GATA2-dependent and region-specific regulation of *Gata2* transcription in the mouse midbrain

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Transcription factor GATA2 is expressed in numerous mammalian tissues, including neural, hematopoietic, cardiovascular and urogenital systems, and yet it plays important roles in the regulation of tissue-restricted gene expression. The *Gata2* gene itself is also under stringent tissue-specific control and multiple *cis*-regulatory domains have been identified in the *Gata2* locus. In this study we sought out and then examined in detail the domains that regulate *Gata2* in the midbrain. We identified two discrete domains in the *Gata2* promoter that direct midbrain expression; these distal 5H and proximal 2H regulatory domains are located 3.0 and 1.9 kbp, respectively, upstream of the transcriptional initiation site. Importantly, both domains contain GATA factor binding sites. Our analyses further revealed that GATA2 is essential for *Gata2* gene expression in the midbrain, whereas GATA3 is not. Both the 2H and 5H domains have the independent ability to activate *Gata2* gene expression in the midbrain superior colliculus, whereas the distal-5H domain is additionally capable of activating *Gata2* transcription in the inferior colliculus. These results demonstrate that two distinct regulatory domains contribute to the *Gata2* gene expression in the mouse midbrain and that *Gata2* midbrain transcription is under positive autoregulation.

Introduction

Genes are expressed in a tightly regulated manner throughout the mammalian development. The collaborative activities of multiple transcription factors are considered to determine cell type-specific gene expression profiles, but it has also been hypothesized that key transcription factors that act as 'master regulators' may exist for the lineage-specific expression of a cluster of genes (Weintraub *et al.* 1989). For example, forced expression of MyoD causes trans-differentiation to myocytes (Weintraub *et al.* 1989) and that of RUNX2/Cbfa1 determines osteoblast differentiation essential for bone formation (Komori *et al.* 1997). Furthermore, GATA1 and PU.1 harbor antagonistic functions in the lineage commitment of hematopoietic progenitors into myeloerythroid vs. myelolymphoid cells, respectively (Arinobu *et al.* 2007). Mutations in these master regulators are often related closely to human diseases (Huard *et al.* 1998; Heinl-Green *et al.* 2005). The regulatory mechanisms elucidating how these master regulators are themselves regulated have been studied extensively, and one common feature is that the master regulators often regulate themselves or they have adopted autoregulation or network inter-regulation between related family members as a standard paradigm. Indeed, it has been reported that MyoD, GATA1 and PU.1 all up-regulate their own transcription (Thayer *et al.* 1989; Nishikawa *et al.* 2003; Okuno *et al.* 2005), and that *RUNX2* gene expression is itself repressed by a RUNX2/Cbfa1 complex (Drissi *et al.* 2000), suggesting that these positive or negative feedback circuits may offer important advantages for regulation of the regulators and specification of cell fate during development.

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The vertebrate GATA factor family consists of six members of zinc-finger transcription factors that usually recognize an (A/T)GATA(A/G) motif in GATA target genes (Yamamoto *et al.* 1990; Ohneda & Yamamoto 2002). Each GATA factor has a different expression profile, and each has been shown to play important roles in cell-type specific gene expression (Burch 2005). Several lines of recent evidence exploiting genetically engineered mice revealed that there exist multiple GATA-binding motifs in the enhancers of the genes encoding GATA1, GATA2, GATA5 and GATA6, and each of these motifs facilitate the autoregulation of GATA gene expression (Burch 2005). For example, GATA1 and GATA2 bind at different times to regulatory domains of the *Gata1* gene to control its hematopoietic expression (reviewed in Ohneda & Yamamoto 2002). Similarly, we also identified an enhancer for the *Gata2* gene that regulates its expression in early hematopoietic cells by recruiting GATA2 and/or GATA3 (Kobayashi-Osaki *et al.* 2005).

In order to fully elucidate the molecular basis of *Gata2* gene expression, we have been analyzing the regulation of *Gata2*. These studies have revealed GATA2 contributions to cell-type specific gene expression and some of the regulatory domains within the gene that control GATA2 function in multiple tissues (Zhou *et al.* 2000; Khandekar *et al.* 2004, 2007; Kobayashi-Osaki *et al.* 2005). Importantly, we recently found that the regulatory motifs controlling the transcription of *Gata2* are dispersed over approximately 413 kbp, even though the six coding exons of *Gata2* span only 7 kbp (Brandt *et al.* 2008). The *Gata2* gene harbors two separate urogenital enhancers at approximately +75 and +113 kbp from the translational initiation site, both of which are necessary for urogenital development (Khandekar *et al.* 2004). Hematopoietic enhancers have also been identified in the promoter proximal region of the gene (Kobayashi-Osaki *et al.* 2005; Grass *et al.* 2006). In addition to these upstream and downstream enhancers, we have also identified enhancers in introns 4 and 5 that dictate endothelial and spinal cord-specific expression of *Gata2*, respectively (Zhou *et al.* 2000; Khandekar *et al.* 2007).

We recently succeeded in visualization of *Gata2* expression in a large battery of tissues where it is normally expressed by means of a green fluorescent protein (GFP) knock-in approach (Suzuki *et al.* 2006). The data showed that GATA2-expressing cells are widely distributed in neurons in the brain, spinal cord and sensory organs. However, the neural enhancer identified in the *Gata2* gene intron 5 supports gene expression specifically in V2 interneurons of the spinal cord (Zhou *et al.* 2000). Therefore, we hypothesized that there should be additional enhancers that regulate *Gata2* expression in the central nervous system (CNS). Indeed, our previous transgenic assays suggested that at least one additional brain enhancer might be located upstream of the *Gata2* gene (Zhou *et al.* 2000).

To identify the putative *Gata2* brain enhancer(s), we employed a standard transgenic mouse strategy. We found that two separate regulatory domains (referred to as the distal-5H and proximal-2H domains) located upstream of the transcriptional start site are both used by midbrain neural cells and hematopoietic cells. Interestingly, the enhancer usage differs between the two anatomically distinct midbrain sub-regions, the superior colliculus (SC) and inferior colliculus (IC). Both enhancers have the ability to independently activate *Gata2* transcription in SC, but the expression in IC depends exclusively on the distal-5H domain. GATA2 activity was also found to be required for enhancer function in the midbrain, indicating that *Gata2* gene expression is positively autoregulated. These results, taken together, demonstrate that the *Gata2* gene expression in CNS is supported by cell-type specific enhancers acting through positive autoregulatory machinery.

Results

Gata2 expression in the developing and adult midbrain

To begin to address the mechanism(s) underlying *Gata2* gene expression in the brain, we first examined reporter gene expression in a GFP knock-in 'EII-KI' mouse line (*Gata2*$^{GFP/+}$ genotype). In this line, GFP was targeted to the *Gata2* locus by homologous recombination (Suzuki *et al.* 2006), in order to monitor endogenous *Gata2* gene expression by GFP. In the mouse brain, GFP expression was first observed in the midbrain at embryonic day 9.5 (E9.5; data not shown), and this expression was maintained at a high level throughout embryogenesis, as at E11.5 (Fig. 1A) and E15.0 (Fig. 1B), after birth (Fig. 1C) and even into the adult stage (data not shown). We also conducted reverse transcriptase-PCR (RT-PCR) analyses, and consistent with the GFP reporter study outlined above, the RT-PCR analysis confirmed that the endogenous *Gata2* gene was exclusively expressed in the midbrain, but not in the cerebrum or cerebellum of the adult brain (Fig. 1D).

GATA2 and GATA3 are expressed in the midbrain in a mutually exclusive manner

GATA3 has also been reported to be expressed in the midbrain (Lakshmanan *et al.* 1999; Nardelli *et al.* 1999; van Doorninck *et al.* 1999) and the current RT-PCR analysis supports this conclusion. As is GATA2, GATA3 appears to be expressed in the midbrain in a tissue-restricted manner

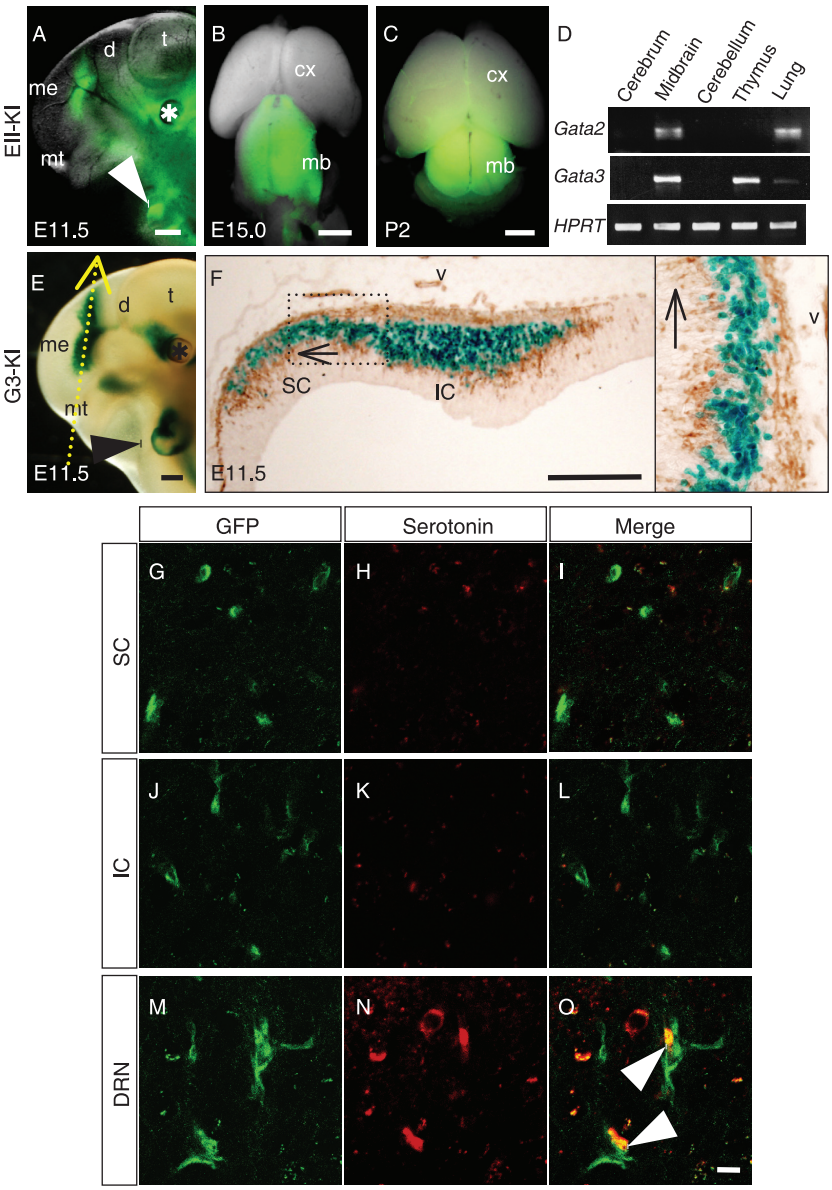

Figure 1 *Gata2* gene expression in the midbrain. (A) GFP expression in the head region of EII-KI mouse at E11.5. The *Gata2* locus derived GFP expression was specifically detected in the mesencephalon (me), metencephalon (mt), otic vesicle (arrowhead) and eye (asterisk), but not in the diencephalon (d) and telencephalon (t). Scale bar is 500 μm (B, C) GFP expression in the EII-KI brain at E15.0 (B) and P2 (C). The midbrain (mb)-specific expression of GFP was observed. cx, cerebral cortex. Scale bars indicate 1 mm (D) RT-PCR analysis of *Gata2* and *Gata3* mRNA expression in the adult brain. Note that midbrain-specific expression of the *Gata2* and *Gata3* mRNAs. cDNA templates from the thymus and lung were used as positive controls for the *Gata3* and *Gata2* expression, respectively. HPRT was used as internal controls. (E) X-gal staining of G3-KI embryonic brain at E11.5. Abbreviations are the same as in (A). Scale bar is 500 μm (F) X-gal (blue) and anti-GFP (brown) double staining of the horizontal section (corresponding to the dotted yellow arrow in E) from the *EII-KI:G3-KI* embryo at E11.5. High magnification view of dotted box is also shown as right panel. Note that both *Gata2* (GFP) and *Gata3* (X-gal) genes are expressed in the superior colliculus (SC) and inferior colliculus (IC) of the midbrain, but the distribution of *Gata2*-positive neurons and *Gata3*-positive neurons are apparently different. *Gata2*-GFP expression was also detected in the blood vessels (v). Arrows indicate the rostral direction. Scale bar indicates 200 μm (G–O) GFP (G, J, M) and serotonin (H, K, N) expression were examined in SC (G–I), IC (J–L) and DRN (M–O) sub-regions of the EII-KI adult midbrain. Merged images of GFP and serotonin expression are shown in I, L and O. Some of cells in DRN are double positive (yellow, arrowheads in O) for *Gata2*-GFP (green) and serotonin (red). Scale bar is 10 μm.

(Fig. 1D). To further examine GATA3 expression, we exploited a *Gata3* gene-modified mouse in which the bacterial *LacZ* gene was knocked into the *Gata3* locus (G3-KI or $Gata3^{LacZ/+}$; Hendriks *et al.* 1999). We used the G3-KI mice for visualization of *Gata3* expression during the embryonic stages, and found that the LacZ expression pattern was quite similar to that of the GFP reporter from the *Gata2* locus (Fig. 1E). In E11.5 embryos, both GATA2 and GATA3 were expressed in the mesencephalon, metencephalon, otic vesicle and eye, but not in the diencephalon or telencephalon (Fig. 1A,E).

As the GFP and LacZ reporter analyses demonstrated concomitant expression of GATA2 and GATA3 in the midbrain, we next addressed whether GATA2 and GATA3 are co-expressed in the same cells or not. For this purpose, we generated double knock-in mutant mice (i.e. *EII-KI:G3-KI*) and analyzed *Gata2*-GFP and *Gata3*-LacZ expression in the same sections. As a result, we found that both *Gata2* (GFP) and *Gata3* (LacZ) were expressed in the superior colliculus (SC) and inferior colliculus (IC) of the midbrain at E11.5. Importantly, however, the GATA2 and GATA3 expression profiles were found to be mutually

exclusive; a majority of midbrain cells expressed either GFP or LacZ, but not both (Fig. 1F). The *Gata2* (GFP)-positive cells (brown in Fig. 1F) were mainly distributed in the inner and outer layers of the mesencephalon, surrounding the *Gata3* (LacZ)-positive middle layer (blue). We also detected *Gata2*-GFP expression in brain vascular endothelial cells (identified by the 'v' in Fig. 1F), confirming our previous observation that endothelial cells expressing *Gata2*-GFP are widely distributed in the mouse (Kobayashi-Osaki *et al.* 2005; Khandekar *et al.* 2007). These results thus demonstrate that although a subset of midbrain cells express GATA2, a different set express GATA3 (Fig. 1F and Supplementary Fig. S1), suggesting that these two GATA factors play different roles in the specification of cellular identity in the mesencephalon. This is in contrast to the observation that GATA2 and GATA3 are co-expressed in the serotonergic neurons of the chick hindbrain (Craven *et al.* 2004).

As a preceding report indicated that GATA2 was expressed in serotonergic neurons (Craven *et al.* 2004), we next examined serotonin production in the midbrain GFP-positive cells by the means of immunohistochemistry using an anti-serotonin antibody. Indeed, there were a number of serotonin or GFP single positive cells in the dorsal raphe nucleus (DRN), and some cells were positive for both serotonin and GFP (Fig. 1M–O). However, the fact that a majority of GFP-positive cells are negative for serotonin production suggests that GATA2 may not play important roles for serotonin synthesis in the DRN. We failed to identify serotonin/GFP double positive cells in the SC or IC sub-regions of the adult mouse midbrain (Fig. 1G–L, respectively). These observations demonstrate that GATA2 is practically not required for serotonin production in the midbrain.

Gata2 expression in the midbrain is regulated by a gene proximal upstream region

To begin to understand the molecular mechanisms by which the unique expression pattern of *Gata2* in the midbrain is achieved, we localized the activity of the *Gata2* gene regulatory domains. As shown in Fig. 2A, the *Gata2* gene harbors two alternative promoters/first exons (IS and IG; Minegishi *et al.* 1998). Therefore, we first examined promoter usage in the midbrain expression of *Gata2* using two independent GFP knock-in mouse lines, that is, EIS-KI and EII-KI (Fig. 2A). The EIS-KI knock-in allele was designed to express GFP exclusively from the IS exon (Fig. 2C). However, the EII-KI allele was designed to recapitulate the entirety of *Gata2* gene expression by inserting the GFP reporter into the translation initiation methionine codon located in exon II. In the latter case GFP expression was also detected in E11.5 midbrain (Fig. 2B). The midbrain GFP expression in the EIS-KI and EII-KI knock-in mice appeared to be virtually identical at all stages we examined (data not shown). RT-PCR analyses confirmed that both the IS and IG promoters are used for *Gata2* expression in the midbrain (Supplementary Fig. S2).

We next examined trans-activation potential of the regulatory domains residing upstream of the IS exon utilizing a transgenic reporter assay. Both 7.0 and 3.1 kbp sequences 5′ to the IS exon appeared to be sufficient to recapitulate endogenous *Gata2* expression in E11.5 CNS, including in the midbrain and spinal cord (Fig. 2A). In the transgenic reporter assays, multiple transgenic lines expressed GFP in hematopoietic tissues (H; fetal liver and AGM) as well as neural tissues (N; brain and spinal cord). The GFP expression patterns in the CNS of 7.0ISGFP and 3.1ISGFP transgenes in E11.5 embryos were virtually identical to that of the EII-KI embryos (compare Fig. 2E, F with 2D). In contrast, GFP expression was not detected in the midbrain of 0.6ISGFP transgenic embryos (0 positive out of 6 founders (F0); Fig. 2A). These results indicate that the enhancer(s) for midbrain and spinal cord expression of *Gata2* is localized within the genomic interval between 3.1 and 0.6 kbp upstream of the IS exon, where the hematopoietic enhancers also reside (Kobayashi-Osaki *et al.* 2005).

We next isolated GFP-positive cells from E10.5 midbrains of 3.1ISGFP transgenic mouse embryos by FACS, and endogenous *Gata2* gene expression was quantified by RT-PCR. FACS analysis showed that 2.1% and 0.9% of total cells in the midbrain and spinal cord, respectively, were positive for GFP (Fig. 2G). This ratio is comparable to the results of immunohistochemical analyses of midbrain sections stained with an anti-GFP antibody (data not shown). The expression of *Gata2* mRNA in the GFP-positive cell fraction was much higher than in the GFP-negative or total cell fractions (Fig. 2G), demonstrating that the expression of 3.1ISGFP transgene is highly correlated with endogenous *Gata2* expression both in the midbrain and spinal cord.

Identification of two regulatory domains for *Gata2* expression in the midbrain

To analyze further the *cis*-acting elements regulating midbrain expression of *Gata2*, the conservation of sequences corresponding to the 3.1IS region among multiple vertebrate genomes was examined using a Blast-like alignment tool (BLAT). The analysis identified two highly conserved sequence domains in the region, and we refer hereafter to the distal and proximal domains as the 5H and 2H domains,

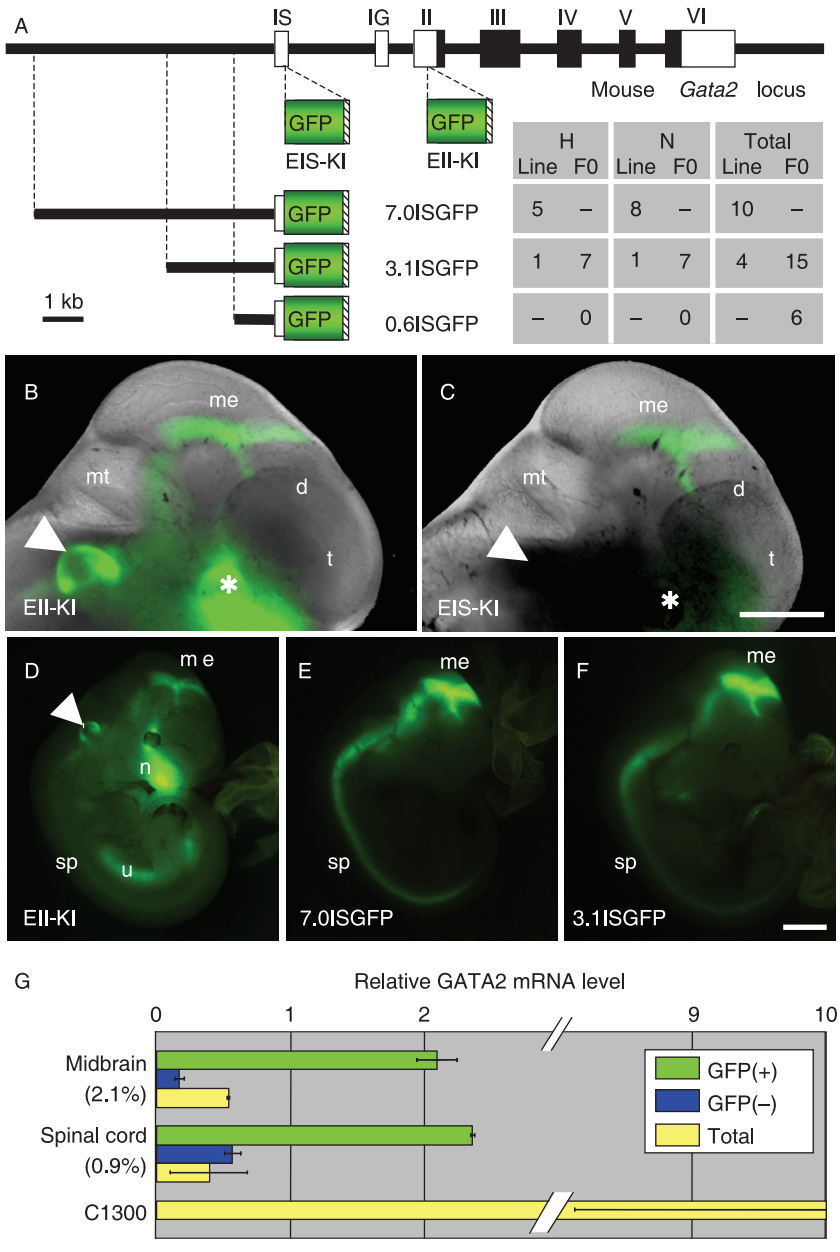

Figure 2 Promoter usage and regulatory domain of the *Gata2* gene in the midbrain. (A) Summary of *Gata2-GFP* transgene reporter assays. Schematic diagrams of the GFP reporter constructs are shown. The mouse *Gata2* gene harbors five translated exons (II–VI) plus two first exons (IS and IG). White and black boxes are the untranslated regions and protein-coding regions of the *Gata2* gene, respectively. Hatched boxes indicate polyadenylation signal sequences for the GFP cassette. Inserted table shows the result of GFP expression analysis at E11.5; the number of the transgenic lines positive for GFP expression in the hematopoietic tissues (H; fetal liver and AGM region) and neural tissues (N; brain and spinal cord) are represented. Total numbers of transgenic mouse lines analyzed are shown along with the results from founder (F0) embryo assays. (B, C) Midbrain (mesencephalon, (me)-specific GFP expression was observed in both EII-KI (B) and EIS-KI (C) embryos at E11.5). Abbreviations are the same as those shown in Fig. 1(A). Scale bar indicates 1 mm (D–F) Comparison of GFP expression among the GFP-reporter transgenic mouse lines at E11.5. (D) EII-KI embryo expressed GFP in the CNS including the mesencephalon (me) and spinal cord (sp) as well as the otic vesicle (arrowhead), nose (n) and urogenital system (u). The GFP expression driven by the *IS* promoter was also observed in CNS of both 7.0ISGFP (E) and 3.1ISGFP (F) transgenic embryos. Scale bar is 1 mm (G) FACS-sorted GFP$^+$ (green bars) and GFP$^-$ (blue bars) cells from the midbrain and spinal cord of 3.1ISGFP transgenic embryos at E10.5. GATA2 mRNA expression levels of these cells were analyzed by the quantitative RT-PCR. Data shows relative GATA2 mRNA level in each cell fraction. The mean expression level of C1300 was set as 10. The percentages of GFP$^+$ cells in each tissue are shown in parentheses. Error bars represent standard deviation.

respectively (Fig. 3A). We previously investigated significance of the distal 5H domain in fetal hematopoietic cells, and identified a 26-bp regulatory element in that domain. We designated the element as the early hematopoietic cell regulatory domain of the *Gata2* gene (*G2-EHRD*), which contains functional binding motifs for GATA factors (Kobayashi-Osaki *et al.* 2005).

To examine whether these two regulatory domains are sufficient for the CNS expression of *Gata2*, two genomic fragments corresponding to the distal 5H or proximal 2H homologies (336 and 359 bp, respectively) were isolated and individually ligated to 0.6ISGFP transgene construct (Fig. 3A). In very good agreement with 3.1ISGFP transgene expression (Fig. 3B–D show E11.5 embryos and 3E depicts an adult mouse), the 5H-ISGFP transgene was expressed in E11.5 midbrain and spinal cord (Fig. 3F–H), and strong GFP expression persisted in the adult midbrain (Fig. 3I). The GFP expression profile of 5H-ISGFP transgenic mice coincides well with those of the 3.1ISGFP transgenic and EII-KI mice (data not shown). This expression

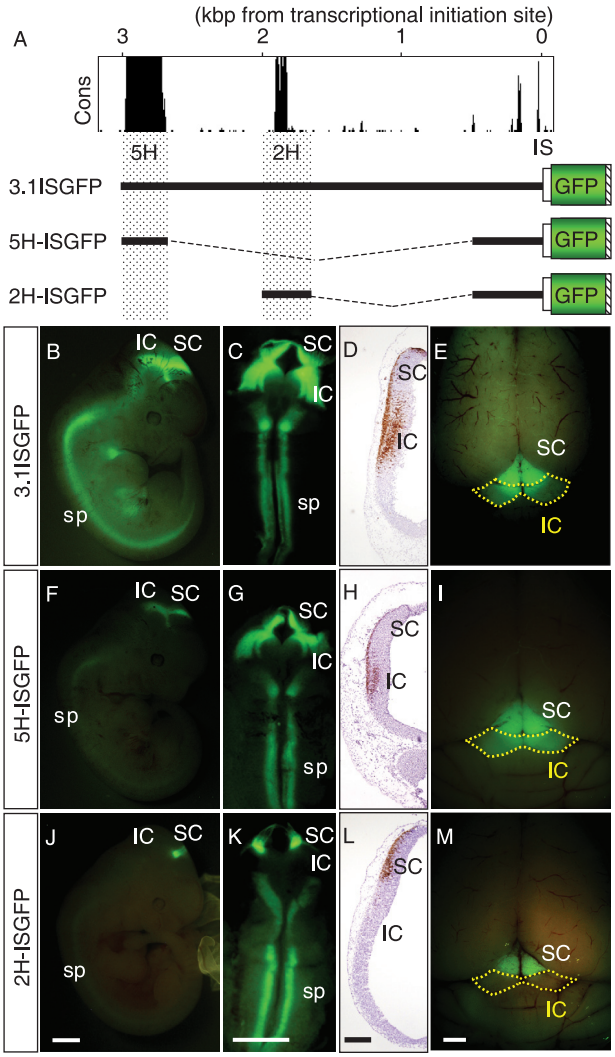

Figure 3 Identification of two regulatory domains playing differential roles in the midbrain-specific *Gata2* gene expression. (A) Sequence conservation (Cons) among 3.1IS regions of the vertebrate *Gata2* genes is displayed as a histogram in which the height reflects the size of the score calculated by BLAT <http://genome.ucsc.edu/cgi-bin/hgBlat>. Two highly conserved domains (5H and 2H, indicated with shaded boxes) exist within 3.1 kbp upstream from the IS exon. To construct 5H-ISGFP and 2H-ISGFP transgenes the 5H and 2H domains were ligated to 0.6ISGFP transgene construct (see Fig. 2A). (B–M) GFP expression profile of 3.1ISGFP (B–E), 5H-ISGFP (F–I) and 2H-ISGFP (J–M) transgenes. Whole mount images of embryos (B, F, J) and backside view of the dissected CNS (C, G, K) at E11.5 are presented as fluorescent images. GFP expression in the IC regions of the adult midbrain is shown by yellow dotted lines in dorsal views of whole brain (E, I, M). Note that GFP expression in the IC is detectable in 3.1ISGFP and 5H-ISGFP embryos, but not in 2H-ISGFP embryo. All three transgenes were expressed in the spinal cord (sp) and SC. Anti-GFP immunohistochemistry (brown color staining) of frozen sections demonstrates the IC-specific expression of 2H-ISGFP transgene in the embryonic midbrain at E11.5 (D, H, L). Expression of 3.1ISGFP and 5H-ISGFP transgenes are observed in both SC and IC (yellow dotted line) of the adult brain (E, I), but 2H-ISGFP expression in the adult brain is restricted in SC (M). The rostral direction is upper side (D, H, L). Scale bars indicate 200 μm (D, H, L) and 1 mm (B, C, E–G, I–K, M).

profile was reproducible in 5 out of 26 embryos bearing the 5H-ISGFP transgene (data not shown), suggesting that the 5H domain is commonly used for the *Gata2* expression in both hematopoietic and neural cells.

We also examined trans-activation potential of the proximal 2H domain by generating 2H-ISGFP transgenic mice. We found that of the 22 transgene-positive embryos, 8 displayed GFP expression in the midbrain and spinal cord at E11.5 (Fig. 3J–L). To our surprise, however, the GFP reporter transgene expression in the IC region of the midbrain was absent in all eight embryos that expressed GFP in the SC sub-region. The IC-specific absence of reporter transgene expression was also observed in the adult midbrain of 2H-ISGFP transgenic mice (Fig. 3M). Taken together, these results identified two discrete midbrain regulatory domains of the *Gata2* gene. The distal 5H domain appears to direct the expression of *Gata2* widely in both the IC and SC, whereas the proximal 2H domain restricts *Gata2* transcription to the SC sub-region.

When GFP fluorescent intensities in the midbrain of 3.1ISGFP and 5H-ISGFP transgenic mice were quantified, there was practically no difference in the ratio of SC to IC fluorescence (Supplementary Fig. S3). The expression level of the 5H-ISGFP transgene was not reduced in the SC sub-region, even though the transgene did not contain the 2H domain (Supplementary Fig. S3). These results suggest that both 5H and 2H domains harbor activity that regulates *Gata2* activity in the SC sub-region, but their functions are neither additive nor synergistic. In the SC, the 5H and 2H domains appear to redundantly activate *Gata2* expression, whereas in the IC region, *Gata2* expression depends exclusively on 5H activity. Thus, *Gata2* gene expression in the SC and IC sub-regions of the midbrain is regulated by a combination of two distinct domains.

GATA factors regulate *Gata2* gene midbrain expression

Alignment of the distal 5H and proximal 2H sequences revealed that several GATA factor binding sites are highly conserved among multiple mammalian genomes (Fig. 4A and Supplementary Fig. S4), suggesting that both the 5H and 2H domains are bound by GATA factors in the midbrain and that they activate *Gata2* gene expression. Based on the conservation of those GATA sites we further surmise that *Gata2* gene expression in the midbrain may be controlled in an autoregulatory manner through these two

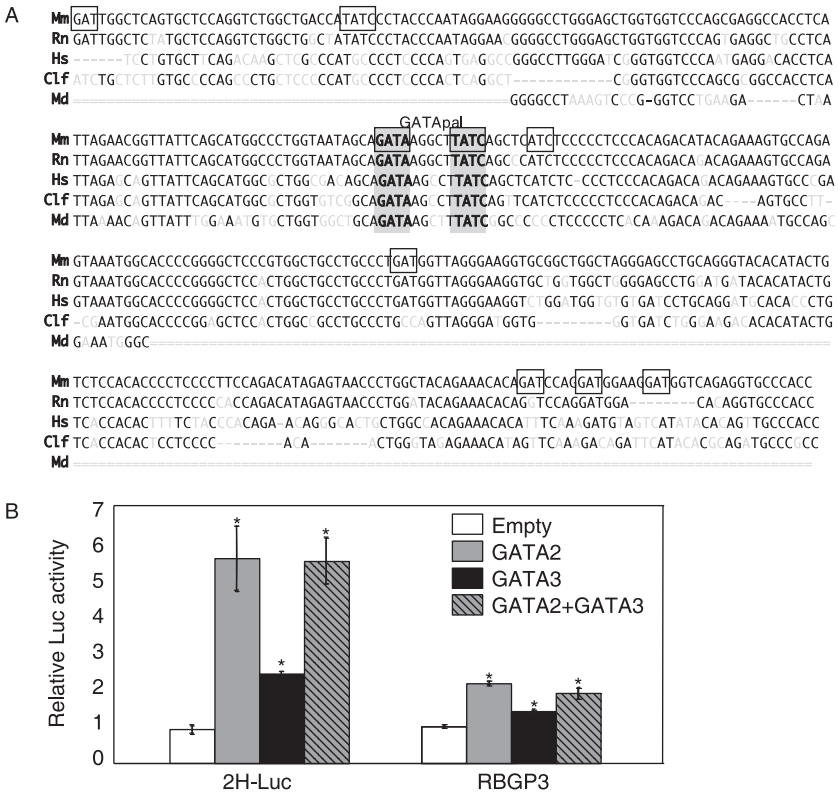

Figure 4 GATA-binding elements in the midbrain regulatory domain of *Gata2*. (A) Comparative aliments of the 2H domain among the mammalian genomic sequences. Conserved sequences among the indicated species are shown by black letters, and open boxes indicate potential GATA boxes. The 2H domain of mouse *Gata2* is between 1977 and 1619 bp upstream from the IS exon transcription initiation site. There is a GATApal (gray boxes) in the 2H domain of all mammalian genomes. Mm, mouse; Rn, rat; Hs, human; Clf, dog; Md, opossum. There are no-bases (–) and gap (=) regions in this alignment. (B) 2H-Luc plasmid was transfected into C1300 neuroblastoma cells without (white bar) or with GATA expression vectors (GATA2, gray bar; GATA3, black bar; both, hatched bar). GATA2 and GATA3 significantly enhanced activity of the 2H domain. pRBGP3 was used as a control basic promoter. Data show relative luciferase units standardized with transfection efficiency, and error bars represent standard deviation. This experiment was carried out in triplicate and repeated three times, and representative data are shown. ⋆$P < 0.01$ compared with basal reporter activities.

distinct domains. In this regard, our previous transgenic reporter analyses proved that GATA motifs in the distal 5H domain are necessary for the *Gata2* gene expression in hematopoietic cells, and biochemical analyses provided additional evidence for binding of the 5H GATA boxes by GATA factors (Kobayashi-Osaki *et al.* 2005).

It has been shown that palindromic GATA motifs, referred to as GATApal, have a higher affinity for GATA factors than does a single GATA binding site (Trainor *et al.* 1996), and we identified a GATApal motif in the 2H domain (Fig. 4A). Direct binding of GATA factors to the 2H GATApal site was confirmed by electrophoretic mobility shift assay (data not shown). When we carried out reporter co-transfection assays using C1300 neuroblastoma cells, which express endogenous GATA2 and GATA3 (Harigae *et al.* 1998), co-transfected GATA2 and GATA3 were each capable of markedly inducing 2H-based luciferase reporter (2H-Luc) expression (Fig. 4B). The induction by GATA factors was also detected, albeit much less, for the basic promoter construct RBGP3 used in this study (Fig. 4B). RBGP3 activity has been shown to be enhanced by co-transfection of GATA factors, as this basic promoter is derived from the rabbit *β-globin* gene and contains a functional GATA box (Igarashi *et al.* 1994). Nonetheless luciferase, when linked to the 2H domain, was expressed at a significantly higher level than RBGP3 alone (Fig. 4B), indicating that the 2H domain mediates the robust transactivation potential of GATA2 and GATA3.

In the reporter co-transfection analysis, expression of the 2H-Luc reporter was induced approximately sixfold by simultaneous expression of GATA2 (Fig. 4B). This level of activation was significantly higher than that achieved by GATA3 co-transfection. No synergistic or additive effects were detected in cells co-transfected both GATA2 and GATA3 (Fig. 4B), suggesting that GATA2 alone is responsible for activation of the *Gata2* gene in neural cells, and that GATA3 cannot activate the 2H midbrain enhancer as efficiently as does GATA2. Several 2H-Luc mutants harboring mutations in the GATApal motif were also examined in these luciferase assays, but no significant change was observed in these mutants when compared to the wild-type 2H reporter (data not shown). We speculate that the other *cis*-acting GATA motifs (9 GATA consensus sites within the 359 bp 2H enhancer, open boxes in Fig. 4A) in the 2H sub-region may compensate for the loss of GATApal activity in these transfection assays.

GATA2 is indispensable for *Gata2* transcriptional activation in the midbrain

In order to determine the role, if any, GATA2 might play in activation of the *Gata2* gene, we crossed heterozygous

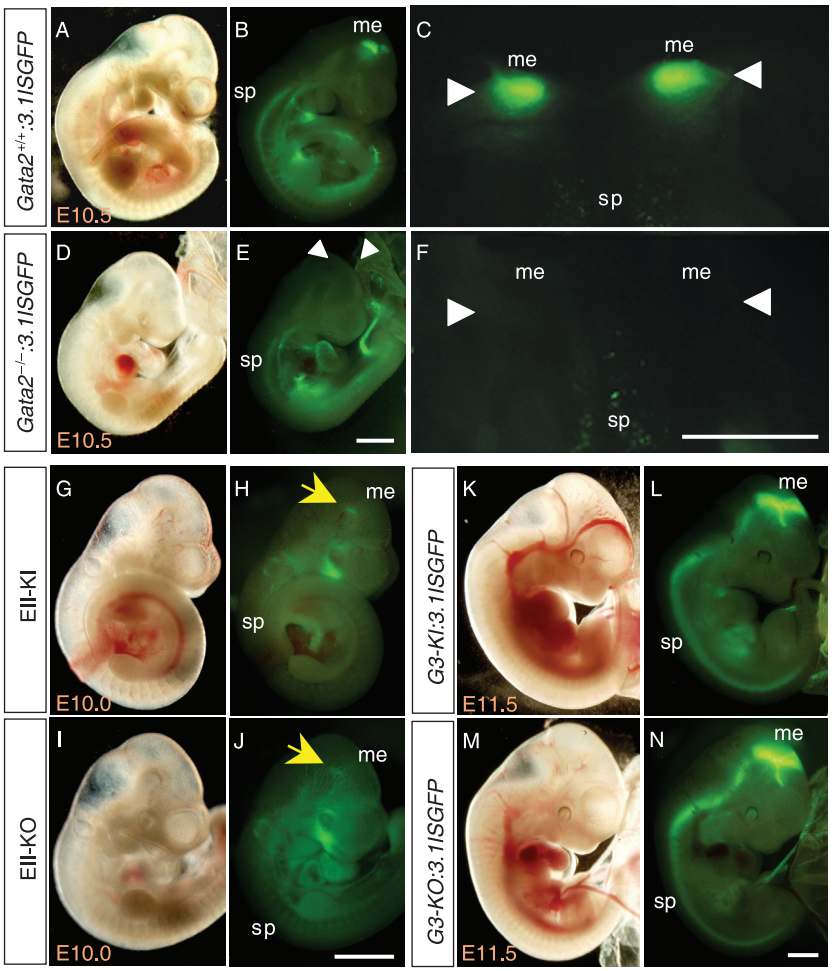

Figure 5 GATA2 is required but GATA3 is dispensable for the expression of 3.1ISGFP transgene in the midbrain. (A–F) Representative bright field (A, D) and fluorescent (B, C, E, F) micrographs of wild-type (A–C) and *Gata2*-null (D–F) embryos owing 3.1ISGFP transgene at E10.5. Higher magnification dorsal views of dissected midbrains are presented in C and F. Abbreviations are as described in the legend to Fig. 2(D). Note that the expression of 3.1ISGFP transgene in the mesencephalon (me) was not detectable in *Gata2*-null embryos (arrowheads in E, F). Scale bars indicate 1 mm (G–J) Representative bright field images (G, I) and fluorescent images (H, J) of EII-KI (G, H) and EII-KO (I, J) embryos at E10.0. The GFP expression in the mesencephalon (me, yellow arrow) was detectable in both EII-KI (H) and EII-KO (J). (K–N) Representative bright field (K, M) and fluorescent (L, N) micrographs of G3-KI (K, L) and G3-KO (M, N) embryos owing 3.1ISGFP transgene at E11.5. The 3.1ISGFP transgene expression was observed in the *Gata3*-null midbrain and the spinal cord. Scale bar, 1 mm; me, mesencephalon; sp, spinal cord.

Gata2-null mutant mice (*Gata2*$^{+/-}$) to mice bearing the 3.1ISGFP transgene. Through subsequent breeding, the 3.1ISGFP transgene was integrated into the *Gata2*$^{-/-}$ mouse genome, so that the GFP expression in the midbrain of the *Gata2*$^{-/-}$ mouse (Fig. 5A–C) could be compared with that of wild-type mice at E10.5 (Fig. 5D–F), just before embryonic demize of the *Gata2*-null embryos (Tsai *et al.* 1994).

We observed that 3.1ISGFP transgene expression in the mesencephalon was missing in *Gata2*$^{-/-}$ embryos, even though spinal cord expression persisted (compare Fig. 5B with 5E). The midbrain-specific loss of expression was confirmed by detailed examination of the histological sections (Fig. 5C,F). We executed analogous experiments employing the 7.0ISGFP transgene, and found that the same disparity of GFP expression was observed (Supplementary Fig. S5). These *in vivo* data thus demonstrate that GATA2 is required for *Gata2* expression in the midbrain and therefore that *Gata2* is under positive autoregulatory control.

We previously observed midbrain GFP expression in 3.1ISGFP transgenic embryos only after E10.5 (Kobayashi-Osaki *et al.* 2005), whereas midbrain fluorescence in EII-KI and EIS-KI mice could be detected by approximately E9.5 (Suzuki *et al.* 2006). This discrepancy suggested that the initiation of *Gata2* expression in the midbrain might require some additional regulatory information that was located outside of the 3.1IS region. An intriguing question here was whether or not GATA2 is also required for the initiation of *Gata2* gene expression in the midbrain. To address this question, we examined the expression of GFP in the midbrain of *Gata2*-null mutant mice (EII-KO) in which GFP gene was knocked into the *Gata2* gene (*Gata2*$^{GFP/-}$). The EII-KO mice were generated through crossing the EII-KI (*Gata2*$^{GFP/+}$) mice with *Gata2*$^{+/-}$ mice. The EII-KO embryos displayed an identical embryonic lethal phenotype to *Gata2*$^{-/-}$ embryos, allowing us to conclude that the EII-KO allele is a genuine null (Tsai *et al.* 1994; Suzuki *et al.* 2006). Importantly, we detected GFP expression in the mesencephalon of EII-KO embryos

at E10.0 (Fig. 5I,J). The GFP expression pattern in EII-KO embryo (Fig. 5I,J) was similar to that in EII-KI embryos at E10.0 (Fig. 5G,H), and no histological abnormality was noticed in the tissue sections of EII-KO midbrain (data not shown). These results thus demonstrate that, although GATA2 is necessary for the stable autoregulation of the *Gata2* gene in the midbrain after E10.5, GATA2 itself is dispensable for the initial activation of the gene in the E9.5 midbrain.

We exploited similar approach to clarify possible compensatory roles for GATA3 in *Gata2* gene expression. For this purpose, we used a *Gata3* knock-in line that also generates a null allele (G3-KI, $Gata3^{LacZ/+}$; Hendriks *et al.* 1999). The study was conducted by intercrossing the G3-KI ($Gata3^{LacZ/+}$) mice with the 3.1ISGFP transgene. We found that 3.1ISGFP transgene was expressed normally in the midbrain of E11.5 embryos even in the homozygous $Gata3^{lacZ/lacZ}$ background (Fig. 5M,N), and the expression profile was indistinguishable from *G3-KI:3.1ISGFP* embryos (Fig. 5K,L). These results, taken together, indicate that GATA3 plays no role in midbrain *Gata2* gene regulation.

Discussion

Physiological significance of GATA2 in the hematopoietic cell differentiation, placenta formation, urogenital system development and pituitary function have been demonstrated exploiting mice defective of the *Gata2* gene expression (Tsai *et al.* 1994; Ma *et al.* 1997; Zhou *et al.* 1998, 2000; Charles *et al.* 2006; Hoshino *et al.* 2008). Similarly, to clarify the regulation governing the *Gata2* gene expression or to understand 'regulation of the regulators,' we have been using a transgenic reporter mouse approach and delineated multiple enhancers directing the gene expression in early hematopoietic cells, urogenital cells and endothelial cells (Minegishi *et al.* 1999, 2003; Zhou *et al.* 2000; Khandekar *et al.* 2004, 2007; Kobayashi-Osaki *et al.* 2005). In this study, we adopted combinatory use of the reporter transgenic mouse lines and reporter knock-in mouse lines, and studied the *Gata2* gene expression in neural cells. We identified two regulatory domains that specify the *Gata2* gene expression in the SC and IC of the midbrain. These two regulatory domains use GATA2 to maintain their activity, but initiation of the gene expression occurs without GATA2. We also found that this GATA box-mediated regulation cannot be replaced by GATA3. These results support our contention that the *Gata2* gene expression in the midbrain is under the positive autoregulatory system.

We found in this study that GATA2 is highly expressed in the midbrain, but not in the cerebrum and cerebellum of adult mouse brain. In the midbrain, cells expressing *Gata2* were distributed in the SC, IC and DRN. Although midbrain function is related to the visual and auditory sensations, details of the function are still elusive (Wallace *et al.* 1993; Krishna & Semple 2000). The GFP transgenic mice used in this study must be a useful means to monitor the midbrain development and also to isolate *Gata2* expressing cells from the midbrain. Especially, the 2H-ISGFP transgene is available as a specific marker for the SC. GATA2 has been suggested to regulate proliferation and differentiation of immature neural progenitor cells. For instance, GATA2 plays a role in maintaining neuronal progenitor cells at quiescent status (El Wakil *et al.* 2006) and GATA2 is essential for development of V2 interneuron in the spinal cord (Zhou *et al.* 2000). On the contrary, this study revealed that virtually no morphological abnormality was observed in the midbrain of *Gata2*-null mice during the time the mice alive. Similarly, neural cell-specific deletion of the *Gata2* gene in mice did not affect development of the midbrain (T. Hoshino and M. Yamamoto, unpublished data). These results indicate that GATA2 is dispensable for the midbrain formation even though it is specifically and highly expressed in there.

A small portion of *Gata2* expressing cells in DRN are found to synthesize serotonin, and this observation is consistent with the previous finding that expression of serotonin biosynthetic enzymes in the hindbrain cells requires GATA2 (Craven *et al.* 2004; Alenina *et al.* 2006). However, most of the *Gata2* expressing cells in the midbrain were negative for serotonin production, suggesting that GATA2 in the midbrain is irrelevant to the serotonin biosynthesis. As GATA3 is also expressed in the midbrain (George *et al.* 1994; Nardelli *et al.* 1999), one plausible hypothesis is that GATA3 may have an activity compensating GATA2 in the midbrain. However, cells expressing *Gata2* is clearly different from those expressing *Gata3* in the midbrain. Thus, our current observation does not support the hypothesis, and further studies are necessary to understand the physiological contribution of GATA2 to the midbrain formation and function. After acceptance of the present paper, a study describing Gata2-expressing midbrain cells was published (Kala *et al.* 2009). This study demonstrates that GATA2 is required for specification of GABAergic neurons but dispensable for proliferation of the midbrain cells. This finding is in agreement with our study showing that Gata2 gene expression is not detectable in serotonergic neurons andis not essential for morphogenesis of the midbrain.

We previously identified two first exons, that is, IS and IG exons, of the *Gata2* gene (Minegishi *et al.* 1998). In this study we found that both exons are used in the midbrain and 3.1 kbp genomic region upstream of the IS exon (3.1IS

region) can direct the midbrain expression of *Gata2* after E10.5. This is consistent with our previous observation that the proximal promoter region of *Gata2* is required for the midbrain expression of the gene (Zhou *et al.* 2000). The 3.1IS region harbors two *cis*-acting domains, distal 5H and proximal 2H, which are highly conserved among the mammalian genomes. Intriguingly, these two regulatory domains play different roles in the SC and IC sub-regions. In SC either 5H or 2H is sufficient to activate the *Gata2* expression, whereas in IC 5H is sufficient for the gene expression but 2H is not. Thus, two *cis*-acting domains in close proximity individually direct the specific gene expression in two sub-regions of one organ. Consistent with this observation, we previously identified two tandem enhancers for the *Gata2* expression in the urogenital system, which direct the gene expression specifically in two separated urogenital regions (Khandekar *et al.* 2004). Similarly, the *SCL* gene was reported to contain two GATA boxes that show differential contributions to the two midbrain sub-regions (Sinclair *et al.* 1999).

There are several GATA boxes in the 5H domain. The 5H domain also serves as an enhancer for the *Gata2* gene expression in the hematopoietic cells, and some of the GATA boxes are important for the *Gata2* gene expression in hematopoietic cells (Kobayashi-Osaki *et al.* 2005). We speculate that through these GATA motifs this domain is used commonly in the midbrain and hematopoietic cells. Similarly, the 2H domain also contains several GATA boxes including a GATApal motif, but the biological activity of the GATA boxes in the 2H domain remains to be clarified.

In this study the loss of function analysis clearly demonstrates that GATA2 is required for the *Gata2* gene expression in the midbrain. GATA2 binds and activates the 5H and 2H domains in transfection analysis. Thus, GATA2 regulates its own gene in the midbrain, and the 5H and 2H domains play central roles in this autoregulatory loop. Supporting this notion, it has been reported that exogenous human GATA2 induces expression of the endogenous *Gata2* gene in chick embryo spinal cord (El Wakil *et al.* 2006). As it is critically important to regulate strictly the expression of master transcription factors, these transcription factors often acquire regulation of their own gene in a manner adopting the positive and/or negative autoregulatory systems. For instance, positive autoregulation for the *MyoD*, *Gata1* and *PU.1* genes, and negative autoregulation for the *Runx2* gene have been reported (Thayer *et al.* 1989; Drissi *et al.* 2000; Nishikawa *et al.* 2003; Okuno *et al.* 2005). This study for the first time reveals a positive autoregulation of the *Gata2* gene *in vivo* using mouse genetics approach, implying that abundant GATA2 is required for midbrain development/function and is produced through the positive feedback system.

The expression of 3.1ISGFP transgene in the mesencephalon was observed after E10.5 (Kobayashi-Osaki *et al.* 2005), suggesting that the autoregulatory system of *Gata2* *via* the 5H and 2H domains starts approximately E10.5. By contrast, the GFP expression in the midbrain of EII-KI and EIS-KI knocked-in mice begins at E9.5, suggesting that the 3.1IS region may not be sufficient for initiating of the midbrain expression of the endogenous *Gata2* gene. Furthermore, GFP expression from the *Gata2* locus of EII-KO mouse lacking the GATA2 expression was detected in the mesencephalon at E10.0. Thus, the regulatory mechanism of the *Gata2* gene may be different in the initiation stage and maintenance stage; the autoregulatory system is working only for the maintenance stage. Apparently, the positive autoregulation system is useful to maintain gene expression at high level, so that use of the system seems to be reasonable after establishment of the initial expression. Indeed, it was recently reported that the expression of Ad4 BP/SF-1 is regulated by positive autoregulation, and before the establishment of autoregulation homeobox transcription factors act to initiate the Ad4 BP/SF-1 gene expression during adrenal development (Zubair *et al.* 2006).

A schematic model summarizing regulatory mechanism of the *Gata2* gene in the midbrain is shown in Fig. 6. This study delineates that the *Gata2* gene regulation system in the midbrain can be divided into two stages, one is the initiation stage and the other is the maintenance stage. In the initiation stage, a factor yet unknown supports initiation of the *Gata2* expression in the mesencephalon of E9.5–E10.5 embryos through binding to a *cis*-acting element located outside of the 7.0IS region. The GATA2-dependency of the *Gata2* gene expression appears approximately E10.5 after switching of the regulatory system to maintain the *Gata2* gene expression at high level by the positive feedback system. In this maintenance stage, the *Gata2* expression is spread in the SC and IC sub-regions. In SC, GATA2 induces the gene expression through binding to both 5H and 2H domains in the promoter proximal region. However, in IC 5H is used exclusively in the gene expression.

In summary, this study demonstrates that high-level expression of the *Gata2* gene in the midbrain is maintained by GATA2 itself in an autoregulatory manner. Two regulatory domains containing several GATA motifs are identified in the promoter proximal region of the gene as the *cis*-regulatory elements playing essential roles in the autoregulatory loop. These two domains are used differentially in the SC and IC sub-regions of the midbrain. These *in vivo* findings support our contention that the autoregulation system is critically important for the strict control of the expression of key transcription factors.

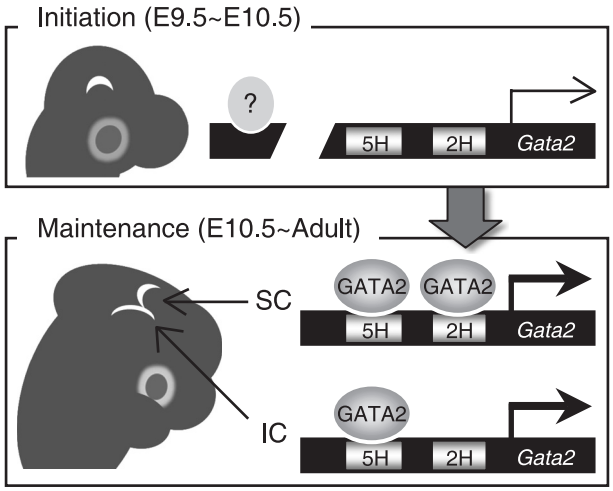

Figure 6 Schematic model of the GATA2-dependent *Gata2* gene expression in the midbrain. At E9.5 the *Gata2* gene expression initiates in the midbrain through contribution of the regulatory element(s) located outside of 7.0IS region and yet unidentified transcription factors. The midbrain expression of *Gata2* is highly maintained after the initiation, and for this maintenance GATA2 binding to the 5H and 2H domains containing highly conserved GATA boxes is essential. Differential mechanisms in the autoregulation of *Gata2* gene between two major domains of the midbrain are demonstrated, that is, both 5H and 2H domains are sufficient to regulate the *Gata2* gene expression in the SC. However, the expression in the IC requires the 5H domain but not the 2H domain.

Experimental procedures

Mice

The EIS-KI (*Gata2*$^{IS\text{-}GFP/+}$ genotype) and EII-KI (*Gata2*$^{GFP/+}$) mouse lines are as previously described (Suzuki *et al.* 2006). The *Gata3*-LacZ knock-in mouse line was as previously described (Hendriks *et al.* 1999) and heterozygous and homozygous mutants were referred to as G3-KI (*Gata3*$^{LacZ/+}$) and G3-KO (*Gata3*$^{LacZ/LacZ}$), respectively. *Gata2*-null mice were kindly provided by Dr Stu Orkin (Tsai *et al.* 1994). The EII-KI and *Gata2*-null mice were crossed to generate EII-KO (*Gata2*$^{GFP/-}$) mice. 7.0ISGFP, 3.1ISGFP, and 5H-ISGFP transgenic lines were as previously reported (Minegishi *et al.* 1999; Kobayashi-Osaki *et al.* 2005). Genotyping was performed by PCR using genomic DNA extracted from the tail of adult mice or a part of embryos as templates (Kobayashi-Osaki *et al.* 2005).

The 0.6ISGFP transgene was prepared from p7.0ISGFP (Minegishi *et al.* 1999) by digesting with *Eco*RI (656 bp from the transcription initiation site of IS exon) and *Hin*dIII. To construct 2H-ISGFP transgene, the 2H domain (–1977 to –1619) was amplified by PCR, and resulting product was ligated to 0.6ISGFP transgene construct. Transgene constructs were isolated from the vector sequences, and purified with QIAquick Gel Extraction Kit (QIAGEN, Venlo, Netherlands). DNA (5 ng/mL) was injected into fertilized mouse eggs using standard protocols as described (Kobayashi-Osaki *et al.* 2005), and embryos and pups harboring the transgene were identified by PCR. All mice were strictly kept in specific pathogen-free conditions and were treated according to the regulations of '*The Standards for Human Care and Use of Laboratory Animals of the University of Tsukuba*.'

Histological analyses

Tissues and embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30–60 min at 4 °C. Fixed samples were embedded in OCT compound (Sakura Finetec, Tokyo) and rapidly frozen in liquid nitrogen, before making sections of 7 μM (for X-gal and immunohistochemical staining) or 50 μM (for immunofluorescence) in thickness.

For X-gal staining, fixed embryos or frozen sections were incubated with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Wako, Osaka, Japan) at 37 °C in the overnight. For immunohistochemistry, frozen sections were treated with 1% hydrogen peroxide to block endogenous peroxidase activity. Before antibody staining, the samples were blocked with 1% goat serum and 0.2% bovine serum albumin in 2% skim milk and 0.1% Triton X-100 in phosphate-buffered saline (PBS-MT). The sections were incubated with rabbit anti-GFP antibody (diluted 1 : 1000, MBL, Nagoya). Specific antibody binding was visualized as brown staining by reaction with diaminobenzidine (250 mg/mL) with 0.01% H_2O_2 after incubation with horseradish peroxidase-conjugated anti-rabbit IgG (BioSource, Camarillo, CA). Hematoxylin was used for counterstaining.

For serotonin immunofluorescence staining, sections were incubated with rabbit anti-serotonin antibody (diluted 1 : 50, Zymed, San Francisco, CA) at 4 °C overnight followed by incubation with biotinylated goat anti-rabbit IgG antibody (diluted 1 : 1000, Vector Laboratories, Burlingame, CA) for 1 h and CY5-streptavidin (diluted 1 : 500, Zymed) for 1 h. After washing, sections were mounted with fluorescent mounting medium (Dako, Glostrup, Denmark) and examined by confocal laser scanning microscopy (Zeiss, Oberkochen, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was isolated from the cerebrum, midbrain, cerebellum, thymus and lung of adult BDF1 mice using Isogen (Nippon Gene, Toyama, Japan) and reverse transcribed by SuperScript II RT (Invitrogen, Carlsbad, CA) and random primers. For semi-quantitative analyses, each cDNA was diluted so that the hypoxanthine phosphoribosyltransferase (HPRT) amplicon was equivalent amongst the test samples. PCR reaction (42 cycles) was performed as follows: 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, using primer pairs G2s (5′-TGCAACACACCACCCGATACC-3′) and G2as (5′-CAATTTGCACAACAGGTGCCC-3′) for *Gata2*, G3RT5′ (5′-TCTCACTCTCGAGGCAGCATGT-3′) and G3RT3′ (5′-GTACCATCTCGCCGCCACAG-3′) for *Gata3* and HPRTs (5′-GTGGTGAAAAGGACCTCT-3′) and HPRTas (5′-ACAG GACTAGAACACCTGC-3′) for *HPRT*.

Quantitative PCR analysis of sorted cells

The midbrain and spinal cord samples from 3.1ISGFP transgenic E10.5 embryos (line 95, Kobayashi-Osaki *et al.* 2005) were dissected, and the tissues were treated with 0.05% trypsin-EDTA at 37 °C for 10 min before dissociation by pipetting. GFP^+ and GFP^- cells in the single cell suspensions were sorted using FACS Vantage (Becton Dickinson, Franklin Lakes, NJ) as described (Kobayashi-Osaki *et al.* 2005). RNA was extracted by RNeasy Mini Kit (QIAGEN) from the sorted cells, and reverse transcribed by SuperScript II RT with random primers. cDNA samples were rendered quantitative RT-PCR analysis of *Gata2* mRNA using ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The sequence of primers and probe were described (Suzuki *et al.* 2003), and the data were normalized to expression level of rRNA (Applied Biosystems).

Comparison of genomic sequences

Conservation of genomic sequences was examined with Mouse BLAT Search in UCSC Genome Browser website <http://genome.ucsc.edu> among vertebrates, that is, mouse, rat, rabbit, human, chimp, rhesus, cow, dog, armadillo, elephant, tenrec, opossum, chicken, xenopus, tetraodon, fugu and zebrafish.

Luciferase reporter analysis

The 2H domain fragment was integrated into pRBGP3 plasmid containing the firefly *Luciferase* gene driven by a basic promoter from the rabbit *β-globin* gene (Igarashi *et al.* 1994) to generate 2H-Luc plasmid. Expression vectors for human GATA2 and human GATA3 under control of the elongation factor-1α promoter, pEF-GATA2 and pEF-GATA3, were as previously described (Yamaguchi *et al.* 1998). C1300 mouse neuroblastoma cells (Dunham & Stewart 1953) were maintained in Dulbecco's modified Eagle medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT), 50-U/mL penicillin and 50-mg/mL streptomycin sulfate. Cells were cultured in 12-well plates, and transfected 0.9 μg of 2H-Luc or pRBGP3 with or without co-transfection of GATA factor expression vectors (50 ng) using FuGene 6 (Roche, Basel, Switzerland). Twenty-four hours after transfection, luciferase activities were determined with Dual-Luciferase reporter assay system (Promega, Madison, WI) following the instructions of the manufacturer, and 0.1 μg of pRL-TK containing the Renilla *Luciferase* gene (Promega) was co-transfected in each well to standardize transfection efficiency. Expression levels of GATA2 and GATA3 proteins in the cultured cells were detected by Western blotting analyses using rat anti-chicken GATA2 monoclonal antibody (RC1.1.1) (Zhou *et al.* 1998) and mouse anti-human GATA3 monoclonal antibody (HG3-31, SANTA CRUZ, Santa Cruz, CA).

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References

Alenina, N., Bashammakh, S. & Bader, M. (2006) Specification and differentiation of serotonergic neurons. *Stem Cell Rev*. **2**, 5–10.

Arinobu, Y., Mizuno, S., Chong, Y., Shigematsu, H., Iino, T., Iwasaki, H., Graf, T., Mayfield, R., Chan, S., Kastner, P. & Akashi, K. (2007) Reciprocal activation of GATA-1 and PU.1 marks initial specification of hematopoietic stem cells into myeloerythroid and myelolymphoid lineages. *Cell Stem Cell* **1**, 416–427.

Brandt, W., Khandekar, M., Suzuki, N., Yamamoto, M., Lim, K.C. & Engel, J.D. (2008) Defining the functional boundaries of the Gata2 locus by rescue with a linked bacterial artificial chromosome transgene. *J. Biol. Chem*. **283**, 8976–8983.

Burch, J.B. (2005) Regulation of GATA gene expression during vertebrate development. *Semin. Cell Dev. Biol*. **16**, 71–81.

Charles, M.A., Saunders, T.L., Wood, W.M., Owens, K., Parlow, A.F., Camper, S.A., Ridgway, E.C. & Gordon, D.F. (2006) Pituitary-specific Gata2 knockout: effects on gonadotrope and thyrotrope function. *Mol. Endocrinol*. **20**, 1366–1377.

Craven, S.E., Lim, K.C., Ye, W., Engel, J.D., de Sauvage, F. & Rosenthal, A. (2004) Gata2 specifies serotonergic neurons downstream of sonic hedgehog. *Development* **131**, 1165–1173.

van Doorninck, J.H., van Der Wees, J., Karis, A., Goedknegt, E., Engel, J.D., Coesmans, M., Rutteman, M., Grosveld, F. & De Zeeuw, C.I. (1999) GATA-3 is involved in the development of serotonergic neurons in the caudal raphe nuclei. *J. Neurosci*. **19**, RC12.

Drissi, H., Luc, Q., Shakoori, R., Chuva De Sousa Lopes, S., Choi, J.Y., Terry, A., Hu, M., Jones, S., Neil, J.C., Lian, J.B., Stein, J.L., Van Wijnen, A.J. & Stein, G.S. (2000) Transcriptional autoregulation of the bone related CBFA1/RUNX2 gene. *J. Cell. Physiol*. **184**, 341–350.

Dunham, L.J. & Stewart, H.L. (1953) A survey of transplantable and transmissible animal tumors. *J. Natl. Cancer Inst*. **13**, 1299–1377.

El Wakil, A., Francius, C., Wolff, A., Pleau-Varet, J. & Nardelli, J. (2006) The GATA2 transcription factor negatively regulates the proliferation of neuronal progenitors. *Development* **133**, 2155–2165.

George, K.M., Leonard, M.W., Roth, M.E., Lieuw, K.H., Kioussis, D., Grosveld, F. & Engel, J.D. (1994) Embryonic expression and cloning of the murine GATA-3 gene. *Development* **120**, 2673–2686.

Grass, J.A., Jing, H., Kim, S.I., Martowicz, M.L., Pal, S., Blobel, G.A. & Bresnick, E.H. (2006) Distinct functions of dispersed GATA factor complexes at an endogenous gene locus. *Mol. Cell. Biol.* **26**, 7056–7067.

Harigae, H., Takahashi, S., Suwabe, N., Ohtsu, H., Gu, L., Yang, Z., Tsai, F.Y., Kitamura, Y., Engel, J.D. & Yamamoto, M. (1998) Differential roles of GATA-1 and GATA-2 in growth and differentiation of mast cells. *Genes Cells* **3**, 39–50.

Heinl-Green, A., Radke, P.W., Munkonge, F.M., Frass, O., Zhu, J., Vincent, K., Geddes, D.M. & Alton, E.W. (2005) The efficacy of a "master switch gene" HIF-1alpha in a porcine model of chronic myocardial ischaemia. *Eur. Heart J.* **26**, 1327–1332.

Hendriks, R.W., Nawijn, M.C., Engel, J.D., van Doorninck, H., Grosveld, F. & Karis, A. (1999) Expression of the transcription factor GATA-3 is required for the development of the earliest T cell progenitors and correlates with stages of cellular proliferation in the thymus. *Eur. J. Immunol.* **29**, 1912–1918.

Hoshino, T., Shimizu, R., Ohmori, S., Nagano, M., Pan, X., Ohneda, O., Khandekar, M., Yamamoto, M., Lim, K.C. & Engel, J.D. (2008) Reduced BMP4 abundance in Gata2 hypomorphic mutant mice result in uropathies resembling human CAKUT. *Genes Cells* **13**, 159–170.

Huard, C., Moisset, P.A., Dicaire, A., Merly, F., Tardif, F., Asselin, I. & Tremblay, J.P. (1998) Transplantation of dermal fibroblasts expressing MyoD1 in mouse muscles. *Biochem. Biophys. Res. Commun.* **248**, 648–654.

Igarashi, K., Kataoka, K., Itoh, K., Hayashi, N., Nishizawa, M. & Yamamoto, M. (1994) Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins. *Nature* **367**, 568–572.

Kala, K., Haugas, M., Lilleväli, K., Guimera, J., Wurst, W., Salminen, M. & Partanen, J. (2009) Gata2 is a tissue-specific post-mitotic selector gene for midbrain GABAergic neurons. *Development* **136**, 253–262.

Khandekar, M., Brandt, W., Zhou, Y., Dagenais, S., Glover, T.W., Suzuki, N., Shimizu, R., Yamamoto, M., Lim, K.C. & Engel, J.D. (2007) A Gata2 intronic enhancer confers its pan-endothelia-specific regulation. *Development* **134**, 1703–1712.

Khandekar, M., Suzuki, N., Lewton, J., Yamamoto, M. & Engel, J.D. (2004) Multiple, distant Gata2 enhancers specify temporally and tissue-specific patterning in the developing urogenital system. *Mol. Cell. Biol.* **24**, 10263–10276.

Kobayashi-Osaki, M., Ohneda, O., Suzuki, N., Minegishi, N., Yokomizo, T., Takahashi, S., Lim, K.C., Engel, J.D. & Yamamoto, M. (2005) GATA motifs regulate early hematopoietic lineage-specific expression of the Gata2 gene. *Mol. Cell. Biol.* **25**, 7005–7020.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S. & Kishimoto, T. (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89**, 755–764.

Krishna, B.S. & Semple, M.N. (2000) Auditory temporal processing: responses to sinusoidally amplitude-modulated tones in the inferior colliculus. *J. Neurophysiol.* **84**, 255–273.

Lakshmanan, G., Lieuw, K.H., Lim, K.C., Gu, Y., Grosveld, F., Engel, J.D. & Karis, A. (1999) Localization of distant urogenital system-, central nervous system-, and endocardium-specific transcriptional regulatory elements in the GATA-3 locus. *Mol. Cell. Biol.* **19**, 1558–1568.

Ma, G.T., Roth, M.E., Groskopf, J.C., Tsai, F.Y., Orkin, S.H., Grosveld, F., Engel, J.D. & Linzer, D.I. (1997) GATA-2 and GATA-3 regulate trophoblast-specific gene expression *in vivo*. *Development* **124**, 907–914.

Minegishi, N., Ohta, J., Suwabe, N., Nakauchi, H., Ishihara, H., Hayashi, N. & Yamamoto, M. (1998) Alternative promoters regulate transcription of the mouse GATA-2 gene. *J. Biol. Chem.* **273**, 3625–3634.

Minegishi, N., Ohta, J., Yamagiwa, H., Suzuki, N., Kawauchi, S., Zhou, Y., Takahashi, S., Hayashi, N., Engel, J.D. & Yamamoto, M. (1999) The mouse GATA-2 gene is expressed in the para-aortic splanchnopleura and aorta-gonads and mesonephros region. *Blood* **93**, 4196–4207.

Minegishi, N., Suzuki, N., Yokomizo, T., Pan, X., Fujimoto, T., Takahashi, S., Hara, T., Miyajima, A., Nishikawa, S. & Yamamoto, M. (2003) Expression and domain-specific function of GATA-2 during differentiation of the hematopoietic precursor cells in midgestation mouse embryos. *Blood* **102**, 896–905.

Nardelli, J., Thiesson, D., Fujiwara, Y., Tsai, F.Y. & Orkin, S.H. (1999) Expression and genetic interaction of transcription factors GATA-2 and GATA-3 during development of the mouse central nervous system. *Dev. Biol.* **210**, 305–321.

Nishikawa, K., Kobayashi, M., Masumi, A., Lyons, S.E., Weinstein, B.M., Liu, P.P. & Yamamoto, M. (2003) Self-association of Gata1 enhances transcriptional activity *in vivo* in zebra fish embryos. *Mol. Cell. Biol.* **23**, 8295–8305.

Ohneda, K. & Yamamoto, M. (2002) Roles of hematopoietic transcription factors GATA-1 and GATA-2 in the development of red blood cell lineage. *Acta Haematol.* **108**, 237–245.

Okuno, Y., Huang, G., Rosenbauer, F., Evans, E.K., Radomska, H.S., Iwasaki, H., Akashi, K., Moreau-Gachelin, F., Li, Y., Zhang, P., Gottgens, B. & Tenen, D.G. (2005) Potential autoregulation of transcription factor PU.1 by an upstream regulatory element. *Mol. Cell. Biol.* **25**, 2832–2845.

Sinclair, A.M., Gottgens, B., Barton, L.M., Stanley, M.L., Pardanaud, L., Klaine, M., Gering, M., Bahn, S., Sanchez, M., Bench, A.J., Fordham, J.L., Bockamp, E. & Green, A.R. (1999) Distinct 5' SCL enhancers direct transcription to developing brain, spinal cord, and endothelium: neural expression is mediated by GATA factor binding sites. *Dev. Biol.* **209**, 128–142.

Suzuki, N., Ohneda, O., Minegishi, N., Nishikawa, M., Ohta, T., Takahashi, S., Engel, J.D. & Yamamoto, M. (2006) Combinatorial Gata2 and Sca1 expression defines hematopoietic stem cells in the bone marrow niche. *Proc. Natl. Acad. Sci. USA* **103**, 2202–2207.

Suzuki, N., Suwabe, N., Ohneda, O., Obara, N., Imagawa, S., Pan, X., Motohashi, H. & Yamamoto, M. (2003) Identification and characterization of 2 types of erythroid progenitors that express GATA-1 at distinct levels. *Blood* **102**, 3575–3583.

Thayer, M.J., Tapscott, S.J., Davis, R.L., Wright, W.E., Lassar, A.B. & Weintraub, H. (1989) Positive autoregulation of the myogenic determination gene MyoD1. *Cell* **58**, 241–248.

Trainor, C.D., Omichinski, J.G., Vandergon, T.L., Gronenborn, A.M., Clore, G.M. & Felsenfeld, G. (1996) A palindromic regulatory site within vertebrate GATA-1 promoters requires both zinc fingers of the GATA-1 DNA-binding domain for high-affinity interaction. *Mol. Cell. Biol.* **16**, 2238–2247.

Tsai, F.Y., Keller, G., Kuo, F.C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F.W. & Orkin, S.H. (1994) An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221–226.

Wallace, M.T., Meredith, M.A. & Stein, B.E. (1993) Converging influences from visual, auditory, and somatosensory cortices onto output neurons of the superior colliculus. *J. Neurophysiol.* **69**, 1797–1809.

Weintraub, H., Tapscott, S.J., Davis, R.L., Thayer, M.J., Adam, M.A., Lassar, A.B. & Miller, A.D. (1989) Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. *Proc. Natl. Acad. Sci. USA* **86**, 5434–5438.

Yamaguchi, Y., Ackerman, S.J., Minegishi, N., Takiguchi, M., Yamamoto, M. & Suda, T. (1998) Mechanisms of transcription in eosinophils: GATA-1, but not GATA-2, transactivates the promoter of the eosinophil granule major basic protein gene. *Blood* **91**, 3447–3458.

Yamamoto, M., Ko, L.J., Leonard, M.W., Beug, H., Orkin, S.H. & Engel, J.D. (1990) Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. *Genes Dev.* **4**, 1650–1662.

Zhou, Y., Lim, K.C., Onodera, K., Takahashi, S., Ohta, J., Minegishi, N., Tsai, F.Y., Orkin, S.H., Yamamoto, M. & Engel, J.D. (1998) Rescue of the embryonic lethal hematopoietic defect reveals a critical role for GATA-2 in urogenital development. *EMBO J.* **17**, 6689–6700.

Zhou, Y., Yamamoto, M. & Engel, J.D. (2000) GATA2 is required for the generation of V2 interneurons. *Development* **127**, 3829–3838.

Zubair, M., Ishihara, S., Oka, S., Okumura, K. & Morohashi, K. (2006) Two-step regulation of Ad4 BP/SF-1 gene transcription during fetal adrenal development: initiation by a Hox-Pbx1-Prep1 complex and maintenance via autoregulation by Ad4 BP/SF-1. *Mol. Cell. Biol.* **26**, 4111–4121.

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Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 Expression of the *Gata2* and *Gata3* genes in the adult midbrain.

Figure S2 Promoter usage of the *Gata2* gene in the midbrain.

Figure S3 GFP fluorescence intensity in SC and IC of *Gata2*-GFP transgenic embryos.

Figure S4 Comparative aliments of the *Gata2*-5H domain among the mammalian genomic sequences, with special emphasis on the GATA boxes in the midbrain regulatory region of the *Gata2* gene.

Figure S5 Midbrain-specific loss of 7.0ISGFP transgene expression in *Gata2*-null embryo.

Additional Supporting Information may be found in the online version of this article.

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