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Transcription factor Gbx2 acts cell-nonautonomously to regulate the formation of lineage-restriction boundaries of the thalamus

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Relatively little is known about the development of the thalamus, especially its differentiation into distinct nuclei. We demonstrate here that *Gbx2*-expressing cells in mouse diencephalon contribute to the entire thalamic nuclear complex. However, the neuronal precursors for different thalamic nuclei display temporally distinct *Gbx2* expression patterns. *Gbx2*-expressing cells and their descendents form sharp lineage-restriction boundaries delineating the thalamus from the pretectum, epithalamus and prethalamus, revealing multiple compartmental boundaries within the mouse diencephalon. Without *Gbx2*, cells originating from the thalamus abnormally contribute to the epithalamus and pretectum. This abnormality does not result from an overt defect in patterning or cell-fate specification in *Gbx2* mutants. Chimeric and genetic mosaic analysis demonstrate that *Gbx2* plays a cell-nonautonomous role in controlling segregation of postmitotic thalamic neurons from the neighboring brain structures that do not express *Gbx2*. We propose that, within the developing thalamus, the dynamic and differential expression of *Gbx2* may be involved in the specific segregation of thalamic neurons, leading to partition of the thalamus into different nuclei.

KEY WORDS: Thalamus, Compartment, Lineage restriction, Fate map, Mouse, Transcription factor, *Gbx2*

INTRODUCTION

The thalamus in mammals is composed of dozens of nuclei, which are aggregates of neurons, and each nucleus displays unique cytoarchitecture and function (Jones, 2007). Some thalamic nuclei project topographically to specific areas of the cortex, having a primary role in processing and relaying sensory input from the periphery to the cortex; other nuclei broadly project to the cortex, regulating the states of consciousness of the cortex (Jones, 2001; Jones, 2007). The thalamus develops from the diencephalic part of the neural tube. Based on morphology and gene expression, the vertebrate diencephalon is transiently divided into three transverse segments called prosomeres 1-3 (p1-3), which are believed to give rise to the pretectum, epithalamus and thalamus, and prethalamus, respectively (Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003). Virtually all thalamic neurons in mice are generated from the alar plate of the p2 segment between embryonic day (E) 10.5 and E16.5 (Angevine, 1970). Between E14.5 and E18.5, the diencephalon is progressively partitioned into discrete neuronal groups that signify their differentiation into nuclei (Jones, 2007). Little is known about the mechanism governing the selective segregation of postmitotic neurons to form the thalamic nuclear complex.

The p2 segment of the diencephalon is defined by the expression of a homeobox gene, *Gbx2*, in the mouse embryo (Bouillet et al., 1995; Bulfone et al., 1993; Miyashita-Lin et al., 1999; Nakagawa and O'Leary, 2001). In both mouse and monkey, *Gbx2* expression is restricted to a subset of thalamic nuclei at birth and in adulthood (Jones and Rubenstein, 2004). Deletion of *Gbx2* in mice leads to an almost complete loss of axonal connections between the cortex and the thalamus (Hevner et al., 2002; Miyashita-Lin et al., 1999). In addition, *Gbx2*-deficient mice exhibit severe defects in histogenesis of the thalamus and loss of a subset of thalamic nuclei, suggesting that *Gbx2* may play an important role in differentiation of thalamic nuclei (Miyashita-Lin et al., 1999; Nakagawa and O'Leary, 2001). However, the molecular and cellular basis of the thalamic defects due to the mutation of *Gbx2* is largely unknown.

The present study examines the development of the thalamus by analyzing the behavior of *Gbx2*-expressing cells in the diencephalon of wild-type and *Gbx2*-mutant embryos. Using a novel *Gbx2-CreER-ires-Egfp* knock-in mouse line to carry out inducible genetic fate-mapping study, we determine the cell fate of *Gbx2*-expressing cells in the diencephalon at different embryonic stages. Our data show that the *Gbx2*-expressing cells and their descendents contribute to the entire thalamic nuclear complex, but not structures that are derived from the pretectum epithalamus and prethalamus, demonstrating that the thalamus is a developmental compartment. We also show that *Gbx2* is essential for maintaining the integrity of the boundaries surrounding the developing thalamus. Finally, we show that *Gbx2* acts cell-nonautonomously in controlling the histogenesis and boundary formation of the thalamus.

MATERIALS AND METHODS

Generation of Gbx2$^{CreER/+}$ mouse line

A *CreER(T2)-ires-Egfp-neo* cassette was inserted into the 5′ untranslated region (UTR) of the *Gbx2* locus by homologous recombination in mouse embryonic stem (ES) cells (Fig. 1A). From 63 G418 resistant cell colonies, seven correctly targeted ES cell clones were identified by Southern blot analysis (Fig. 1B). Germline chimeras were generated from two independent ES clones, which provided the identical phenotype. The *neo* cassette, which was flanked by two *Frt* sites, was removed in vivo by the *hACTB-FLP* transgene (Rodriguez et al., 2000). The new *Gbx2* knock-in allele is designated as *Gbx2^{CreER}*.

Mouse breeding and genotyping

Mice were maintained on an outbred CD1 genetic background (Charles River Lab, Wilmington, MA). Noon of the day on which the vaginal plug was found was designated as E0.5. For inducible genetic fate mapping, *Gbx2$^{CreER/+}$*; *R26R$^{-/-}$* males, homozygous for the *Cre* reporter *R26R*

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(Soriano, 1999), were bred with wild-type or *Gbx2*$^{+/-}$ females (Wassarman et al., 1997). Four to six milligrams of tamoxifen (Sigma) in corn oil (20 mg/ml) was administered by oral gavage to pregnant females as described previously (Li and Joyner, 2001). Genotypes of mice were determined by PCR analysis. For PCR analysis of mosaic deletion, 200 μm brain slices were obtained by vibratome sectioning, and the thalamus, which is demarcated by EGFP, of *Gbx2*$^{CreER/F}$; *R26R*$^{+/-}$ embryos at E16.5 was dissected under a fluorescent stereoscope. The following primers were used to distinguish the floxed and deletion *Gbx2* alleles (Fig. 7I,J): Gbx2-F1, GTTCGCTCCACAGCCACT; Gbx2-R, TGCTTGGATGTCCACATC-TAGG.

Generation of Gbx2-deficient ES cells and chimera analysis

Gbx2$^{+/flox\text{-}neo}$ ES cells, which were described previously (Li et al., 2002), were cultured with a high concentration of G418 (6 mg/ml). We screened 150 colonies and identified four *Gbx2*$^{flox\text{-}neo/flox\text{-}neo}$ ES cell clones by Southern blot analysis. Transient transfection of *Cre* into *Gbx2*$^{flox\text{-}neo/flox\text{-}neo}$ ES cells resulted in *Gbx2*-deficient ES cells (*Gbx2*$^{-/-}$). To generate chimeras, *Gbx2*$^{-/-}$ or control *Gbx2*$^{+/flox\text{-}neo}$ ES cells were injected into blastocysts that carried the *ROSA26* gene trap insertion, which expresses β-galactosidase (β-gal) ubiquitously (Friedrich and Soriano, 1991).

β-galactosidase, BrdU labeling, immunofluorescence and in situ hybridization

Embryos or brains were processed for in situ hybridization as described previously (Guo and Li, 2007). Standard X-gal staining was used to examine β-gal activities (Nagy et al., 2003). BrdU labeling was performed as described previously (Li et al., 2002). Pregnant females were injected intraperitoneally with 100 μg BrdU per gram of body weight 1.5 hours before they were sacrificed. Detailed protocols are available in the Li lab website (http://www.genetics.uchc.edu/lilab/Pages/Protocols.html). Antibodies used in the study are the following: rabbit anti-GFP (Invitrogen), mouse anti-BrdU (BD), mouse anti-TuJ1 (Covance) and Alexa fluorescent secondary antibodies (Invitrogen).

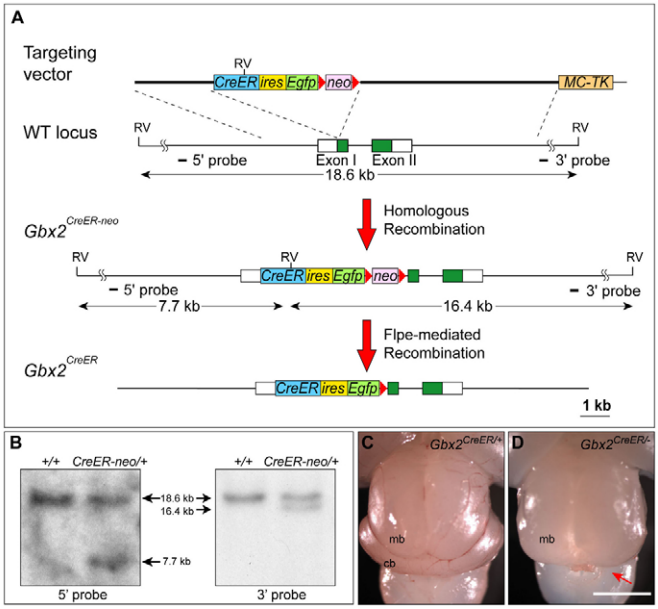

Fig. 1. Generation of a *Gbx2-CreER-ires-Egfp* knock-in allele. (**A**) Schematic representation of gene targeting strategy. The thick line in the targeting vector represents *Gbx2* genomic DNA with an insertion of *CreER-ires-Egfp-neo cassette* at the 5′ untranslated region of the mouse *Gbx2* gene. The selectable marker, *neo*, is flanked by two *Frt* sites (red triangles). (**B**) Southern blot identification of targeted ES cell clones using the 5′ and 3′ probes after digestion of genomic DNA with *Eco*RV restriction enzyme (RV). (**C**,**D**) Dorsal view of E18.5 *Gbx2*$^{CreER/+}$ (C) and *Gbx2*$^{CreER/-}$ (D) brains. Arrow indicates the absence of the cerebellum in *Gbx2*$^{CreER/-}$ embryos. cb, cerebellum; mb, midbrain. Scale bar: 1.7 mm.

RESULTS

Generation of a knock-in mouse line with simultaneous expression of CreER and EGFP recapitulating endogenous Gbx2 expression

To gain more insight into the function of *Gbx2*, we generated a knock-in mouse line *Gbx2*^{CreER}, in which *CreER-ires-Egfp* was targeted into the 5′ UTR of the *Gbx2* gene (Fig. 1A). As the *CreER-ires-Egfp* cassette contains polyadenylation signals and stop codons, we expected that the insertion would result in a null mutation of *Gbx2*. Indeed, homozygous *Gbx2*$^{CreER/CreER}$ or compound heterozygous *Gbx2*$^{CreER/-}$ mutants, which contain a previously characterized *Gbx2*-null allele, exhibited an identical phenotype to *Gbx2*$^{-/-}$ mutants, demonstrating that *Gbx2*^{CreER} is a null allele (Miyashita-Lin et al., 1999; Wassarman et al., 1997) (Fig. 1C,D and see below).

In *Gbx2*$^{CreER/+}$ embryos, *CreER* transcripts and EGFP proteins were detected in the same domain as the endogenous *Gbx2* expression between E10.5 and E16.5 (Fig. 2A-C and data not shown). In the diencephalon of *Gbx2*$^{CreER/+}$ embryos at E12.5, EGFP was detected in cells in the mantle zone and their axons, which traversed through the prethalamus toward ventral telencephalon (Fig. 2C,D,F,H). To determine if *Gbx2*-expressing cells in the diencephalon are postmitotic, we performed colocalization studies of EGFP and BrdU in *Gbx2*$^{CreER/+}$ embryos with BrdU pulse (1.5 hour) labeling at E12.5. EGFP and BrdU signals were found largely mutually exclusive (Fig. 2D-E″). We also performed colocalization analysis of EGFP and the mitotic marker phosphorylated histone H3 (pH3) on serial coronal sections of the thalamus of E12.5 *Gbx2*$^{CreER/+}$ embryos by confocal microscopy (Hendzel et al., 1997). At the rostral and caudal level, EGFP- and pH3-positive cells were largely segregated (data not shown). At the middle level, pH3 signals were detected in a broad domain outside the ventricular layer of the thalamus, with some positive cells embedding in the EGFP-positive domain (Fig. 2F). We did detect a few pH3-positive cells with weak GFP signals (Fig. 2G-G″). However, the majority of the pH3-positive cells were negative for EGFP. Furthermore, examination of a marker for postmitotic neuronal precursors, neuronal class III β-tubulin (TuJ1), revealed that EGFP was completely colocalized with TuJ1 in the diencephalon of *Gbx2*$^{CreER/+}$ embryos at E11.5 (Fig. 2F-G″). These data demonstrate that *Gbx2* is primarily expressed in the neuronal precursor cells that have exited the cell cycle in the diencephalon.

The Gbx2-expressing cells and their descendents form a self-contained compartment corresponding to the entire thalamus

To examine the fate of *Gbx2*-expressing cells in the diencephalon, we performed inducible genetic fate mapping by combining *Gbx2*^{CreER} and the *R26R* reporter alleles (Soriano, 1999). In *Gbx2*$^{CreER/+}$*; R26R* embryos, Cre-mediated recombination at the *R26R* locus in cells that express activated CreER will result in permanent expression of β-gal in these cells and all their descendents (Joyner and Zervas, 2006). Activation of CreER is achieved by administration of tamoxifen to pregnant females carrying *Gbx2*$^{CreER/+}$*; R26R* embryos. We administered tamoxifen at E10.5, when *Gbx2* is already expressed in the diencephalon (Nakagawa and O'Leary, 2001), and assessed embryos 24 hours

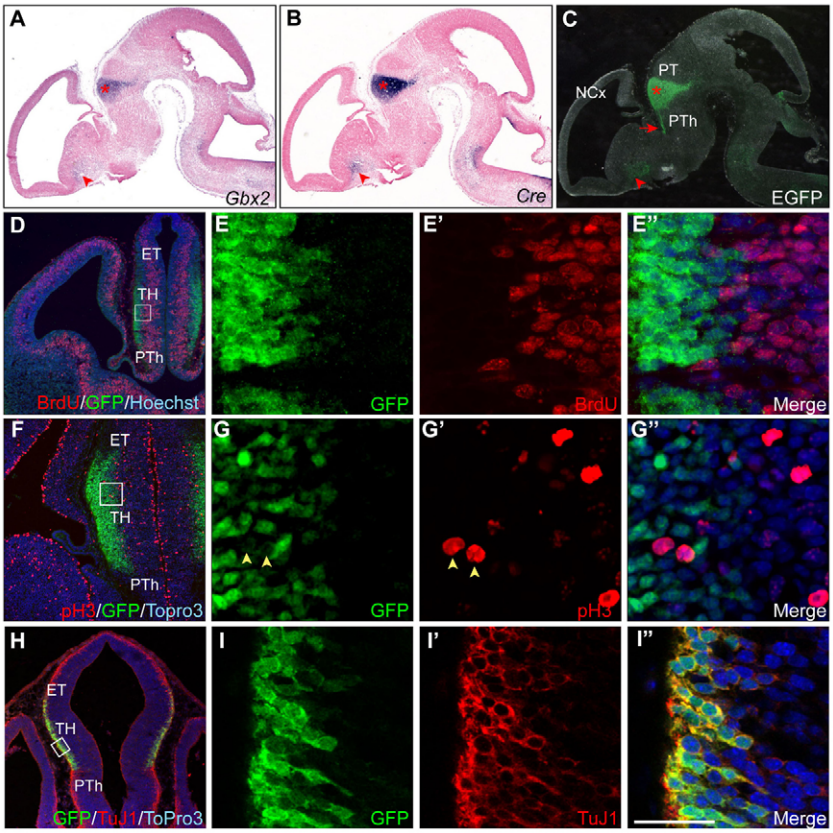

Fig. 2. Expression of CreER and EGFP recapitulates the endogenous *Gbx2* in *Gbx2*$^{CreER/+}$ embryos. (**A-C**) Expression of *Gbx2* (A) and *CreER* (B) transcripts, and EGFP proteins (C) on adjacent sagittal brain sections of *Gbx2*$^{CreER/+}$ embryos at E12.5. The expression in the thalamus and the medial ganglionic eminence is marked by the asterisk and arrowhead, respectively. The arrow indicates thalamic axonal processes. (**D-E″**) Double-immunofluorescence analysis of BrdU and GFP on coronal brain sections of *Gbx2*$^{CreER/+}$ embryos at E12.5. The boxed area in D is magnified in E-E″. (**F-G″**) Confocal images of immunofluorescence of pH3 and GFP on coronal brain sections of *Gbx2*$^{CreER/+}$ embryos at E12.5. The boxed area in F is magnified in G-G″. Note that most of the pH3-positive cells are negative for GFP, whereas two pH3-positive cells that display weak GFP immunoreactivity are marked by the yellow arrowheads. (**H-I″**) Confocal images of immunofluorescence of GFP and TuJ1 on coronal brain sections of *Gbx2*$^{CreER/+}$ embryos at E11.5. The boxed area in H is magnified in I-I″. ET, epithalamus; mb, midbrain; Ncx, neocortex; PT, pretectum; PTh, prethalamus; TH, thalamus. Scale bars: 1034 μm in A-C; 400 μm in D; 300 μm in F; 600 μm in H; 50 μm in E-E″,G-G″,I-I″.

later to delineate the initially marked cohort of *Gbx2*-expressing cells. β-Gal-positive cells were detected in the diencephalon, anterior hindbrain and spinal cord of *Gbx2*$^{CreER/+}$*; R26R* embryos (Fig. 3B and data not shown). This pattern of β-gal expression was remarkably similar to that of *Gbx2* expression at E10.75, demonstrating that the activation of CreER by tamoxifen faithfully labels *Gbx2*-expressing cells in *Gbx2*$^{CreER/+}$*; R26R* embryos (Fig. 3A). No β-gal-positive cells were detected in *Gbx2*$^{CreER/+}$*; R26R* embryos ($n \geq 10$) without tamoxifen administration, demonstrating that Cre activity from the *Gbx2*^{CreER} allele is tamoxifen-dependent (see Fig. S1A in the supplementary material). In agreement with previous reports (Hayashi and McMahon, 2002; Zervas et al., 2004), we found that labeling of *Gbx2*-expressing cells in the diencephalon was largely restricted to a window of 6 to 36 hours after tamoxifen administration (see Fig. S1B-E in the supplementary material).

We next examined the fate of *Gbx2*-positive cells in the diencephalon marked at E10.5. Analysis of β-gal activity in whole-mount brains at E18.5 showed that marked cells were confined to the presumptive thalamus in *Gbx2*$^{CreER/+}$ embryos (Fig. 3C). Histological analysis revealed that the fate-mapped cells contributed broadly to the thalamus and formed remarkably sharp boundaries delineating the thalamus from the neighboring brain structures (Fig. 3D-F). Examination of X-gal and Nissl staining on adjacent sections revealed that the anterior and posterior boundaries delineated by β-gal-positive cells strictly coincided with the histological borders demarcating the thalamus from the pretectum and the prethalamus (Fig. 4E,G). Furthermore, the β-gal-positive cells defined a clear dorsal border separating the thalamus from the epithalamus, with only a few marked cells in the lateral habenular nuclei (Fig. 4M,O). The ventral border of the thalamus was also clearly defined by the marked cells, except for some marked cells in the nucleus of Darkschewitsch (Fig. 4E), which is presumably derived from the basal plate of p1 and p2 (Puelles and Rubenstein, 2003).

Thalamic cells that express Gbx2 at different stages form distinct groups of thalamic nuclei

Previous data on *Gbx2* expression in the developing thalamus between E10.5 and postnatal day 2 (P2) have suggested that *Gbx2* is specifically expressed and maintained in subsets of thalamic neurons that form the anterior and medial groups of thalamic nuclei (Jones and Rubenstein, 2004; Nakagawa and O'Leary, 2001). Surprisingly, we found that the marked descendents of *Gbx2*-expressing cells labeled by tamoxifen administration at E10.5 broadly contribute to the thalamus in *Gbx2*$^{CreER/+}$*; R26R* embryos (Fig. 3C-F). To resolve this apparent inconsistency, we sought to examine if *Gbx2*-expressing cells at other stages might have preferential contribution to particular thalamic nuclei.

As CreER is active within a window of 6-36 hours after the administration of tamoxifen, we gave tamoxifen at E9.5, and determined the contribution of the initial *Gbx2*-expressing thalamic cells in *Gbx2*$^{CreER/+}$*; R26R* mice at P15, when various thalamic nuclei can be identified by Nissl histology (Caviness and Frost, 1980; Jones, 2007). The marked cells were found in the lateral-posterior and ventral thalamic nuclei groups (L, LGd, VM, VL, VB, Pom, VMb, LP and MG), but not the anterior and medial thalamic nuclei group (Fig. 5A-C; Table 1). The fate-mapped cells labeled at E10.5 were present in most of the thalamic nuclei in the caudal and lateral regions of the thalamus, whereas the rostromedial-most nuclei contained a dramatically reduced number of β-gal-positive cells (Fig. 5D-F; Table 1). When tamoxifen was administered at E15.5, β-gal-positive cells were found in the anterior (AD, AM and

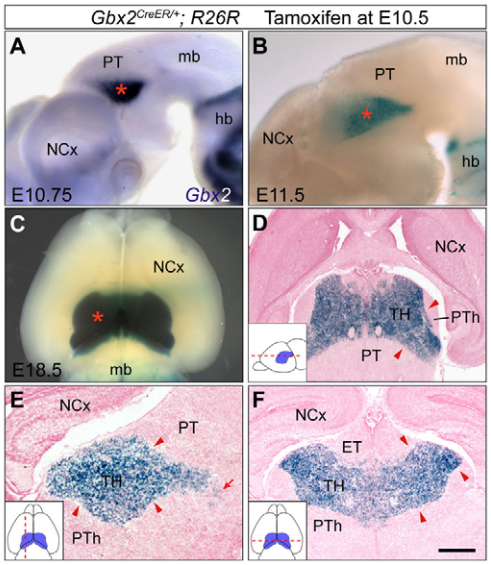

Fig. 3. The thalamus is a developmental compartment. (**A**) Whole-mount in situ hybridization showing that *Gbx2* expression demarcates the alar plate of p2 (asterisk) at E10.75. (**B**) Whole-mount X-gal staining of *Gbx2*$^{CreER/+}$; *R26R* embryo at E11.5 showing the initial population of *Gbx2*-expressing cells (asterisk) labeled at E10.5. (**C-F**) Analysis for β-gal activity in whole-mount (C), horizontal (D), sagittal (E) and coronal (F) sections of *Gbx2*$^{CreER/+}$; *R26R* embryos at E18.5 after administration of tamoxifen at E10.5. Insets in D-F indicate the sectioning plane; the arrowheads mark the sharp border; the arrow indicates a few marked cells in the nucleus of Darkschewitsch. ET, epithalamus; hb, hindbrain; mb, midbrain; Ncx, neocortex; PT, pretectum; PTh, prethalamus; TH, thalamus. Scale bars: 400 μm in D-F.

AV) and medial (Ce, Cl, MD, Pc, PT, PV and Re) thalamic nuclei, and were largely absent from posterior and ventral nuclei groups except for MG and LP (Fig. 5G-I; Table 1). Therefore, our data show that, while all thalamic nuclei are derived from the *Gbx2* lineage, the precursors for different groups of thalamic nuclei display distinct temporal expression patterns of *Gbx2*.

Gbx2 is required for forming the dorsal and posterior, but not the anterior and ventral, borders of the thalamus

The remarkably sharp *Gbx2*-lineage borders led us to investigate if *Gbx2* is required for the boundary formation by fate mapping *Gbx2*-transcribing cells in *Gbx2*$^{CreER/-}$; *R26R* embryos, which lack *Gbx2* function (Fig. 1C,D). When tamoxifen was administered to pregnant *Gbx2*$^{+/-}$ females that were mated with *Gbx2*$^{CreER/+}$; *R26R*$^{-/-}$ males at E10.5, the distribution of β-gal-positive cells in the diencephalon was indistinguishable between *Gbx2*$^{CreER/+}$; *R26R* and *Gbx2*$^{CreER/-}$; *R26R* embryos at E11.5 and E12.5 (Fig. 4A-B,I-J; and data not shown). These results demonstrate that the initial transcription of the *Gbx2* locus in thalamic precursors is independent of Gbx2 protein activity. This enabled us to examine whether the marked thalamic cells continued to be restricted to the thalamic compartment without *Gbx2*.

We found that the morphology of the thalamus in *Gbx2*$^{CreER/-}$; *R26R* was severely disrupted after E14.5, similar to that found in *Gbx2*$^{-/-}$ mutants (Miyashita-Lin et al., 1999). The thalamus, which was demarcated by the marked descendents of *Gbx2*-transcribing cells labeled at E10.5, was apparently reduced in the mediolateral dimension but expanded in the ventrodorsal dimension, resulting in an abnormal shape (Fig. 4D,F,L,N). A large number of marked descendants of *Gbx2*-transcribing cells labeled at E10.5 were across the dorsal and posterior borders of the thalamus expanding into the epithalamus and the pretectum, respectively, in *Gbx2*$^{CreER/-}$; *R26R* embryos at E14.5 (Fig. 4D, and data not shown). The marked cells that crossed the lineage border were mainly found in the lateral habenular nuclei (Fig. 4N,P) and the anterior part of the pretectum (Fig. 4F,L) in the mutants at E18.5. In contrast to the dorsal and the posterior borders, the anterior and ventral borders of the thalamus were much less affected, with the fate-mapped cells demarcating a clear thalamus-prethalamus boundary in *Gbx2*$^{CreER/-}$; *R26R* embryos at E18.5 (Fig. 4F,N). In agreement with the cell-fate mapping data, histological analysis by Nissl staining revealed that the dorsal and posterior, but not the anterior and ventral, borders of the thalamus were disrupted in *Gbx2*$^{CreER/-}$; *R26R* embryos (Fig. 4G-H,Q-R). Collectively, our data demonstrate that *Gbx2* is required for the formation of the dorsal and posterior boundaries separating the thalamus from the epithalamus and the pretectum, respectively. However, a *Gbx2*-independent mechanism is involved in the development of the anterior and ventral boundaries of the thalamus.

Loss of Gbx2 does not result in a major patterning defect in the diencephalon

The severe disruption in the histogenesis and the dorsal and posterior borders of the thalamus in *Gbx2* mutants prompted us to examine by marker analysis if *Gbx2* is required for maintaining the fate of thalamic cells. At E12.5, the expression domains of *Gbx2* and *Dlx2/5* demarcate the thalamus and prethalamus, respectively, while *Shh* is expressed in the zona limitans intrathalamica (ZLI) at the interface between the thalamus and prethalamus (Bulfone et al., 1993) (Fig. 6A-D). In agreement with a previous study (Miyashita-Lin et al., 1999), the transcripts of truncated *Gbx2* were detected in the same domain in the lateral wall of the diencephalon in *Gbx2*$^{CreER/-}$ embryos at E12.5 as that found in wild-type embryos (Fig. 6A,E). Furthermore, in *Gbx2*$^{CreER/-}$ embryos at E12.5, *Dlx2/5* and *Shh* were each detected in the same domain as those in wild-type embryos (Fig. 6E-H). *Lhx1, Pax3* and *Pax7* are expressed in the pretectum at E13.5 (Fig. 6I,J; and data not shown). In addition, *Lhx1* is also expressed in the ZLI (Fig. 6I). Again, no difference in *Lhx1, Pax3* and *Pax7* expression was observed in *Gbx2*$^{-/-}$ embryos (Fig. 6L,M; data not shown). Examination of another pretectum marker, *Bhlhb4*, which encodes a basic helix-loop-helix transcription factor (Bramblett et al., 2002), showed that its expression was also normally restricted to the anterior pretectum in *Gbx2*$^{-/-}$ embryos at E13.5 as in wild type (Fig. 6K,N). Together, our data suggest that the abnormal histogenesis and the disruption of thalamic boundaries in *Gbx2* mutants do not result from obvious defects in patterning or cell-fate specification in the diencephalon.

Gbx2 plays a cell-nonautonomous function in the formation of thalamic boundaries

Differential affinities for cell-cell interactions have been proposed as a basic mechanism for separating cells into distinct compartments (Irvine and Rauskolb, 2001; Kiecker and Lumsden, 2005). Indeed, members of the Cadherin family and other cell adhesion molecules are expressed in stripes or patches in the diencephalon with their expression coinciding with prosomeric borders or developing thalamic nuclei (Gao et al., 1998; Mackarehtschian et al., 1999; Redies et al., 2000; Yoon et al., 2000). We therefore probed the possibility that *Gbx2* controls

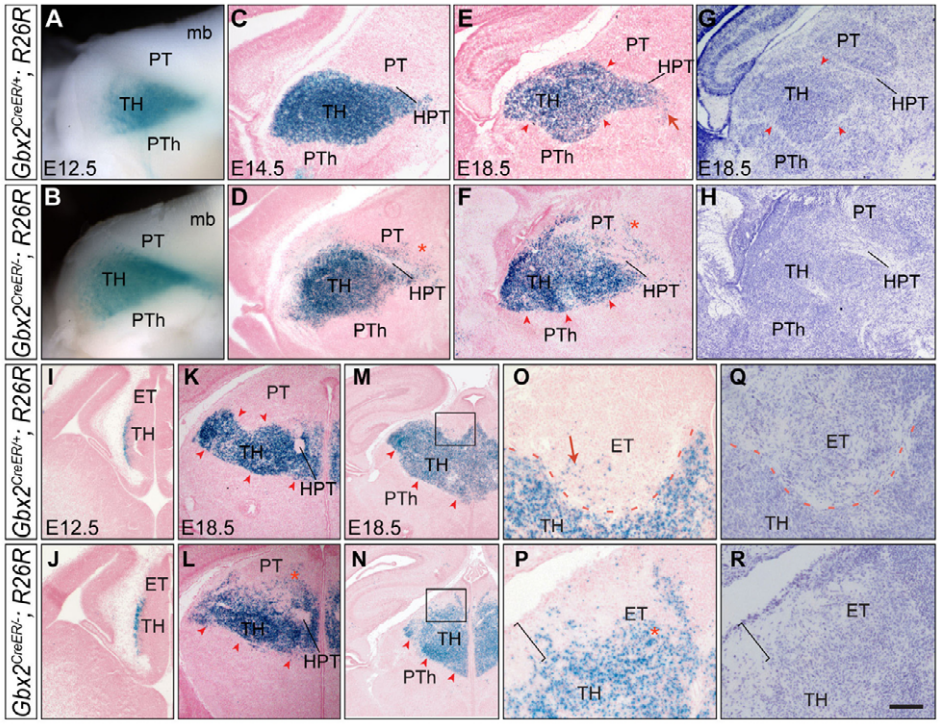

Fig. 4. Loss of *Gbx2* disrupts the dorsal and posterior borders of the thalamus. (**A-F**) After tamoxifen administration at E10.5, X-gal staining of whole-mount (A,B) and sagittal brain sections of *Gbx2*$^{CreER/+}$ and *Gbx2*$^{CreER/-}$ embryos (C-F) at different stages, as indicated. (**G**,**H**) Nissl analysis of immediate adjacent sections of E and F, respectively. (**I-P**) After tamoxifen administration at E10.25 (I-L) or E10.5 (M-P), X-gal analysis of coronal brain sections of *Gbx2*$^{CreER/+}$ and *Gbx2*$^{CreER/-}$ embryos at E12.5 (I,J) and E18.5 (K-P). The boxed area in M and N is magnified in O and P. (**Q**,**R**) Nissl analysis of adjacent sections of O and P, respectively. The arrowheads indicate the lineage-restriction boundaries; the asterisks indicate cells that violate compartment boundaries; the red dashed line indicates the border between the thalamus and lateral habenular nucleus; the arrow indicates marked cells in the nucleus of Darkschewitsch (E) and the lateral habenlar nucleus (O). Note that the HPT is enlarged in *Gbx2* mutants (H and L), and that the region under the pial surface contains sparse cells in the mutants (bracket in P and R), probably due to abnormal accumulation of neuritis. ET, epithalamus; HPT, habenular-peduncular tract; mb, midbrain; Ncx, neocortex; PT, pretectum; PTh, prethalamus; TH, thalamus. Scale bars: 265 μm in C,D; 250 μm in E-H; 350 μm in I,J; 450 μm in K-N; 200 μm in O-R.

thalamic boundaries by regulating expression of these cell surface molecules. On serial coronal sections of E14.5 brain, *Cdh6* expression was detected in the medial part of the thalamus, and its expression domain becomes a narrow band in the lateral region with its dorsal and posterior limits clearly delineating the thalamus from the epithalamus and the pretectum, respectively (Fig. 7A; data not shown). In *Gbx2* mutants at E14.5, the dorsal border of *Cdh6* expression was indiscernible, although diffuse expression of *Cdh6* persisted in the presumptive thalamus (Fig. 7C). *Efna5* encodes a member of the EphrinA ligand family. By interacting with EphA receptors, EphrinA ligands mediate cell segregation in rhombomeres of vertebrate hindbrains (Xu et al., 1999). In the E14.5 diencephalon, *Efna5* is expressed in four transverse stripes flanking the p1-2 and p2-3 borders, respectively (Fig. 7B). Without *Gbx2*, the expression of *Efna5* in the thalamus was lost, whereas the two transverse bands of *Efna5* expression in the pretectum and the prethalamus were unaffected (Fig. 7D). These data appear to be consistent with a possible role of *Gbx2* in regulating cell adhesive properties of thalamic neurons.

To determine if loss of *Gbx2* indeed alters cell adhesion in the thalamus, we performed chimera experiments. We reasoned that an alteration in cell-adhesive properties due to loss of *Gbx2* would lead to abnormal mixing of the *Gbx2*-mutant and wild-type cells in chimeric embryos. Chimeric embryos were generated by injecting *Gbx2*$^{+/-}$ or *Gbx2*$^{-/-}$ ES cells into the blastocysts that were heterozygous for the ubiquitous *ROSA26 lacZ* reporter allele so that the host cells could be identified by β-gal activity (Friedrich and Soriano, 1991). Surprisingly, we found that *Gbx2*$^{-/-}$, like *Gbx2*$^{+/-}$ cells, were present throughout the thalamus and intermingled with the host cells in the thalamus of the chimeric embryos at E16.5 (Fig. 7E-H). By contrast, the mutant cells were segregated from the wild-type cells and aggregated specifically in the cerebellum of the chimeric embryos (Fig. 7F). These observations demonstrate that there are distinct cellular requirements for *Gbx2* in the thalamus and the cerebellum. In the cerebellum, *Gbx2* appears to act cell-autonomously in regulating cell adhesion. However, in the thalamus, the normal cell mixing suggests that wild-type and mutant cells have similar cell-adhesive properties. Alternatively, wild-type cells may rescue the defect of cell adhesion of *Gbx2*$^{-/-}$ cells in the thalamus. Consistent with a possible cell-nonautonomous role of *Gbx2*, we found that, in sharp contrast to those found in *Gbx2*-null mutants, the morphology and the histological borders of the thalamus were remarkably normal in the chimeras that were composed of *Gbx2*$^{-/-}$ and wild-type cells (Fig. 7F).

We next investigated whether the mutant thalamic neurons are prevented from dispersing into the epithalamus or the pretectum in chimeric embryos. We performed genetic mosaic analysis by

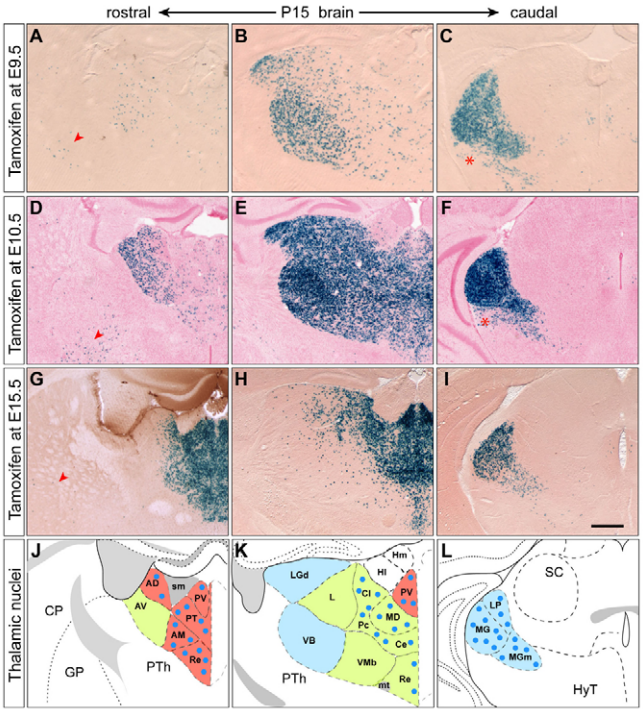

Fig. 5. Descendents of *Gbx2*-expresssing cells at different developmental stages populate distinct thalamic nuclei. (**A-I**) X-gal staining of coronal sections at the rostral, middle and caudal levels of the thalamus of *Gbx2*$^{CreER/+}$; *R26R* mice at P15 after administration of tamoxifen at E9.5 (A-C), E10.5 (D-F) and E15.5 (G-I). The asterisk indicates some marked cells outside the medial geniculate nucleus. The arrowheads indicate marked cells that originate from *Gbx2*-expressing cells in the medial ganglionic eminence, in the caudateputamen and globus pallidus. (**J-L**) Schematic summary of five classes of thalamic nuclei formed by temporally distinct *Gbx2*-expressing cells. The nuclei marked by light blue, light green and red represent nuclei formed by the initial wave (between E9.5 and E10.5), second wave (between E10.5 and E11.5) and the final wave (E15.5) of *Gbx2*-expressing cells, respectively. The nuclei in which *Gbx2* expression is maintained to postnatal stages are indicated by blue dots. CP, caudateputamen; GP, globus pallidus; HyT, hypothalamus; MGE, medial ganglionic eminence; SC, superior colliculus. See Table 1 for abbreviations of thalamic nuclei. Scale bar: 400 μm.

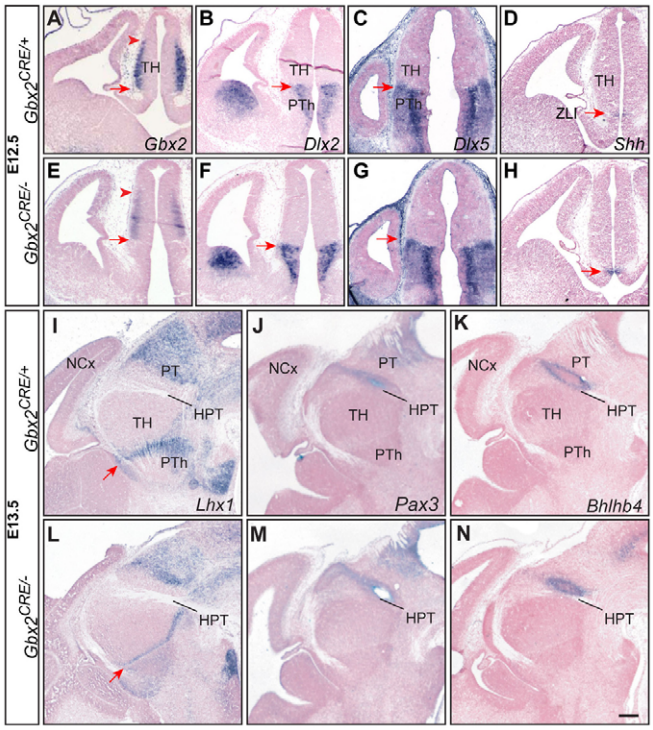

Fig. 6. Loss of *Gbx2* does not result in obvious defects in patterning of the diencephalon. (**A-H**) In situ hybridization assay on coronal sections of *Gbx2*$^{CreER/+}$ (A-D) and *Gbx2*$^{CreER/-}$ embryos (E-H) at E12.5 with different markers for the thalamus, prethalamus and ZLI as indicated. (**I-N**) Analysis of markers as indicated for the pretectum on sagittal sections of *Gbx2*$^{CreER/+}$ (I-K) and *Gbx2*$^{CreER/-}$ embryos (L-N) at E13.5. The border between the thalamus and the pretectum is demarcated by the cell-free zone, corresponding to the habenulopeduncular tract. The arrowheads indicate the border between the epithalamus and the thalamus; the arrows mark the ZLI. ET, epithalamus; HPT, habenulopeduncular tract; NCx, neocortex; PT, pretectum; PTh, prethalamus; TH, thalamus. Scale bar: 220 μm in A-H; 200 μm in I-N.

combining the *Gbx2*^{CreER} allele with a conditional *Gbx2* deletion allele, *Gbx2*^{F} (Li et al., 2002). Taking advantage of the mosaic manner of CreER-mediated recombination (Joyner and Zervas, 2006), we expected that administration of tamoxifen at E10.5 would produce a genetically mosaic thalamus composed of *Gbx2*$^{CreER/F}$ (*Gbx2* heterozygous – wild type in phenotype) and *Gbx2*$^{CreER/–}$ (*Gbx2* null) cells in *Gbx2*$^{CreER/F}$; *R26R* embryos, whereas the *Gbx2*$^{CreER/–}$ cells would be probably marked by β-gal (Fig. 7I). PCR analysis of microdissected thalamic tissues showed that the administration of tamoxifen at E10.5 indeed produced a genetically mosaic thalamus composed of *Gbx2*$^{CreER/F}$ and *Gbx2*$^{CreER/–}$ cells in *Gbx2*$^{CreER/F}$; *R26R* embryos at E16.5 (Fig. 7J). Significantly, the morphology of the thalamus was largely normal in the genetic mosaic embryos that contained a significant number of β-gal-positive cells in the thalamus (n=11) (Fig. 7K and inset in Fig. 7O). The labeled descendants of *Gbx2*-expressing cells were restricted to the thalamic compartment in *Gbx2*$^{CreER/F}$; *R26R* embryos at E14.5 and E18.5, similar to those found in *Gbx2*$^{CreER/+}$; *R26R* embryos (compare Fig. 7K, inset in Fig. 7O with Fig. 3E,F; Fig. 4E,F, and Fig. 4M-P). Mosaic embryos that contained stronger β-gal activity in the thalamus did exhibit a mild defect in the morphology of the thalamus in *Gbx2*$^{CreER/F}$ embryos (n=7; Fig. 7L). These results suggest that in the presence of wild-type cells, the dorsal and posterior boundaries of the thalamus are rescued in the genetic mosaic embryos.

Finally, we examined if the expression of *Efna5* is rescued in the presence of wild-type cells in the thalamus of chimeric and mosaic embryos. In contrast to that in *Gbx2*-null mutants, *Efna5* is expressed at the dorsal and the caudal borders of the thalamus in both chimeric or mosaic embryos at E16.5 (Fig. 7M-P). Interestingly, the expression domains of *Efna5* appear to be expanded in the chimeric and mosaic embryos. Taken together, the results of our chimeric and genetic mosaic analysis demonstrate that Gbx2 proteins function cell-nonautonomously in controlling the histogenesis and the boundary formation of the thalamus.

Table 1. Differential contribution of *Gbx2*-expressing cells at different stages to thalamic nuclei

			Tamoxifen administration		
	Thalamic nuclei		E9.5	E10.5	E15.5
Anterior group	Anterior dorsal nucleus	AD	–	++	++
	Anterior medial nucleus	AM	–	++	+++
	Anterior ventral nucleus	AV	–	+++	+
	Lateral nucleus	L	+	+++	+
Medial group	Medial dorsal nucleus	MD	+	++	+++
	Paraventricular nucleus	PV	–	+	+++
	Paratenial nucleus	PT	–	+	+++
	Reunions nucleus	Re	+	++	++
Intralaminar group	Central medial nucleus	Ce	+	++	+++
	Central lateral nucleus	Cl	+	+++	++
	Paracentral nucleus	Pc	+	+++	+++
	Parafasicular nucleus	PF	++	+++	–
Ventral nuclei	Ventrobasal nucleus	VB	++	+++	–
	Ventral lateral nucleus	VL	++	+++	–
	Ventral medial nucleus	VM	++	+++	+
	Ventral medial basal nucleus	VMb	++	+++	–
Posterior group	Lateral posterior nucleus	LP	++	+++	+++
	Posterior complex, medial division	Pom	+++	+++	+
Lateral and medial geniculate complex	Dorsal nucleus of lateral geniculate body	LGd	++	+++	+
	Principle nucleus of the medial geniculate body	MG	+++	+++	++
	Magnocellular nucleus of the medial geniculate body	MGm	+++	+++	++

+++, large numbers of labeled cells.
++, moderate number of labeled cells.
+, a few cells.
–, none or few labeled cells.
Nomenclature and classification of thalamic nuclei follow Caviness and Frost (Caviness and Frost, 1980), and Jones (Jones, 2007).

DISCUSSION

Gbx2-expressing cells and their descendents define the lineage-restriction boundaries of the thalamus

The prosomeric model proposed by Puelles and Rubenstein has provided us with an important conceptual framework for understanding the development of the forebrain (Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003). However, it remains controversial whether the prosomeres represent true development compartments that are units of cell lineage restriction similar to rhombomeres in the vertebrate hindbrain (Figdor and Stern, 1993; Larsen et al., 2001; Zeltser et al., 2001). According to the prosomeric model, the thalamus is generated from the alar plate of the p2 segment, presumably from the population of cells that express *Gbx2* (Bouillet et al., 1995). In this study, we demonstrate that the *Gbx2*-expressing cells and their descendents contribute to the entire thalamus. Significantly, the fate-mapped *Gbx2*-expressing cells form lineage-restriction boundaries delineating the thalamus not only from the pretectum and prethalamus, which correspond to the p1 and p3 segments, but also from the epithalamus, which is presumably derived from the same p2 segment. Our results have thus revealed the presence of a hitherto unknown developmental compartment, which is defined by the expression domain of *Gbx2*, within the diencephalon. Interestingly, a few marked cells were often detected in the lateral habenular nucleus and the nucleus of Darkschewitsch, which are presumably derived from the epithalamus and basal plate, respectively, of the p2 segment. These data suggest that the intra-prosomeric boundaries within p2 may be less stringent compared with inter-prosomeric boundaries.

It is worth noting that our current study primarily analyzed the developmental fate of *Gbx2*-expressing cells, which are mostly postmitotic neuronal precursors. We are not certain whether the progenitors in the ventricular zone of p2 obey the same lineage-restriction boundaries as *Gbx2*-expressing cells. However, based on the kinetics of tamoxifen induction, we expect that both *Gbx2*-expressing cells and the progenitors that are committed to express *Gbx2* would be labeled in a window of 36 hours after the administration of tamoxifen. Therefore, the lineage-restriction boundaries revealed by our fate mapping of *Gbx2*-expressing cells should apply to cells in the mantle zone as well as those committed progenitors in the ventricular zone of the p2 segment.

Compartment boundary restriction and nuclear formation in the thalamus

The developmental compartment defined by the *Gbx2* lineage contrasts with the known compartments in the vertebrate hindbrain and telencephalon, where the postmitotic cells in the mantle zone are known to be able to cross rhombomeric or the pallial-subpallial boundaries, although their progenitors in the proliferating zone are restricted to a cell-tight compartment (Fishell et al., 1993; Wingate and Lumsden, 1996). It has been postulated that compartmental boundaries are mainly required for a proliferating cell population with labile cell fates, whereas boundary restriction becomes dispensable for postmitotic cells, as their fates are specified (Kiecker and Lumsden, 2005). Therefore, the confinement of the *Gbx2*-expressing cells and their descendents, which are mainly postmitotic, within the thalamic compartment may serve a different function from those compartments. Interestingly, we observed that the borders of the *Gbx2* lineage marked at E10.5 were progressively sharpened between E14.5 and E16.5 (see Figs 3 and 4), coinciding with the initial parceling of the dorsal thalamus (Jones, 2007). We speculate that the lineage restriction of postmitotic *Gbx2*-positive thalamic cells may underlie the formation of thalamic nuclei. By fate mapping *Gbx2*-expressing cells at E9.5, E10.5 or E15.5, we have identified five groups of the thalamic nuclei (summary in Fig. 5J-L). The initial *Gbx2*-expresing cells (around E10.5) give rise to most of the principal relay nuclei, such as LGd, VB, LP and MG. However, *Gbx2*

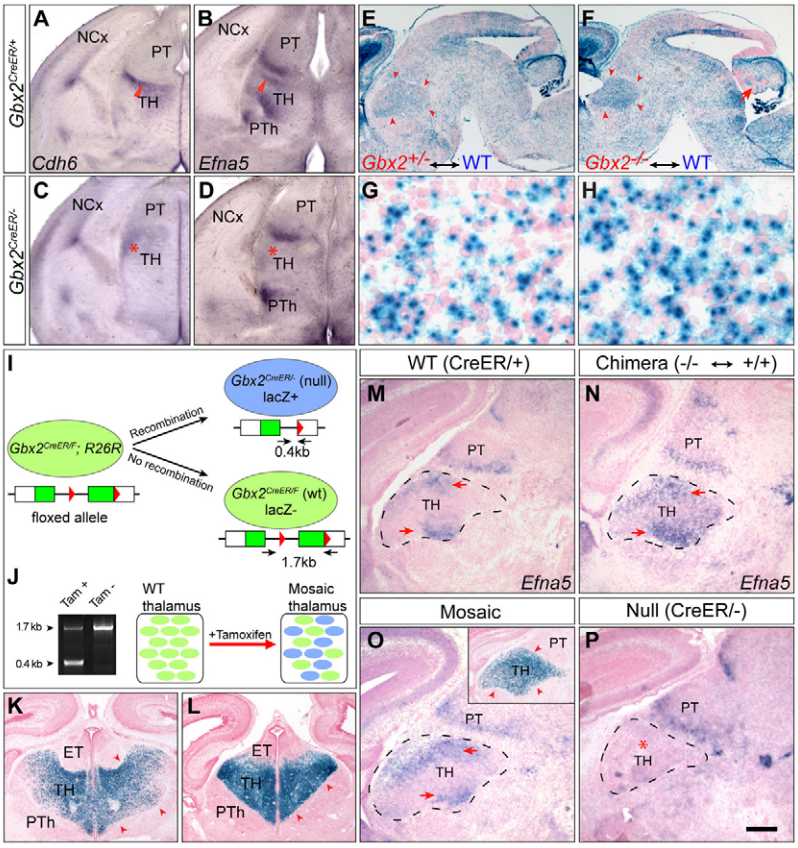

Fig. 7. *Gbx2* plays a cell-nonautonomous role in controlling the thalamic lineage boundaries. (**A-D**) In situ hybridization analysis of *Cdh6* and *Efna5* expression on the coronal brain sections of $Gbx2^{CreER/+}$ and $Gbx2^{CreER/-}$ embryos at E14.5. The arrowhead indicates the expression in the thalamus; the asterisk marks the diminished *Cdh6* and lost *Efna5* expression in the mutant embryos. (**E-H**) X-gal and Fast Red staining of sagittal brain sections of E16.5 chimeric embryos composed of wild-type (blue) and ES-derived cells (pink) of genotype $Gbx2^{+/-}$ (E) or $Gbx2^{-/-}$ (F). The arrow indicates aggregates of $Gbx2^{-/-}$ cells in the cerebellum; the arrowheads indicate the sharp thalamic borders. (G,H) Magnified view of the thalamus in E and F. Note that the $Gbx2^{-/-}$ cells, like $Gbx2^{+/-}$, extensively intermingle with the host cells in the thalamus. (**I**) Schematic diagram illustrating the generation of genetic mosaics using CreER-mediated deletion of *Gbx2* in the thalamus of $Gbx2^{CreER/F}$; *R26R* embryos. The arrows indicate the primers for PCR analysis to detect the floxed (1.7 kb) and deletion (0.4 kb) alleles of *Gbx2*. (**J**) PCR analysis of microdissected thalamus of $Gbx2^{CreER/F}$; *R26R* embryos. Note that the thalamus contains both $Gbx2^{CreER/F}$ and $Gbx2^{CreER/-}$ cells after tamoxifen administration, but only $Gbx2^{CreER/F}$ cells without tamoxifen. (**K**,**L**) X-gal staining of coronal brain sections of two $Gbx2^{CreER/F}$; *R26R* embryos with different levels of β-gal activity at E18.5 after tamoxifen administration at E10.5. The arrowheads mark the sharp borders of the fate-mapped *Gbx2* lineage. (**M-P**) In situ hybridization of *Efna5* on sagittal brain sections of $Gbx2^{CreER/+}$ (M), $Gbx2^{-/-}$wild-type chimera (N), $Gbx2^{CreER/F}Gbx2^{CreER/-}$ mosaic (O), and $Gbx2^{CreER/-}$ (P) embryos at E16.5. Inset in O shows X-gal staining of a sagittal section of the mosaic embryo. The arrows indicate the restored expression of *Efna5* in the chimeric and mosaic embryos; the asterisk indicates the absence of *Efna1* expression in the thalamus of $Gbx2^{-/-}$ embryo. ET, epithalamus; Ncx, neocortex; PT, pretectum; PTh, prethalamus; TH, thalamus; WT, wild type. Scale bars: 400 μm in A-D; 450 μm in E,F; 29 μm in G,H; 380 μm in K; 200 μm in L-O.

expression is downregulated in LGd and VB (designated as group I nuclei) after E10.5, and persists in LP and MG (group II). The second wave of *Gbx2*-expressing cells (E10.5-E11.5) gives rise to many association nuclei, such as AV, L, Cl, MD, Pc, Ce, VMb and Re, and relay nucleus L. Among these nuclei, *Gbx2* expression is maintained in MD and Cl, Pc and Ce (group III), and lost in L and VMb (group IV). The last wave of *Gbx2*-expressing cells (E15.5) gives rise to the most anteromedial nuclei, AD, AM, PV and Re (group V), where *Gbx2* expression persists into postnatal stages. Therefore, the precursors for distinct groups of thalamic nuclei display dynamic and distinct temporal patterns of *Gbx2* expression, although all thalamic neurons are derived from the *Gbx2* lineage. These observations suggest that the expression of *Gbx2* itself allows the thalamus as a whole to be segregated from the neighboring structures, which never express *Gbx2*. Within the thalamus, however, the dynamic and differential expression of *Gbx2* may lead to segregation of *Gbx2*-positive neurons from *Gbx2*-negative neurons, which have not yet started or have lost *Gbx2* expression.

Specific requirements for Gbx2 in the formation of the dorsal and caudal lineage boundaries of the thalamus

Birth-dating analysis using [^{3}H]-thymidine autoradiography has previously demonstrated an 'outside-in' gradient, with earlier-born neurons being displaced outside by later-born neurons in the diencephalon (Angevine, 1970). This arrangement of thalamic neurons according to their time of origin raises the question of whether the cell-tight thalamic compartment revealed by the current study is simply a result of lack of cell movement in the developing

thalamus. To further investigate the distribution of thalamic neurons that are generated at different stages, we marked *Gbx2*-expresing cells by administrating tamoxifen at E9.5 (presumed to label postmitotic thalamic neurons born between E10 and E12) and performed BrdU pulse labeling at E13.5 (presumed to label neurons born around E13.5) in $Gbx2^{CreER/+}$; $R26R^{+/-}$ embryos. In agreement with the previous birth-dating analysis, X-gal positive cells were mostly found in the lateral region, whereas the majority of BrdU-positive cells were found in the medial region. However, these two populations of cells were not exclusively segregated, with many later-born BrdU-positive cells intermingled with earlier-born X-gal-positive cells (see Fig. S2 in the supplementary material). These observations suggest that, at least to a certain extent, cell mingling does occur within the developing thalamus. Significantly, we demonstrate that, without *Gbx2*, cells originating from the thalamus no longer obey the lineage restriction at the dorsal and the posterior borders of the thalamus, expanding to the lateral habenular nucleus and the pretectum. Collectively, these observations strongly support the argument that the postmitotic thalamic neurons are normally restricted to the dorsal and posterior thalamic boundaries by an active *Gbx2*-dependent mechanism, rather than by a lack of cell movement.

In addition to the defect of the boundaries, we observed abnormally widened habenulopeduncular tract and accumulation of neurites under the pial surface of the thalamus in *Gbx2* mutant embryos (Fig. 4F,H,P,R). However, as shown on the coronal sections at the anterior and posterior levels, the thalamus-epithalamus and the thalamus-pretectum borders are completely disrupted, including at places distant from the habenulopeduncular tract (Fig. 4L,N,P). These data suggest that the abnormality of the habenulopeduncular tract cannot completely explain the aberrant dispersal of thalamic cells into the epithalamus and the pretectum. Importantly, we have found that *Gbx2* acts cell-autonomously in the control of axonal outgrowth of thalamic neurons (L.C. and J.Y.H.L., unpublished data).

In contrast to the dorsal and caudal thalamic boundaries, the lineage boundaries at the anterior and ventral borders of the thalamus are maintained in the absence of *Gbx2*. Therefore, the formation of the anterior and ventral borders of the thalamus is independent on *Gbx2*. Interestingly, it has been demonstrated that the ZLI, which defines the p2/3 border, represents a lineage-restriction boundary and a signaling center, which expresses secreted factor, such as *Shh* and *Fgf8*, to regulate the development of both thalamus and prethalamus (Kataoka and Shimogori, 2008; Kiecker and Lumsden, 2004; Vieira et al., 2005). A recent study showed that the ZLI represents a unique lineage restriction compartment depending on *Lfng* activity (Zeltser et al., 2001). We show that the expression of *Shh* and *Lhx1* at the ZLI is unaffected in *Gbx2* mutants, demonstrating that *Gbx2* is not essential for development of the p2/p3 border (Fig. 6D,H,I,L).

A cell-nonautonomous role of Gbx2 in the regulation of lineage-restriction boundary of the thalamus

Given that the expression of *Cdh6* and *Efna5* is disrupted in the thalamus of *Gbx2* mutant embryos, we were surprised to discover that *Gbx2*-deficient and wild-type cells intermix normally in the thalamus of chimeric and genetic mosaic embryos. Our data strongly suggest that *Gbx2* plays a cell-nonautonomous role in the formation of the thalamic boundaries. First, we found that the morphology and the histological border of the thalamus are remarkably normal in the chimeric and mosaic embryos that contain a significant percentage (not less than 50%) of *Gbx2*-deficient cells, as judged by PCR and β-gal expression. It is remarkable that mosaic embryos that contained strong β-gal activity in the thalamus did exhibit a mild defect in the morphology of the thalamus in $Gbx2^{CreER/F}$ embryos. These results indicate that administration of tamoxifen indeed leads to deletion of *Gbx2*, and deletion of *Gbx2* after E10.5 can still recapitulate the defect of *Gbx2*-null cells. Therefore, the rescue observed in the mosaic embryos is unlikely to be due to the residual Gbx2 proteins produced before CreER-mediated deletion occurs. The mild phenotype in the mosaic embryo with strong β-gal activity also suggests that a certain percentage of wild-type cells may be required for the rescue of the mutant phenotype. Second, in $Gbx2^{CreER/F}$; *R26R* embryos with mosaic deletion of *Gbx2* at E10.5, the marked descendents of *Gbx2*-expressing cells become normally restricted to the thalamic compartment. It is reasonable to assume that a significant number of the marked cells in $Gbx2^{CreER/F}$; *R26R* embryos have lost *Gbx2* due to CreER-mediated recombination. The absence of β-gal-positive cells in either the epithalamus or the pretectum demonstrates that the presence of wild-type cells rescues the lineage-restriction boundaries of the thalamus in mosaic $Gbx2^{CreER/F}$; *R26R* embryos. We did not detect a bias of the wild-type cells being at the boundaries of the thalamus in chimeric embryos, arguing against the possibility that the wild-type cells may form border cells to restore the boundary. Finally, we found that *Efna5* expression is restored in the thalamus of chimeric and mosaic embryos at E16.5. Because of the unavailability of suitable antibodies, we were unable to determine whether *Efna5* is expressed in *Gbx2*-deficient cells in the chimeric or mosaic embryos. Nevertheless, the restored expression of *Efna5* demonstrates that the dorsal and caudal borders of the thalamus are rescued in the chimeric and mosaic embryos. Collectively, our data demonstrate that *Gbx2* acts cell-nonautonomously in regulating formation in the thalamic boundary. As Gbx2 is a transcription factor and presumably acts within the thalamic cells, we postulate that *Gbx2* may regulate an extracellular signaling pathway, which in turn mediates the cell-nonautonomous role of *Gbx2* in controlling boundary formation in the thalamus.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/8/1317/DC1

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