene and HSE construct	No. of embryos with indicated type of expression (% of total)				n^a
	Shifted	Repressed	Normal	Ectopic	
Otx2					
HSE-Gbx2	7 (19)		29 (81)	0 (0)	36
HSE–Gbx2HD-Tle4	26 (44)		32 (46)	0 (0)	58
HSE-Gbx2 Δ29N	0 (0)		17 (50)	17 (50)	34
Fgf8					
HSE-Otx2		8 (36)	14 (64)	0 (0)	22
HSE–Otx2HD-Tle4		20 (46)	23 (44)	0 (0)	43
HSE–Otx2 Δ149–182		0 (0)	25 (74)	9 (26)	34
Gbx2					
HSE-Otx2		20 (54)	17 (46)		37
HSE–Otx2 Δ149–182		12 (43)	16 (57)		28

[a] Total number of GFP-positive embryos used for in situ hybridization.

sion of *Fgf8* gene products (2). As expected, the overexpression of full-length *Otx2* clearly reduced the expression of *Fgf8* (Fig. 7F and Table 2). The *Otx2* mutant lacking the eh1-like motif failed to down-regulate *Fgf8* expression and even produced small, ectopic patches of *Fgf8* transcripts (Fig. 7G and Table 2). Therefore, *Otx2* represses *Fgf8* in a *Tle*-dependent manner. This contrasts with its effects on *Gbx2*, which in our experiments appear to be independent of the corepressor.

Our coimmunoprecipitation and colocalization experiments with Otx2/Gbx2 and Tle4 suggested a medium affinity interaction of these proteins. Covalent bonding of the transcription factors in a fusion protein with the corepressor should, therefore, enhance Groucho-dependent repression effects. To test this hypothesis, we fused Tle4 with the DNA binding domains of Gbx2 (Gbx2HD-Tle4) and Otx2 (Otx2HD-Tle4) and misexpressed them in medaka embryos. Both fusion constructs exhibited repressive functions on the same target genes as the full-length proteins (Table 2), thereby further supporting a corepressor-mediated function. Furthermore, the repressive effects of Otx2 on *Fgf8* and of Gbx2 on *Otx2* were strongly enhanced by using Tle4 fusion constructs (Table 2 and see Fig. S1 in the supplemental material).

The function of Groucho/Tle proteins can be blocked efficiently by misexpression of the short family member *Aes*, which interferes with the function of the Q-domain (43, 55, 57). Similarly, the interaction of proteins with the WD40 repeats should specifically be blocked by overexpression of the WD40 domain. For the experiments, we selected a DsRed-WD40 fusion protein, which gave better results in colocalization experiments compared to the isolated WD40 domain. Misexpression of this protein indeed resulted in phenotypes reminiscent of *Aes* injection experiments in medaka (6; B. Bajoghli, N. Aghaallaei, D. Soroldoni, and T. Czerny. submitted for publication). The eyes and the midbrain were smaller (see Fig. S1 in the supplemental material) and heart looping was affected (data not shown). Injection of an eh1-deleted *Gbx2* had resulted in ectopic activation of *Otx2* (see above). We therefore tested whether dominant negative interference at the level of the WD40 interaction similarly resulted in a derepression of the endogenous *Gbx2*. Indeed, we observed ectopic activation of *Otx2* in 12% of the embryos ($n = 25$) (see Fig. S1 in the supplemental material). Taken together, the in vivo experiments demonstrate the involvement of Tle proteins in the genetic interactions that form the isthmic organizer.

DISCUSSION

Specifications within the neural tube result from complex interactions involving different classes of transcription factors that share common boundaries (reviewed in references 34, 54, 59, and 70). Many of these factors can recruit Groucho cofactors for their transcriptional regulation. This is the case for Engrailed (32), Pax2, Pax5, (13, 23), and Otx (52) proteins, which are involved in regional patterning along the anteroposterior body axis and the dorsoventrally expressed gene products, Pax6 and Nkx (48). Here, we analyzed the interaction between Gbx2 and Groucho/Tle proteins. The results of our coimmunoprecipitation and colocalization essays are supported by a number of studies. First, *Gbx* and *Tle* genes are coexpressed in the vertebrate hindbrain in mouse (39) and fish embryos (5, 43, 69). Second, other homeodomain-containing factors like Engrailed, Goosecoid, Nkx/Nk, and Hesx/Anf interact with Groucho corepressor proteins (21, 32, 48). Third, phenotypes caused by the ectopic expression of full-length *Gbx2* in *Xenopus* can be phenocopied by fusion proteins of the DNA-binding homeodomain of Gbx2 and the Groucho interacting repression domain of engrailed (28, 66). We could extend these data by demonstrating Groucho-dependent repression of *Gbx2* in medaka embryos.

Tle-dependent transcriptional repression of Gbx2 and Otx2. Groucho binding transcription factors can be classified according to their transcriptional properties. One group comprises strong repressors like Hairy-related basic helix-loop-helix proteins (50) and the homeodomain proteins En and Gsc (32, 33). Other DNA-binding partners for Groucho proteins are known to act as both transcriptional repressors and activators, such as Tcf/Lef, the nuclear targets of canonical Wnt signaling (14, 57). We found that Gbx2 acts as a repressor when fused to Gal4. A major part of this repression is mediated by Groucho/Tle proteins. On the other hand, induction of reporter gene activity has been reported for Gbx2 at the *myelomonocytic growth factor* minimal promoter of chick, which contains additional AP-1 binding sites (40).

In contrast, the *Drosophila* Dorsal protein is known to activate the transcription of specific target genes in vitro (20) but has also been shown to exhibit Groucho-dependent repression in vivo (22). The Dorsal protein therefore appears to intrinsically activate, but can repress transcription with the binding of specific cofactors (26). When we fused Otx2 to the heterologous Gal4 DNA-binding domain, it exerted transcriptional activation. This is in agreement with cell culture studies in which Otx protein could activate a promoter containing Bicoid target sites (52, 60). Interestingly, in vivo studies in *Xenopus* suggest that transcriptional repression by Otx2 is critical for positioning of the MHB (28). Thus, Otx2 appears to belong to a class of transcription factors that are converted from activators to repressors by the recruitment of Groucho proteins.

The interaction between Gbx2/Otx2 and Groucho proteins is mediated by eh1-like motifs and the WD40 repeat domain. The physical interaction between Gbx2 and Tle4 appears to be direct, since it is detected in assays using bacterially produced proteins and in vitro translated Gbx2 preparations. This association depends on an eh1-like motif of Gbx2, which is located within a conserved N-terminal domain. eh1-related motifs have been found in various classes of homeodomain-containing proteins, including En, Gsc, Nkx/Nk, Msx/Msh, Hesx/Anf, and UNC-4 and have been shown to be critical for both repression and Groucho recruitment (21, 32, 33, 48, 61, 68). Deletion of this motif in Gbx2 and Otx2 strongly reduced signals in coimmunoprecipitation assays and prevented redistribution of the proteins in colocalization studies. Moreover, it is essential for Groucho-dependent transcriptional repression of both Gbx2 and Otx2 (Fig. 4).

The WD40 repeat domain is thought to represent an ancient eukaryotic protein-protein interaction surface resulting from tandem repeats of a 40-amino-acid motif that form a β-propeller structure (38, 49). The WD40 repeat domain of Groucho has been shown to participate in interactions with the eh1 motif of Engrailed proteins (31, 32) and is required for interactions involving Hesx1 (21) and Cbfa1/Runx2 (45). In yeast

two-hybrid assays, the Six domain of Six3 interacts with the WD40 domain of Tle1 and Tle3 (37, 43). In our experiments, a Tle4 mutant lacking this domain was unable to mediate transcriptional repression by Gbx2 and Otx2 in cell culture and failed to associate with these transcription factors in colocalization assays. Similar arguments based on the absence of activity in mutants have been previously used to infer essential functions of the WD40 domain in other interactions (21, 32). Although coimmunoprecipitation experiments using the isolated domain so far failed (21), we detected a WD40-mediated interaction for both Gbx2 and Otx2. The success of these experiments might be attributed to an increased detection sensitivity using the 18-copy myc tag.

Since our cell culture colocalization assay turned out to be highly sensitive for the detection of full-length Groucho protein interactions in vivo, we used it for the isolated WD40 domain as well. Initial experiments using GFP fusion proteins were negative (data not shown), but the application of the DsRed protein was successful. Red-WD40 protein relocated from the cytoplasm to the nucleus when cotransfected with En2 in almost 100% of the cells, whereas a single point mutation in the eh1 region of En2(F65E) completely abolished this effect (Fig. 5 G and H). Interestingly, DsRed is an obligate tetramer, in contrast to Gfp (71), and multimerization has previously been shown to be important for the repressive function of Groucho proteins (17). The assay gave positive results also for Gbx2 and Otx2 although at a reduced efficiency (Fig. 5I and J and Table 1). Altogether, we found that the WD40 repeat domain is not only essential but also sufficient for interactions with the eh1 motifs of Gbx2 and Otx2.

Development of a sensitive colocalization assay for the detection of protein-protein interactions in the nucleus. A number of methods are used to demonstrate protein-protein interactions, such as coimmunoprecipitation and pull-down assays; however, the presence of "sticky" domains within the interacting proteins may limit the sensitivity of these assays. While Engrailed proteins reliably coimmunoprecipitated Tle4 in our experiments (data not shown), considerable optimization of conditions was necessary to detect specific interactions with Otx2 and Gbx2 above background. This might reflect weaker interactions between Groucho corepressors and Gbx2 and Otx2 compared to those involving Engrailed. In addition, we observed considerable nonspecific binding of individual Tle4 domains (data not shown), suggesting that we reached the sensitivity limits of the assay. We therefore searched for an alternative method to analyze protein-protein interactions. We looked for an in vivo method to avoid artifacts of in vitro conditions. The relocation of binding partners to different intracellular compartments has been used before to demonstrate Groucho protein interactions (57). We observed that Tle4 proteins localize to different intranuclear positions compared to DNA-binding transcription factors, whereas coexpression leads to a redistribution of Tle4. In a previous experiment, AML proteins also targeted Tle to a speckled pattern within the nucleus (30). Using fluorescently labeled proteins, this approach provides an exceptionally detailed analysis of such interactions. After refining the experimental conditions, we detected interactions in nearly 100% of the cells with the strong binding partners, Engrailed, and Hes1. For Otx2 and Gbx2, we detected interactions in a large number of the cells. In addition, specific mutations within the interaction motifs completely abolished these effects (Fig. 3G and I). Our in vivo assay therefore provides highly specific detection of protein-protein interactions for experiments using transient transfection.

Interactions between Otx2/Gbx2 and Groucho/Tle proteins regulate the genetic network of the mid-hindbrain organizer in fish embryos. The midbrain-hindbrain organizer is a signaling center that orchestrates development in the midbrain and the anterior hindbrain primordia. One of the earliest events in the development of the anterior central nervous system is its subdivision into anterior *Otx2*-positive and posterior *Gbx2*-positive domains. These homeobox genes antagonize each other (11, 35, 46, 66), and this reciprocal relationship is critical for the positioning of the MHB. The molecular basis of this interaction, however, is not yet known. Fusion protein experiments in *Xenopus* suggest that transcriptional repression rather than activation underlies the mutual antagonism of Otx2 and Gbx2 (28). A physical interaction between Otx proteins and Tle4 was also recently demonstrated (52), but the consequences of this association on the positioning of the MHB have not been investigated.

mRNA injection of *Gbx2* has previously been shown to produce truncations in anterior neural structures, such as the forebrain and eyes (36, 66). This early phenotype is not dependent on Groucho corepressors in medaka, as N-terminal deletions of Gbx2 including the eh1-like interaction domain do not affect the results of mRNA injection experiments (data not shown). However, by shifting the onset of Gbx2 expression to a later developmental stage using a heat shock-inducible system (7), we observed specific effects on MHB positioning. The Otx2 and Gbx2 expression domains, as well as their common boundary, shifted to a more anterior position (particularly on the dorsal side of the embryo) (Fig. 7B). A similar anterior shift of the MHB region was previously observed in mouse mutants with reduced levels of *Otx* gene products (1). This suggests that *Otx2* gene function is down-regulated by *Gbx2* activity.

Our inducible misexpression system was also instrumental for the *Otx2* experiments since overexpression of this gene is known to block the convergent extension movements of gastrulation (47). In these experiments, heat shock activation of *Otx2* resulted in significantly reduced *Gbx2* expression. These effects occurred primarily in the anterior-most expression domain of Gbx2, directly at the MHB and abutting the *Otx2*-positive territory. Interestingly, the position of the MHB was not affected in these experiments. These misexpression experiments therefore reproduce the reciprocal repression of the two homeodomain transcription factors in MHB specification, as seen in other experimental systems.

The effect of *Gbx2* on *Otx2* was dependent on Groucho protein interactions since misexpression of Gbx2-Tle4 fusion proteins extended the phenotype of wild-type *Gbx2*, whereas an expression construct lacking the eh1 motif did not show repression (Table 2). On the contrary, misexpression of the *Gbx2* mutant resulted in ectopic expression of *Otx2* in a region posterior to the *Otx2*-positive territory (Fig. 7H), suggesting a switch of *Gbx2* repressing functions to activation of its target gene, *Otx2*. These data are in agreement with our misexpression experiments using the WD40 do-

main to block interaction of the endogenous Gbx2 protein with the corepressor. Furthermore, overexpression of the dominant negative *Aes* in medaka embryos caused ectopic expression of *Otx2* in the hindbrain (43).

The reciprocal *Otx2* misexpression experiments did not, however, indicate corepressor dependence (Table 2). Interestingly, *Otx2* and Otx2-Tle4 misexpression resulted in the repression of *Fgf8* in the MHB, which was not the case for the *Otx2* deletion construct (Fig. 7F and Table 2). *Fgf8* plays a pivotal role in isthmic organizer activity (reviewed in reference 58), and *Otx* gene products have been shown to regulate *Fgf8* expression. Thus, in Otx1$^{-/-}$ Otx2$^{+/-}$ mouse embryos, the *Fgf8* expression domain was abnormally enlarged in the presumptive diencephalon (1). Therefore, a critical level of *Otx* gene activity is required to repress *Fgf8* expression in the midbrain. Our experiments suggest that Groucho proteins play an important role in this process.

Taken together, our misexpression studies in medaka embryos fully support the hypothesis that specific interactions between *Gbx2* and *Otx2* are crucial for normal MHB development. Groucho proteins regulate the transcriptional repression exerted by *Gbx2* on *Otx2* in the MHB, as well as the repression of *Fgf8* by *Otx2* in this critical region.

ACKNOWLEDGMENTS

We thank Viktoriya Titova and Dmitri Frolkine for the cloning of DNA constructs and Daniele Soroldoni for help with in situ experiments. Furthermore, we thank Jochen Wittbrodt, Siew-Lan Ang, Wolfgang Wurst, and Gail Martin for providing cDNAs; Margarita Kalogeropoulos for initiating the coimmunoprecipitation experiments; and Carl Bumba for critical reading of the manuscript. Medaka genomic sequence information was provided for use in this publication by the National Institute of Genetics and the University of Tokyo.

This work was supported by the Austrian Science Fund (grant P15185), the Italian Association for Cancer Research, and the MRC (grant G0400410).

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