**Gbxc2 regulates thalamocortical axon guidance by modifying the LIM and Robo codes**

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**SUMMARY**
Combinatorial expression of transcription factors forms transcriptional codes to confer neuronal identities and connectivity. However, how these intrinsic factors orchestrate the spatiotemporal expression of guidance molecules to dictate the responsiveness of axons to guidance cues is less understood. Thalamocortical axons (TCAs) represent the major input to the neocortex and modulate cognitive functions, consciousness and alertness. TCAs travel a long distance and make multiple target choices en route to the cortex. The homeodomain transcription factor Gbx2 is essential for TCA development, as loss of Gbx2 abolishes TCAs in mice. Using a novel TCA-specific reporter, we have discovered that thalamic axons are mostly misrouted to the ventral midbrain and dorsal midline of the diencephalon in Gbx2-deficient mice. Furthermore, conditionally deleting Gbx2 at different embryonic stages has revealed a sustained role of Gbx2 in regulating TCA navigation and targeting. Using explant culture and mosaic analyses, we demonstrate that Gbx2 controls the intrinsic responsiveness of TCAs to guidance cues. The guidance defects of Gbx2-deficient TCAs are associated with abnormal expression of guidance receptors Robo1 and Robo2. Finally, we demonstrate that Gbx2 controls Robo expression by regulating LIM-domain transcription factors through three different mechanisms: Gbx2 and Lhx2 compete for binding to the Lmo3 promoter and exert opposing effects on its transcription; repressing Lmo3 by Gbx2 is essential for Lhx2 activity to induce Robo2; and Gbx2 represses Lhx9 transcription, which in turn induces Robo1. Our findings illustrate the transcriptional control of differential expression of Robo1 and Robo2, which may play an important role in establishing the topography of TCAs.

**KEY WORDS:** Thalamocortical projections, Transcription factor, LIM domain proteins, Robo, Axon guidance, Mouse

**INTRODUCTION**
The assembly of neural circuits in the central nervous system exhibits remarkable precision. Significant progress has been made in our understanding of the extrinsic signaling mechanisms that regulate axon pathfinding, as well as intrinsic determinants, such as transcription factors, that control the identity of neurons (O’Donnell et al., 2009). However, far less is understood about the transcriptional control of intrinsic responsiveness to guidance cues that lead to proper wiring of the brain.

The thalamus plays a pivotal role in integrating and processing visual, auditory, sensory and motor information to and from the cortex (Jones, 2007). During embryogenesis, thalamic axons, known as thalamocortical axons (TCAs), have to navigate along a complex path to reach the cerebral cortex. In mouse embryos, TCAs first grow rostrally through the prethalamus, reaching the diencephalon and telencephalon (Métin and Godement, 1996; Molnar et al., 1998; Seibt et al., 2003; Powell et al., 2008). Genetic studies have demonstrated that Slit-Robo signaling plays an important role in the navigation of TCAs in the ventral telencephalon (Bagri et al., 2002; López-Bendito et al., 2007; Bielle et al., 2011a; Bielle et al., 2011b). Furthermore, it has been shown that Slit1 is involved in establishing the topography of TCAs in the ventral telencephalon (Bielle et al., 2011b). Robo2 is broadly expressed in the developing thalamus, whereas the expression of Robo1 is mainly restricted to the medial part of the thalamus (Bagri et al., 2002; López-Bendito et al., 2007). The differential expression of Robo1 and Robo2 in distinct groups of thalamic neurons may be important for the navigation and topographic sorting of TCAs.

Members of the LIM-homeodomain (LIM-HD) and basic helix-loop-helix (bHLH) transcription factor families are expressed in different domains of the thalamus, suggesting that these proteins may form combinatorial transcriptional codes to impart neuronal identity and connectivity to thalamic neurons (Nakagawa and O’Leary, 2001). It has recently been shown that LIM-HD factor Lhx2 directly binds to putative regulatory elements in the Robo1 and Robo2 loci, and negatively regulates their transcription (Marcos-Mondejar et al., 2012). However, Lhx2 generates the different expression patterns of Robo1 and Robo2 in the thalamus by the same inhibitory regulation remains unclear. Expression of Gbx2 (gastroduodenal brain homeobox gene 2) is initiated in postmitotic neurons of the mouse thalamus at E10.5 (Bulfone et al., 1993). Deletion of Gbx2 causes severe defects in the histogenesis of the thalamus and an almost complete loss of TCAs (Miyashita-Lin et al., 1999; Hevner et al., 2002). We have recently shown that.

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although all thalamic neurons express Gbx2 during development, the onset and duration of Gbx2 expression are highly variable among different thalamic nuclei (Chen et al., 2009). These observations underscore the importance of Gbx2 in the regulatory network that coordinates thalamic neuron specification and connectivity.

In this study, we have examined Gbx2 function in controlling TCA development. We demonstrate that Gbx2 determines the initial directional outgrowth of TCAs into the prethalamus and their subsequent pathfinding to the cortex by regulating their responsiveness to guidance cues. We show that the guidance errors of Gbx2-deficient TCAs were associated with mis-regulation of Robo1 and Robo2 expression. We have identified Lmo3 as a putative direct transcriptional target of Gbx2. Our genetic and molecular biological data collectively demonstrate that Gbx2 regulates the LIM transcriptional codes comprising Lhx2, Lhx9 and Lmo3, which subsequently control the differential expression of Robo1 and Robo2 in the thalamus.

MATERIALS AND METHODS

Mouse and tissue preparation

All animal procedures described herein were approved by the Animal Care Committee at the University of Connecticut Health Center. All mouse strains were maintained on an outbred genetic background. Noon of the day on which a vaginal plug was detected was designated as E0.5 in the staging of embryos. The knock-in Gbx2creER allele and Gbx2mined conditional mutant allele (Gbx2*) have been described previously (Li et al., 2002; Chen et al., 2009). To perform conditional deletion of Gbx2, 4-6 mg of tamoxifen (Sigma, St Louis, MO, USA) in corn oil was administered to pregnant females carrying Gbx2creER/F; R26RlacZ by oral gavage. Two other mouse lines harboring the following mutant alleles were used in the study: Lhx2−/−, a null allele of the Lhx2 gene (Porter et al., 1997), and Lmo3lacZ, which is a lacZ knock-in and also a null allele of the Lmo3 gene (Tse et al., 2004).

Embryonic mouse brains were dissected in cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) for 40 minutes. Brains were cryoprotected, frozen in OCT freezing medium (Sakura Finetek) and sectioned.

Histochemistry, immunofluorescence and in situ hybridization

Standard protocols were used for X-gal histochemistry, immunofluorescence and in situ hybridization, as described previously (Chen et al., 2010). Detailed protocols are available on the Li Laboratory website (http://lilab.uchc.edu/protocols/index.html). Primary antibodies used in the study were as follows: rabbit anti-GFP (Invitrogen), rat anti-GFP (Nacalai Tesque), rabbit anti-Lhx9 (Santa Cruz), mouse anti-Neft (2H3-DSHB), and rabbit anti-Robo1 and rabbit anti-Robo2 (kind gifts from Dr Atsushi Tamada, Niigata University, Japan). Alexa fluorescent secondary antibodies (Invitrogen) were used.

Chimeric embryos were generated by aggregation of morula, which resulted from intercrosses between Gbx2+/−; R26RlacZ+/−; R26RFP/− embryos that were given tamoxifen at E10.5 so that the thalamic neurons and their processes were labeled with RFP. Brain explants were co-cultured with aggregates of 293T cells transfected with CMV-EGFP alone or CMV-EGFP and CMV-SLIT2 in Matrigel (BD Biosciences). After 48 hours, the cultures were mounted on slides and imaged. Axon outgrowth originating from proximal and distal quadrants was determined by measuring the total RFP fluorescence associated with the axon-occupied area using ImageJ software (NIH) as described previously (Brose et al., 1999). Statistical difference between proximal and distal areas was determined by chi-square test.

RESULTS

Gbx2 determines the initial trajectory of TCAs

We have previously generated a Gbx2creER/F; R26RlacZ/GFP:Gbx2creER/F; R26RlacZ/Gfp:Gbx2creER/F; R26RlacZ/Gfp:Gbx2 allele, which contains a creER-IRES-EGFP sequence inserted into the 5′-untranslated region of the Gbx2 locus so that the expression of both creER and EGFP mimics the endogenous Gbx2 expression (Chen et al., 2009). In embryos carrying the Gbx2creER/F; R26RlacZ/GFP:Gbx2 allele, TCAs were specifically labeled by EGFP, providing an excellent tool with which to study TCA development. Immunohistochemistry for GFP showed that TCAs extended rostrally through the prethalamus reaching the diencephalic- telencephalic border in Gbx2creER/F; R26RlacZ/GFP:Gbx2 embryos at E12.5 (Fig. 1A). Strikingly, the majority of GFP+ neurons projected dorsally and caudally, and few GFP+ axons were found in the prethalamus in E12.5 Gbx2creER/F; R26RlacZ/GFP:Gbx2 embryos, which are deficient for Gbx2 (Fig. 1B). In Gbx2creER/F; R26RlacZ/GFP:Gbx2 embryos at E14.5, immunofluorescence for GFP and neurofilament (NF) showed that TCAs were specifically labeled by GFP and GFP immunoreactivity was absent from the fasciculus retroflexus (FR) tract, which originates from the habenula (Fig. 1C). However, in Gbx2creER/F; R26RlacZ/GFP:Gbx2 embryos, GFP+ thalamic axons were mostly misrouted and abnormally present in the FR tract (Fig. 1D). To rule out the possibility that the misrouted GFP+ axons resulted from ectopic expression of GFP in Gbx2creER/F; R26RlacZ/GFP:Gbx2 embryos, we performed retrograde labeling by inserting DiI and DiA crystals in the ventral midbrain and dorsal midline of the diencephalon, respectively. In control embryos, few thalamic neurons were labeled by either DiI or DiA (supplementary material Fig. S1A-B,E). By contrast, in Gbx2creER/F; R26RlacZ/GFP:Gbx2 embryos, DiI and DiA backfilled neurons were abundantly found inside the thalamus, in additional to their normal presence in the habenula, pretectum and prethalamus (supplementary material Fig. S1C-D,F). In agreement with the previous findings (Miyashita-Lin et al., 1999; Hevner et al., 2002), TCAs were mostly absent in the cortex of Gbx2creER/F; R26RlacZ/GFP:Gbx2 embryos at E16.5 (Fig. 1F). Therefore, our data demonstrate that Gbx2 is essential for determining the initial trajectory of TCAs. In the absence of Gbx2, most thalamic axons abnormally project to the ventral midbrain along the FR tract or dorsal midline of the diencephalon, resulting in the loss of TCAs.
**Gbx2 controls thalamic efferents**

*Gbx2* expression persists in many thalamic neurons (Jones and Rubenstein, 2004; Chen et al., 2009). To investigate whether prolonged *Gbx2* expression is required for TCAs to properly navigate to the cortex, we deleted *Gbx2* by administering tamoxifen to *Gbx2<sup>creER<sup>+</sup></sup>* embryos at E12.5. *Gbx2* is also expressed in the medial ganglionic eminence (MGE). (C,D) Double immunofluorescence for GFP and neurofilament (NF) on sagittal sections of *Gbx2<sup>creER<sup>+</sup></sup>* (C) and *Gbx2<sup>creER<sup>−</sup></sup>* (D) embryos at E14.5. (E,F) Immunofluorescence for GFP on coronal sections of *Gbx2<sup>creER<sup>+</sup></sup>* (E) and *Gbx2<sup>creER<sup>−</sup></sup>* (F) embryos at E16.5. Arrows indicate normal TCAs; arrowheads show aberrant projections. ETH, epithalamus; fr, fasciculus retroflexus; IPN, interpeduncular nucleus; Ncx, neocortex; Pt, pretectum; PTh, prethalamus; Th, thalamus. Scale bar: 400 μm in A,B; 423 μm in C,D; 332 μm in E,F.

**Gbx2 continues to regulate the navigation of TCAs after they pass the prethalamus**

*Gbx2* expression is required for TCAs to properly navigate to the cortex. To test whether prolonged *Gbx2* expression is required for TCAs to navigate to the cortex, we deleted *Gbx2* by administering tamoxifen to *Gbx2<sup>creER<sup>+</sup></sup>* embryos at E13.5, when most TCAs have passed the prethalamus. The conditional knockout (CKO) embryos are designated as *Gbx2<sup>CKO</sup>*. Interestingly, removing *Gbx2* at E9.5 or E13.5 resulted in abnormal trajectories of TCAs within the diencephalon, as found in *Gbx2<sup>creER<sup>+</sup></sup>* embryos (Fig. 2B; Fig. 3D), indicating that *Gbx2* controls the axonal trajectory of newly generated thalamic neurons at various stages. Additional guidance defects of TCAs were detected in E15.5 *Gbx2<sup>CKO</sup>* embryos that were given tamoxifen at E13.5. In these mutants, many GFP<sup>+</sup> fibers failed to enter the ventral telencephalon and instead projected towards the ventral midline of the hypothalamus (Fig. 2D). Furthermore, some GFP<sup>+</sup> fibers abnormally crossed over the TCA bundle at the entry of the internal capsule (Fig. 2F). As described previously (Lund and Mustari, 1977; Rakic, 1977), TCAs mostly remain in the subplate before invading the overlying cortical plate in control embryos at E15.5 (Fig. 2G). However, increased number of TCAs were found in the cortical plate in *Gbx2<sup>CKO</sup>* mutants at E15.5 (Fig. 2H). Taken together, these results demonstrate that, in addition to its crucial role in regulating the initial trajectory of TCAs, *Gbx2* continues to regulate the navigation of TCAs and the timing to innervate the cortical plate.

**Gbx2 controls the intrinsic responsiveness of thalamic axons to guidance cues**

We have previously demonstrated that *Gbx2* has a non-cell-autonomous function in development of the thalamus (Chen et al., 2009). We thus investigated the cell autonomy of *Gbx2* function in TCA development. Because of the mosaic nature of creER-mediated recombination induced by tamoxifen, the thalamus in *Gbx2<sup>creER<sup>+</sup></sup>; R26<sup>R<sup>lacZ</sup></sup>/+ embryos that were given tamoxifen consisted of a mixture of *Gbx2<sup>creER<sup>+</sup></sup>* (functionally wild type) and *Gbx2<sup>creER<sup>−</sup></sup>* (Gbx2-deficient) cells as described previously (Chen et al., 2009) (supplementary material Fig. S2). Using a RFP cre-reporter line (Madisen et al., 2010), we showed that RFP<sup>+</sup> axons, which were derived from recombined neurons (presumably *Gbx2* deficient), abnormally projected to dopaminergic neurons in the ventral midbrain via the FR tract (Fig. 3A,B). Furthermore, thalamic efferents with abnormal trajectories appeared to originate from recombined cells, whereas non-recombined cells gave rise to normal TCAs in E13.5 *Gbx2<sup>creER<sup>+</sup></sup>; R26<sup>R<sup>lacZ</sup></sup>/+ embryos that received tamoxifen at E9.5 (Fig. 3D). These observations suggest a cell-autonomous requirement for *Gbx2* in controlling the trajectory of thalamic axons. Because of the lack of anti-*Gbx2* antibodies, we could not directly identify the individual *Gbx2*-deficient thalamic neurons in the mosaic embryos. To follow the behavior of *Gbx2*-deficient neurons, we generated chimeric embryos by aggregating *Gbx2<sup>creER<sup>−</sup></sup>* embryos with wild-type ES cells. In control chimeric embryos that were composed of wild-type and *Gbx2<sup>creER<sup>−</sup></sup>* cells, *Gbx2<sup>creER<sup>−</sup></sup>* neurons (GFP<sup>+</sup>) projected rostrally into the prethalamus at E11.5 (Fig. 3E-G). A few processes that did extend into the epithalamus traveled only for a short distance and became tangled (Fig. 3F). These observations suggest that loss of *Gbx2* expression is required for TCAs to properly navigate to the cortex.

We thus investigated whether *Gbx2* continues to regulate the intrinsic responsiveness of TCAs to guidance cues. *Slit1* and *Slit2*, which encode secreted guidance proteins, are known to regulate TCA entry and navigation into the dorsal telencephalon (Bagri et al., 2002). Explant assays have shown that Slit2 inhibits the outgrowth of thalamic axons and acts as a repellant to thalamic axons (Bonnin et al., 2007; López-Bendito et al., 2007; Braisted et al., 2009). To investigate whether loss of *Gbx2* alters the responsiveness of TCAs to *Slit2*, we performed explant co-culture experiments. As expected, control (GFP<sup>+</sup>) thalamic axons displayed avoidance to *Slit2*-expressing cells (Fig. 3K,M). However, *Gbx2<sup>creER<sup>−</sup></sup>* thalamic neurons mostly failed to respond to *Slit2* (Fig. 3L,M). Altogether, our results demonstrate that *Gbx2* regulates the intrinsic responsiveness of TCAs to guidance cues.
**GbxA regulates the expression of Robo1 and Robo2 in thalamic neurons**

The guidance defects at the junction between the diencephalon and the telencephalon in GbxA-CKO mutants share similarities to those described in mutants lacking Slit or Robo genes (Bagri et al., 2002; López-Bendito et al., 2007). Furthermore, the failure to respond to Slit2 suggests that loss of GbxA may alter Robo expression in the thalamus. As described previously (Bagri et al., 2002; López-Bendito et al., 2007), although both Robo1 and Robo2 are expressed in the medial region of the thalamus, only Robo2 transcripts are present in the lateral wall of the thalamus (Fig. 4A-B'). In the absence of GbxA, Robo1 transcripts and proteins were abnormally detected in the lateral area of the thalamus, as well as in the misrouted thalamic axons in the epithalamus (Fig. 4C-C'). Robo2 transcripts and proteins were greatly reduced in the thalamus without GbxA (Fig. 4D-D'). In control embryos at E15.5, most of the GFP\(^+\) TCAs displayed only low levels of Robo1 (Fig. 4E). By contrast, the expression of Robo1 proteins was noticeably enhanced in TCAs within the cortex of GbxA-CKO embryos (Fig. 4F). Significantly, enhanced Robo1 expression was also detected in the TCAs that precociously invaded the cortical plate in GbxA-CKO embryos (Fig. 4F), demonstrating a cell-autonomous requirement for GbxA in repressing Robo1 expression. Therefore, GbxA is essential for establishing the distinct expression patterns of Robo1 and Robo2 in the developing thalamus.

**Lmo3 is a direct transcriptional target of GbxA**

As it has been shown that Robo1 and Robo2 are direct transcriptional targets of Lhx2 (Marcos-Mondejar et al., 2012), we studied the interaction between Lhx2 and GbxA in regulating Robo1 and Robo2. We found that the expression of Lhx2 was unaltered in the thalamus lacking GbxA at E12.5 (data not shown), demonstrating that GbxA does not regulate Lhx2 at the mRNA level. The function of LIM-HD transcription factors can be negatively regulated by LIM-domain-only (LMO) proteins, which compete for LIM-HD obligate co-factor, LIM domain-binding protein (Ldb) (Bach, 2000). Significantly, loss of GbxA led to upregulation of Lmo3, one of the four members of mammalian LMO genes (Tse et al., 2004). Lmo3 is normally expressed in the epithalamus and pretectum, and was ectopically expressed in the thalamus of GbxA\(^{+/+}\) embryos at E12.5 (Fig. 5A,B). In E12.5 GbxA\(^{+/+}\) embryos that received tamoxifen at E10.5, ectopic Lmo3-expressing cells displayed a ‘salt-and-pepper’ pattern of expression in the thalamus (Fig. 5C). This pattern of ectopic expression is consistent with a cell-autonomous function of GbxA in repressing Lmo3 in mosaic mutant thalami.

To investigate whether GbxA may directly regulate Lmo3 transcription, we searched for putative GbxA binding sequences in the evolutionarily conserved genomic DNA sequence upstream of the Lmo3 promoter using rVISTA (Loots et al., 2002; Berger et al., 2008). Two GbxA-binding sites were identified within a highly conserved 510 bp DNA sequence located 1.2 kb upstream of the Lmo3 transcription start site (Fig. 5E). Electrophoretic mobility shift assay (EMSA) showed that GbxA specifically bound to these two putative binding sites (Fig. 5F; data not shown). Therefore, GbxA is required to inhibit Lmo3 transcription in the thalamus probably by binding to the Lmo3 promoter.

**Lmo3 is a feedback inhibitor of Lhx2**

Interestingly, GbxA and Lhx2 proteins have almost identical DNA-binding sequences (Berger et al., 2008), and similar temporospatial expression patterns in the thalamus (Nakagawa and O’Leary, 2001; Lakhina et al., 2007). These observations raise the interesting possibility that GbxA and Lhx2 might have opposing functions in regulating the transcription of Lmo3 by competing for binding to the same sequence in the Lmo3 promoter. Using a luciferase reporter assay in P19 cells, we showed that Lhx2 robustly activated the Lmo3 promoter, while GbxA efficiently inhibited the activity of Lhx2 (Fig. 5G,H). The transactivation was specific to Lhx2 and Lhx5, whereas other homeodomain transcription factors that were tested had no activity in the same assay (Fig. 5G). Moreover, mutating any of the two putative GbxA/Lhx2-binding sites completely abolished the activation by Lhx2 (Fig. 5G). This is in agreement with the report that LIM-HD transcriptional complexes
bind to DNA with two ‘half-binding sites’ (Thaler et al., 2002; Lee et al., 2008). Finally, we showed that, in the presence of Lmo3 protein, Lhx2 failed to activate the transcription of the Lmo3 promoter, demonstrating that Lmo3 can inhibit the transcriptional activity of Lhx2 (Fig. 5G).

To determine whether Lhx2 is indeed responsible for inducing Lmo3 in the Gbx2-deficient thalamus, we generated Gbx2creER/creER; Lhx2–/– double mutants. In support of our model, ectopic expression of Lmo3 in the thalamus was greatly reduced in Lmo3Gbx2creER/creER; Lhx2–/– embryos at E12.5 (Fig. 5D). Furthermore, in both Lhx2–/– and Gbx2creER/creER, Lhx2–/– embryos, Lmo3 expression was mostly lost in the ventral telencephalon (Fig. 5D; data not shown), demonstrating a requirement of Lhx2 in regulating Lmo3 expression was mostly lost in the ventral telencephalon (Fig. 5D; data not shown), demonstrating a requirement of Lhx2 in regulating Lmo3 expression.

Ectopic expression of Lmo3 contributes to the loss of Robo2 in Gbx2-deficient thalamus

Based on the above model (Fig. 5I), we made the following two predictions: (1) inactivation of Gbx2 would lead to functional disruption of Lhx2 through ectopic expression of Lmo3; (2) removal of Lmo3 would restore defects caused by the disrupted Lhx2 function in Gbx2 mutants. To test the first prediction, we examined whether loss of Gbx2 or Lhx2 leads to similar changes in Robo1/2 expression. The previous study has only examined Robo1/2 expression in the Lhx2-deficient thalamus at E14.5 (Marcos-Mondejar et al., 2012). Therefore, we compared the initial expression of Robo1/2 in Gbx2 and Lhx2 mutant thalami at E12.5. Similar to that found at later stages, ectopic expression of Robo1 was found in the lateral-most region of the thalamus lacking either Gbx2 or Lhx2 (Fig. 6A-C). Interestingly, the level of Robo2 expression was reduced in the thalami in Lhx2–/– embryos, similar to that found in Gbx2creER/– embryos, at E12.5 (Fig. 6D-F). These findings are consistent with our model that Gbx2 is essential for Lhx2 function in the regulation of Robo1 and Robo2 expression in the thalamus.

We next examined whether removal of Lmo3 could restore Lhx2 function as well as the expression of Robo1/2 in Gbx2 mutants. We used an Lmo3-null allele, Lmo3<sup>3mo2</sup> (Tse et al., 2004), to generate Gbx2 and Lmo3 double mutants. In agreement with our prediction, the expression of Robo2 was mostly restored in the thalamus of Gbx2<sup>2creER/creER</sup>; Lmo3<sup>3mo2z</sup> Lhx2<sup>–/–</sup> embryos at E13.5 (Fig. 6I,J). Unexpectedly, both Gbx2<sup>2creER/–</sup> and Gbx2<sup>2creER/creER</sup>; Lmo3<sup>3mo2z</sup> embryos displayed similar ectopic expression of Robo1 in the lateral wall of the thalami at E13.5 (Fig. 6G,H). Our results demonstrate that the ectopic expression of Lmo3 in Gbx2 mutants...
contributes to the loss of Robo2, but not to the ectopic expression of Robo1.

**Gbx2 negatively regulates the transcription of Lhx9 in the thalamus**

The failure to restore Robo1 expression in Gbx2creER/creER; Lmo3lacZ/lacZ embryos suggests that other factors in addition to Lmo3 and Lhx2 are involved in mediating Gbx2 function to regulate Robo1 in the thalamus. It has been reported that forced expression of Lhx2 leads to downregulated expression of Lhx9, as well as Robo1, in the thalamus (Marcos-Mondejar et al., 2012). This prompted us to investigate whether Lhx9 may act downstream of Gbx2 or Lhx2 in regulating Robo1 expression. We therefore first examined whether expression of Lhx9 is altered due to loss of either Lhx2 or Gbx2. As described previously (Nakagawa and O’Leary, 2001), Lhx9 is broadly expressed but its transcripts are clearly absent in the lateral-most area of the thalamus at E12.5 (Fig. 7A). Without Lhx2 or Gbx2, the expression domain of Lhx9 was expanded to the lateral wall of the thalamus at E12.5 (Fig. 7B,C). Immunohistochemistry revealed the same abnormal expression of Lhx9 in the thalamus lacking Lhx2 or Gbx2 (data not shown). Therefore, both Lhx2 and Gbx2 are required to inhibit Lhx9 expression in the lateral-most region of the thalamus.

To investigate the potential regulation of Robo1 by Lhx9 in the developing thalamus, we performed in situ hybridization to compare the expression of Robo1 and Lhx9 on adjacent sections of control embryos at E13.5. Compared with the expression domain of Robo1, Lhx9 transcripts were detected in cells closer to the ventricular surface (Fig. 7E-J), suggesting that Lhx9 is induced before the onset of Robo1 transcription. Robo1 and Lhx9 transcripts were mostly absent from the lateral-most area. Importantly, although the negative expression domains changed in size at different rostral-caudal levels of the thalamus, these two genes displayed an almost identical negative domain at any given position (Fig. 7E-J). In the absence of Gbx2, the expression domains of Robo1 and Lhx9 were similarly expanded to the lateral-most area of the thalamus (Fig. 7E’-J’). Finally, we found that removing Lmo3 had no effect on the ectopic expression of Robo1 and Lhx9 in Gbx2-deficient thalamus (Fig. 7E”-J”). Therefore, Lhx2 and Gbx2 both negatively regulate Lhx9. Our expression analyses suggest that Lhx9 may be responsible for the induction of Robo1, and the deregulation of Lhx9 may contribute to the ectopic expression of Robo1 in the thalamus lacking Gbx2.

**DISCUSSION**

In this study, by taking advantage of the EGFP expression that specifically labels TCAs in mice carrying the Gbx2creER allele, we have uncovered a hitherto unknown TCA phenotype of Gbx2 mutants. We demonstrate that Gbx2 regulates the intrinsic responsiveness of TCAs, at least in part, by regulating the expression of Robo1 and Robo2 receptors. We identified an opposing activity between Gbx2 and Lhx2 in their direct control of Lmo3 transcription. We showed that Lmo3 acts as a feedback inhibitor of Lhx2. Using genetic and molecular biological studies, we have identified the mechanisms by which Gbx2 regulates the LIM code in controlling Robo1 and Robo2 expression in thalamic neurons.

**Regulation of the initial trajectory of TCAs**

Although it is known that Gbx2 is essential for the formation of TCAs, the mechanism underlying the Gbx2 function has not been determined (Miyashita-Lin et al., 1999; Hevner et al., 2002). We show here that a significant proportion of thalamic efferents are misrouted caudally and dorsally in Gbx2creER/embryos (Fig. 1; supplementary material Fig. S1). Furthermore, conditionally deleting Gbx2 between E9.5 and E14.5 caused similar aberrant trajectories of thalamic efferents (Fig. 2B; Fig. 3B,D; data not...
shown). Therefore, Gbx2 plays a crucial role in establishing the initial axonal trajectory of the newly generated thalamic neurons at different embryonic stages.

Genetic studies have identified numerous molecules that are essential for TCA development (López-Bendito and Molnar, 2003; Seibt et al., 2003; Powell et al., 2008; Dwyer et al., 2011). However, mutant mice lacking any of these molecules apparently do not display the trajectory defect found in Gbx2 mutants, indicating that Gbx2 controls an unknown pathway in regulating the initial trajectory of TCAs. In the absence of Gbx2, many thalamic efferents are fasciculated with the FR tract and project to the ventral midbrain (Fig. 1B,D; Fig. 3B). The projection pattern of Gbx2-deficient thalamic neurons is remarkably similar to that of habenular neurons (Quina et al., 2009). The habenula and thalamus are both derived from the alar plate of a single diencephalic segment, called prosomere 2 (Rubenstein et al., 1994). We have previously shown that Gbx2 is exclusively expressed in the thalamus, primarily in both neural precursors that are about to exit cell cycle and postmitotic neurons (Chen et al., 2009). Therefore, Gbx2 may be important for assigning distinct characteristics, such as TCA connectivity, to postmitotic neurons arising from the alar plate of prosomere 2.

Fig. 5. Lmo3 is a transcriptional target of Gbx2 and Lhx2. (A-D) In situ hybridization of Lmo3 on coronal sections of E12.5 embryos of indicated genotypes. Gbx2creER embryos were given tamoxifen at E10.5. Arrowheads indicate ectopic expression of Lmo3 in the thalamus; asterisks show lack of Lmo3 in the thalamus and ventral telencephalon in Gbx2creER/Lhx2–/– embryos; broken lines demarcate the dorsal-caudal border of the thalamus. (E) Identification of putative Gbx2-binding sites in highly conserved genomic sequence of the mouse Lmo3 promoter region with VISTA genome browser. (F) EMSA analysis of in vitro binding of Gbx2 to the Lmo3 promoter. Arrows indicate bands corresponding to the protein-DNA complex and super-shifted complex. (G,H) Luciferase reporter analyses of the activation of the Lmo3 promoter by Lhx2 and other transcription factors (G), and the inhibition of Lhx2 by Gbx2 (H). The key shows Lmo3-luciferase (GL3) constructs without or with point mutations at the putative Gbx2/Lhx2-binding sites. Co-transfection of Gbx2, as little as 1/100 the amount of Lhx2, can significantly inhibit Lhx2 activity, and mutating either or both binding sites abolishes the activation of the Lmo3 promoter by Lhx2. Data are mean±s.d. Asterisks indicate significant differences (***P<0.001, *P<0.05; Student’s t-test). (I) The regulation of the LIM code by Gbx2 in thalamic neurons. Gbx2 normally occupies the Lmo3 promoter, preventing Lhx2 from activating Lmo3 transcription; in the absence of Gbx2, Lhx2 binds to Lmo3 promoter and leads to production of Lmo3 proteins, which in turn inhibit Lhx2 function by disrupting the Lhx2-Ldb transcription complex. Scale bar: 250 μm in A-D.
Pathfinding defects of TCAs and abnormal Robo expression in the absence of Gbx2

By using chimera and mosaic analyses, we demonstrate that Gbx2 acts cell-autonomously to control the extension of TCAs into the prethalamus. Furthermore, deleting Gbx2 after the TCAs have passed the prethalamus led to TCA guidance defects and accelerated invasion into the cortical plate (Fig. 2J). As conditional deletion using creER creates genetic mosaicism (supplementary material Fig. S2) (Chen et al., 2009; Sunmonu et al., 2011), the guidance defects in Gbx2-CKO mutants thus reflect a cell-autonomous function of Gbx2. These in vivo studies, together with the explant assays (Fig. 3K-M), clearly demonstrate that Gbx2 regulates the outgrowth and pathfinding of TCAs by modifying their responsiveness to guidance cues en route to the neocortex.

To control TCA guidance, Gbx2 probably regulates guidance receptors and intracellular signaling in thalamic neurons and TCAs. In the current study, we have mainly focused on Robo1 and Robo2 because of their important roles in TCA development. The guidance defects of TCAs outside the diencephalon in Gbx2-CKO mutants share similarities with those reported in mouse embryos lacking Slit1 and Slit2 or Robo1 and Robo2 (Bagri et al., 2002; Andrews et al., 2006; López-Bendito et al., 2007), as well as in Lhx2-CKO mutants that exhibit ectopic expression of Robo1 (Marcos-Mondejar et al., 2012). Therefore, altered expression of Robo1 and Robo2 probably accounts for the guidance defects found in the telencephalon in Gbx2-CKO mutants. Furthermore, studying the regulation of Robo1/2 has allowed us to define an intricate genetic network regulated by Gbx2 in the developing thalamus. Significantly, it has recently been shown that Slit/Robo interactions play an important role in the topographic sorting of TCAs in the ventral telencephalon (Bielle et al., 2011a). It has been demonstrated that differential expression of Robo1 and Robo2 (robo and lea – FlyBase), which interact with Slit signaling from the midline, determines the lateral position of post-crossing commissural and longitudinal axons in Drosophila (Rajagopalan et al., 2000; Simpson et al., 2000). Similarly, the differential expression of Robo1 and Robo2 in different thalamic nuclei may form a Robo code to interpret Slit signals in establishing the TCA topography in the ventral telencephalon. We have previously demonstrated that different thalamic nuclei display distinct and dynamic expression of Gbx2 (Chen et al., 2009). Therefore, regulating Robo1 and Robo2 receptor levels through Gbx2 activity in TCAs derived from different thalamic nuclei may provide an important mechanism with which to establish the topographic projections of TCAs.

Regulation of the LIM code by Gbx2 leads to differential expression of Robo1 and Robo2 in thalamic neurons

In Drosophila wing disc, the Lhx2 homolog Apterous induces expression of LMO protein, which in turn represses the activity of Apterous (Milán et al., 1998). In the current study, we have identified a similar feedback mechanism between Lhx2 and Lmo3 in the developing thalamus. Furthermore, we demonstrate that Gbx2 blocks Lmo3 transcription by competing for the binding of Lhx2 to the Lmo3 promoter. Therefore, Gbx2 exerts its regulatory role in the developing thalamus in part by regulating an evolutionarily conserved Apterous-Lmo feedback loop. We show that the positive regulation of Lhx2 activity by Gbx2 is essential for Robo2 expression in the thalamus (Fig. 7K). In contrast to Lhx2, Lhx9 is negatively regulated by Gbx2 at the transcriptional level. Although definitive evidence awaits further study, our data suggest that Lhx9 may mediate Gbx2 function in regulating the expression of Robo1 (Fig. 7K). We found that the expression patterns of Lhx9 and Robo1 in the thalamus almost completely mirrored each other, not only in wild type but also in Gbx2 mutant embryos (Fig. 7E-J'). Furthermore, removing Lmo3 had no effects on the ectopic expression of either Lhx9 or Robo1 in the Gbx2-deficient thalamus (Fig. 7E'-J'). Altogether, these observations suggest that Lhx9 positively regulates Robo1 expression, and that the mis-regulation of Lhx9

Fig. 6. Gbx2 positively regulates Lhx2 activity in the control of Robo1 and Robo2 expression. In situ hybridization on coronal sections of E12.5 (A-F) and E13.5 (G-J) embryos. RNA probes and genotypes are indicated to the left and on the top. Broken lines demarcate the dorsal-caudal border of the thalamus; arrows indicate the ectopic expression of Robo1 in the MZ of the thalamus; asterisks show the reduction of Robo2; the arrowhead indicates the rescued Robo2. Scale bar: 250 μm in A-J.

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in Gbx2-deficient thalamus may be responsible for the ectopic expression of Robo1 in the same region.

It has been reported that Lhx2 negatively regulates Robo1 and Robo2, probably by direct transcriptional regulation (Marcos-Mondejar et al., 2012). As Lhx2 negatively regulates the expression of Lhx9 (Fig. 7B), the mis-regulation of Lhx9 may contribute to the altered expression of Robo1 following the manipulations of Lhx2 in the previous study. In contrast to our current findings, it was shown that gain or loss of function of Lhx2 resulted in the reduction or increase, respectively, in the level of Robo2 expression. Differences in experimental approaches may contribute to this apparent discrepancy. For example, the previous study examined Robo2 expression only at E14.5, while we have focused on the initial expression of Robo2 in the thalamus. In the current study, we examined Robo2 transcripts in the thalamus in global Lhx2-KO mutants.

In summary, our results demonstrate that Gbx2 regulates the expression of Robo2 in the thalamus by repressing Lmo3 and consequently promoting Lhx9 transcription in this region. For example, the previous study examined Robo2 expression only at E14.5, while we have focused on the initial expression of Robo2. Importantly, in the previous study, deletion of Lhx2 was achieved by tamoxifen-induced deletion using the Gbx2creER knock-in mouse line after E10.5, whereas gain of function was performed by in utero electroporation at E13.5. As the expression of Lhx2 and Gbx2 are initiated in the thalamus around the same time at E10.5, those manipulations will only change Lhx2 expression after its initially normal expression. Furthermore, those manipulations will alter Lhx2 expression in thalamic neurons in a mosaic manner. In the current study, we examined Robo2 transcripts in the thalamus in global Lhx2-KO mutants.

Concluding remarks

Here, we have identified at least three different mechanisms through which Gbx2 modifies the LIM transcriptional code: (1) Gbx2 regulates Lhx2 function by repressing its inhibitor Lmo3; (2) Gbx2 competes for binding of Lhx2 to the same DNA sequences of the Lmo3 promoter; (3) Gbx2 negatively regulates the transcription of Lhx9. Although Gbx2 and Lhx2 antagonize each other when regulating Lmo3 expression, they both promote Robo2 expression. Therefore, the interaction between Gbx2 and Lhx2 must be highly context dependent. It is conceivable that there are many other Lmo3-like or Robo2-like transcriptional targets that are regulated by both Gbx2 and Lhx2 in the developing thalamus. The relative stoichiometries of Gbx2 and Lhx2 are thus important in conferring distinct identity and connectivity of thalamic neurons.
During embryogenesis, the thalamus is gradually parcellated into multiple nuclei. Each nucleus has distinct cytoarchitecture and function, and projects to a specific set of cortical areas. Gbx2, Lhx2 and Lhx9 are expressed in discrete and overlapping domains of the thalamus, and different thalamic nuclei display distinctive expression patterns of Gbx2 (Nakagawa and O'Leary, 2001; Chen et al., 2009). Gbx2 and LIM-HD transcription factors may thus constitute the core of the regulatory network that define the diverse identity and connectivity of thalamic nuclei.

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Competing interests statement
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Supplementary material availability online at Supplementary material

Supplementary material available online at

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