

# Identification and characterization of alternative promoters of zebrafish *Rtn-4/Nogo* genes in cultured cells and zebrafish embryos

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## ABSTRACT

In mammals, the Nogo family consists of Nogo-A, Nogo-B and Nogo-C. However, there are three *Rtn-4/Nogo*-related transcripts were identified in zebrafish. In addition to the common C-terminal region, the N-terminal regions of *Rtn4-n/Nogo-C1*, *Rtn4-m/Nogo-C2* and *Rtn4-l/Nogo-B*, respectively, contain 9, 25 and 132 amino acid residues. In this study, we isolated the 5'-upstream region of each gene from a BAC clone and demonstrated that the putative promoter regions, P1-P3, are functional in cultured cells and zebrafish embryos. A transgenic zebrafish *Tg(Nogo-B:GFP)* line was generated using P1 promoter region to drive green fluorescent protein (GFP) expression through Tol2-mediated transgenesis. This line recapitulates the endogenous expression pattern of *Rtn4-l/Nogo-B* mRNA in the brain, brachial arches, eyes, muscle, liver and intestines. In contrast, GFP expressions by P2 and P3 promoters were localized to skeletal muscles of zebrafish embryos. Several GATA and E-box motifs are found in these promoter regions. Using morpholino knockdown experiments, *GATA4* and *GATA6* were involved in the control of P1 promoter activity in the liver and intestine, while *Myf5* and *MyoD* for the control of P1 and P3 promoter activities in muscles. These data demonstrate that

zebrafish *Rtn4/Nogo* transcripts might be generated by coupling mechanisms of alternative first exons and alternative promoter usage.

## INTRODUCTION

The inability of mammalian central nervous system (CNS) axons to regenerate after spinal cord injury can partly be attributed to inhibitory proteins associated with CNS myelin (1,2). These myelin proteins include Nogo, myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein (3). The human *nogo* gene encodes three isoforms, termed Nogo-A, -B and -C, by alternative promoter usage and alternative RNA splicing. All three isoforms share a common C-terminal region composed of two transmembrane domains and an extracellular domain with a short 66-amino acid (aa) residue (Nogo-66), which binds to the Nogo-66 receptor (4). The human Nogo-A protein is the longest variant and contains three regions: the N-terminal 1–180 aa, the middle 181–1004 aa (NogoA-specific) and the C-terminal 1005–1192 aa containing the Nogo-66 domain. Nogo-B is an isoform lacking 181–1004 aa of Nogo-A, while Nogo-C lacks residues 1–1004 and has an extra 12-aa residue at the N-terminus.

Currently, Nogo-A is a known myelin-associated inhibitor of axonal regeneration after spinal injury (5), whereas Nogo-B was suggested to play important roles in the control of tumor progression (6,7) as well as regulating

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vascular remodelling (8). However, less is known about the function of Nogo-C. Moreover, a human Nogo-B was demonstrated to bind to a new receptor, NgBR, which is expressed in endothelial cells (9). Although Nogo-B is not essential for normal vessel development, local overexpression of Nogo-B elicited a beneficial effect on vascular injury-induced neointimal expansion (10).

In contrast to mammals, many axons in zebrafish regrow in the CNS after a lesion (11). Although growth cones of goldfish retinal axons can cross fish CNS myelin, they collapse when they come in contact with mammalian CNS myelin. These data suggest that fish axons recognize neurite growth inhibitors associated with mammalian CNS myelin, such as Nogo-A, but fish CNS myelin is devoid of such inhibitors (12). The absence of the *Nogo-A* gene in the zebrafish genome was reported (13).

Three zebrafish *Rtn4*/Nogo-related transcripts were cloned (13–15). They were first named *Rtn4-l*, *Rtn4-m* and *Rtn4-n* (13), followed by other names, Nogo- $\alpha$ , Nogo- $\beta$  and Nogo- $\gamma$  (14). They have identical C-terminal regions of 187 aa, which contain two transmembrane domains and one Nogo-66 domain and show high degrees of aa identity to human and rat Nogo-related proteins. The variable N-terminal regions of zebrafish *Rtn4*/Nogo-related proteins contain 132-, 25- and 9-aa residues, respectively. Indeed, *Rtn4-l* and Nogo- $\alpha$  are homologous to human and mouse Nogo-B with 43% identity, while *Rtn4-m*/Nogo- $\beta$  and *Rtn4-n*/Nogo- $\gamma$  are homologous to mammalian Nogo-C. Thus, there are two isoforms of Nogo-C in zebrafish. The short form can be called *Rtn4-n*/Nogo- $\gamma$ /Nogo-C1, while the long form can be called *Rtn4-m*/Nogo- $\beta$ /Nogo-C2.

Moreover, these transcript variants use different translation start sites, and have distinct expression patterns of transcripts. The *Rtn4-l*/Nogo-B and *Rtn4-n*/Nogo-C1 transcripts were expressed ubiquitously in all tissues examined. The *Rtn4-m*/Nogo-C2 mRNA was also expressed in all tissues except for the ovary, but was more abundant in the muscle. During development, the *Rtn4-l*/Nogo-B mRNA was expressed in eye, midbrain, brachial arch, muscle, liver and intestine, while *Rtn4-m*/Nogo-C2 mRNA was present in forebrain, midbrain, hindbrain, brachial arch, muscle and intestine (13–15). These data suggest that the expressions of alternative splice variants of *Rtn4*/Nogo genes can be differentially regulated by alternative promoters. Currently, little is known about coupling mechanisms of alternative first exons and alternative promoter usage in zebrafish *Rtn4*/Nogo genes. Herein, we report the isolation and characterization of three alternative promoters of zebrafish *Rtn4*/Nogo genes. The results of the luciferase analysis in cultured cells and transgenic zebrafish expressing the green fluorescent protein (GFP) driven by their upstream regions revealed that these three alternative promoters are biologically functional. Thus, the expressions of zebrafish *Rtn4*/Nogo transcripts might also be regulated by alternative splicing coupled with the use of alternative promoters, which is similar to the transcriptional regulation of mammalian Nogo genes.

## MATERIALS AND METHODS

### Cell cultures

Monkey kidney fibroblast COS-1 cells and mouse muscle myoblast C2C12 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (HyClone, UT, USA), penicillin G (50 U ml<sup>-1</sup>), streptomycin (50  $\mu$ g ml<sup>-1</sup>) and L-glutamine (2 mM) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

### Fish

Zebrafish were raised and maintained under standard conditions. Embryos were incubated at 28°C, and different developmental stages were determined according to the description in the *Zebrafish Book* (16).

### Cloning of the P1, P2 and P3 promoter regions of Nogo-related genes

All three promoter regions were amplified by a polymerase chain reaction (PCR) using the BAC clone, DKEY-103K8, as a template. Three primer sets, 5'-GGT ACCAAGCTTTGATTGGTTAATTGTAA-3' and 5'-GTCGACAGAAACG TTATTTTAAAG-3', 5'-GGTACC AGATCTCAAAAAGTCTTTAAAAA-3' and 5'-GTCGACCGGTCATATATCCAGTCAGGGGTC-3', and 5'-GGTACCAGATCTGTGTAAAAAGGTCTGCCG-3' and 5'-GTTCGACCTGCGATCAGACAGCCTG GGCC AA-3' were, respectively, used to amplify the P1 region (–4885 to –13), the P2 region (–3230 to –1) and the P3 region (–3014 to –1). The forward and reverse primers, respectively, contained *KpnI* and *SalI* sites. The PCR product was digested with *KpnI* and *SalI* and cloned into the pGL3-Basic vector (Promega, Madison, WI, USA). Other deletion constructs from P1, such as –3028/–13, –2617/–13, –2028/–13 and –782/–13 were similarly generated by a PCR using primers as follows: –3028, 5'-GGTACC GCGCAGTAGGTTAGTGCTGTT GCCTCACA-3'; –2617, 5'-GGTACCCTGCAGAACC GGTGGTACTTTACGTC-3'; –2028, 5'-GGTACCT AATTAATCAGGGGTCACCACAGTGGAA-3'; and –782, 5'-GGTACC GGGCCCCATAGCTGGATTGCT TAGGGCC-3'. Similarly, other deletion constructs from P2 (–2564/–1 and –1213/–1) (as follows: –2564, 5'-GGT ACCTCGCGACAACATAATTAATTCCACTTTG-3' and –1213, 5'-GGTACCTTAATTAATTTTTTTTAAAG AAAGAAACT-3') and P3 (–1292/–1 and –757/–1) (as follows: –1292, 5'-GGTACCATTAAATTGATCAAA AGTGACATGCAA-3' and –757, 5'-GGTACCTTAAT TAAA GCCAGTCTTACCTCAATGC-3') were similarly generated by a PCR with suitable primer sets containing the *KpnI* and *SalI* sites.

To construct the GFP reporter plasmids, a similar strategy was used as described above for the luciferase reporter constructs except using primers carrying the *SalI* and *ApaI* sites. PCR products were digested with *SalI* and *ApaI*, and subcloned into the pT2KXIG $\Delta$ in vector digested with the same restriction enzyme sets (17).

### Search for transcription factor binding sites in the promoter region

Putative transcription factor binding sites in the P1, P2 and P3 promoter regions were identified by the TRANSFAC 6.0 data base using Transcription Element Search Software (18).

### Transactivation assay

Transfections were performed in 12-well plates. One microgram of promoter DNA and 0.5  $\mu\text{g}$  of a pSV- $\beta$ -galactosidase were transfected using a Lipofectamine kit (Invitrogen Life Technologies, CA, USA) into COS-1 or C2C12 cells. Cells were harvested at 48 h after transfection and assayed for luciferase and  $\beta$ -galactosidase activity using a kit from Promega. Luciferase activity was normalized to  $\beta$ -galactosidase activity in cell lysates and expressed as an average of three independent experiments.

### Microinjection of expression constructs into zebrafish embryos

The reporter plasmids were linearized, and DNA concentrations were adjusted to 100  $\mu\text{g ml}^{-1}$  in a 0.1 M KCl solution containing 0.5% phenol red. Microinjection and image capture were performed as described before (19). Injected embryos were observed by fluorescence microscopy.

To obtain stable transgenic fish, embryos at the one-cell stage were co-injected with 10  $\text{ng } \mu\text{l}^{-1}$  of either P1(−4885/−13)-GFP or P3(−3014/−1)-GFP or P3(−1292/−1)-GFP plasmid, 5  $\text{ng } \mu\text{l}^{-1}$  of capped Tol2 transposase mRNA and 0.1% phenol red as previously described (20). The pCS-TP plasmid (21) encoding the transposase was linearized with *NotI* and used as a template for *in vitro* transcription with mMessage mMachine (Ambion, Foster City, CA, USA), according to the manufacturer's instructions.

To generate germline transgenic zebrafish, the injected embryos were raised to adulthood and 3-month-old fish (F0) was crossed to wild-type fish. F1 embryos were examined by fluorescence microscopy to identify germline-transmitted F0 founders. Positive F1 embryos were raised to adulthood and were then screened in the same way to estimate copy numbers of the transgene and to establish stable transgenic lines. Three independent lines (F2 and more advanced generations) were obtained.

### Morpholino injections

Respective morpholino oligonucleotides (MOs) were synthesized by Gene Tools (Philomath, OR, USA). The MO was dissolved in Danieau solution containing 0.5% phenol red to 0.3 mM and 3.2 ng per embryo was injected into embryos at the one- to two-cell stage. MO sequences are: *myod*, 5'-ATATCCGACAACCTCCATCTTTTTTGG-3'; *myf5*, 5'-TCTGGGATGTGGAGAATACGTCCAT-3'; *GATA4*, 5'-TCCACAGGTGAGCG A TTATTGCTCC-3' and *GATA6*, 5'-CGATGGCCAGGGTCTGATACATGTC-3'.

### In situ hybridization

Digoxigenin-labelled antisense RNA probes were generated by *in vitro* transcription using linearized *Rtn4-l/Nogo-B*, *Rtn4-n/Nogo-C1* and *Rtn4-m/Nogo-C2* plasmids as templates. Whole-mount *in situ* hybridization was performed as previously described (19). Specific primers for Nogo-B (5'-GTTGAAACTGCTGCACTGT CGTTCAC-3' and 5'-GCACGAAGAGTTACATT TAT ATGGCGC-3'), Nogo-C1 (5'-GGGACTTTTTGTTTTGA AATTTGACCGGA AC-3' and 5'-CTTGTGTTGGAATC CATCTCGCTCATCTGCG-3'), Nogo-C2 (5'-GTCCTG TCCTGCTCCAGCCAGAGAC-3' and 5'-CCTGTTCT TCCCAGTGTTTA ATCTCC-3'), L-FABP (5'-CACAG GCTGATGGCGTTCCTGTTGAAATAT-3' and 5'-GC TTTACAATCATGAGATGCGTCTGCTGAT-3'), i-FABP (5'-TCTGTCA TCATCATGACCTTCAACGG GACC-3' and 5'-GATAACAGTTTAAGCCCTCT TGA AAATCCT-3'), GFP (5'-CGTAAACGGCCACAAGTT CAGCGTGTCCGG-3' and 5'-GGCGGTCACGAACT CCAGCAGGACCATGTG-3') were used to amplify probe templates.

### Cryosectioning and immunohistochemistry

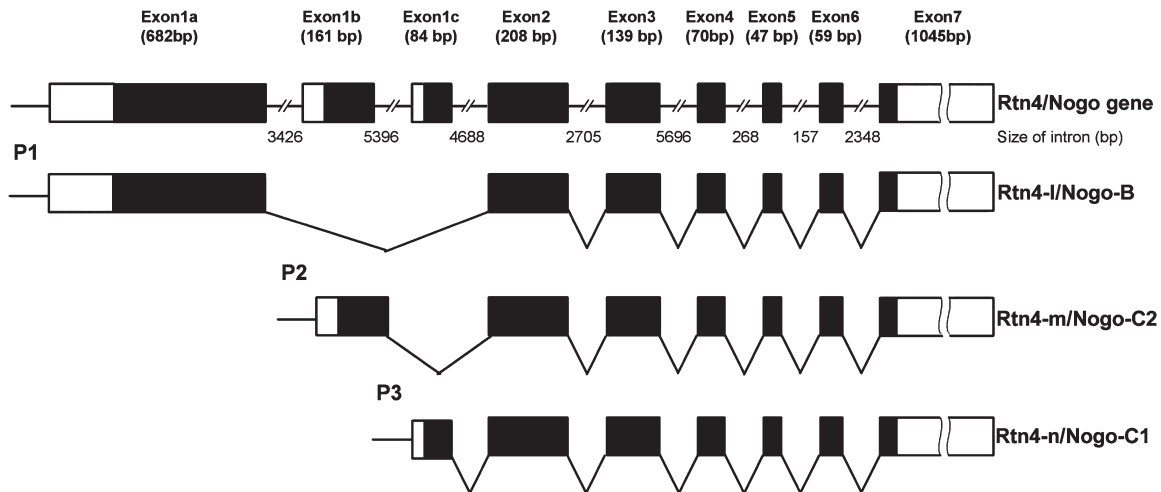
Standard protocol was used for cryosectioning. Sections of 30  $\mu\text{m}$  thick were cut and collected on a Leica CM1900 Cryostat (Leica, Wetzlar, Germany). Monoclonal antibodies F59 (antislow MyHC; 1:10) and EB165 (antifast MyLC; 1:200) were obtained from Developmental Studies Hybridoma Bank (University of Iowa, IA, USA). Rabbit anti-GFP (1:200) was obtained from Abcam (Cambridge, MA, USA). For brown staining, embryos were incubated with peroxidase-tagged secondary antirabbit antibodies (1:200) (BioRad, Hercules, CA, USA) and stained with Fast DAB (Sigma, CA, USA). Embryos were mounted between cover slips for viewing and analysed using Zeiss Axioplan 2 microscope (Thornwood, NY, USA).

## RESULTS

### Analysis of P1 promoter activity in cultured cells and zebrafish embryos

We used those *Rtn4/Nogo* complementary (c) DNAs as bait to perform an online BLAST search of the GenBank data base and matched nine non-contiguous regions in 219 514 bp of the DKEY-103K8 zebrafish BAC clone. Subsequently, we compared sequences between this BAC clone and each zebrafish *Rtn4/Nogo* cDNA. The result indicated that all three *Rtn4/Nogo* cDNAs were contained within nine putative exons and eight introns spanning ~27 kb. They had identical sequences derived from exons 2 to 7. The N-terminal regions of *Rtn4-l/Nogo- $\alpha$ /Nogo-B*, *Rtn4-m/Nogo- $\beta$ /Nogo-C2* and *Rtn4-n/Nogo- $\gamma$ /Nogo-C1* were, respectively, encoded by exons 1a, 1b and 1c. The putative 5'-upstream promoter regions of exons 1a, 1b and 1c were, respectively, designated P1, P2 and P3 (Figure 1).

Analysis of the P1 region (−4885 to −13 relative to the AUG translation initiation site of *Rtn4-l/Nogo-B*) revealed



**Figure 1.** Three *Rtn4/Nogo*-related transcripts were generated by alternative promoter usage and alternative RNA splicing. Genomic organization of the zebrafish *Rtn4/Nogo* gene was shown. Exons are indicated by boxes numbered 1–7. Solid boxes indicate the *Rtn4/Nogo* coding region, whereas open boxes represent the 5'- and 3'-untranslated regions. Introns and the 5'-flanking regions are indicated by solid lines. All three isoforms have identical sequences derived from exons 2 to 7 but not exon 1. Exon 1a was used for *Rtn4-l/Nogo-B*, exon 1b for *Rtn4-m/Nogo-C2* and exon 1c for *Rtn4-n/Nogo-C1*. The 5'-upstream promoter regions of each exon 1 are, respectively, designated P1, P2 and P3.

numerous putative binding sites for transcription factors, such as Nkx2.5, c-Ets-1, SP1, AP1, AP4 and GATA (Figure 2A, panel c), 24 E-boxes (panel b) and four myocyte enhancer factor-2 (MEF-2) motifs (panel d). No typical TATA box was found. The P1 region, -4885 to -13, corresponded to nucleotides 72 272–77 144 of the DKEY-103K8 BAC clone.

To test the activity of the P1 promoter *in vitro*, a large fragment (-4885 to -13) and four deletions were fused to the luciferase reporter gene in the pGL3-Basic vector. As shown in Figure 2B, the luciferase activity of pGL3-P1(-4885/-13) was about 2.5- and 2-fold higher than those of pGL3-P1(-3028/-13) in COS-1 and C2C12 cells, respectively, suggesting that the distal 1.8-kb upstream region contained positive regulatory sequences in both cell types.

We also used zebrafish embryos and a GFP reporter gene to investigate the promoter activity *in vivo* (22). As shown in Table 1, embryos injected with P1(-4885/-13)-GFP predominantly displayed GFP expression in muscles (81.7%) and skin (48.7%), moderately in neurons (25.2%) and less so in the heart (12.2%), circulating blood cells (13.0%) and notochord (4.4%). One zebrafish embryo injected with P1(-4885/-13)-GFP is presented (Figure 3, panels a and b) to show the GFP signal in muscles, skin and heart. Embryos injected with P1(-3028/-13)-GFP and P1(-2028/-13)-GFP displayed similar GFP expression patterns to those embryos injected with P1(-4885/-13)-GFP, but with lower expression rates (Table 1). These data are consistent with results of the luciferase activity assay in cultured cells. Moreover, the expression pattern in a variety of tissues of zebrafish embryos injected with P1(-3028/-13) was similar to the endogenous expression pattern of the *Rtn4-l/Nogo- $\alpha$ /Nogo-B* transcript, which is expressed in the brain, eyes, muscles, brachial arches, liver and intestines (14).

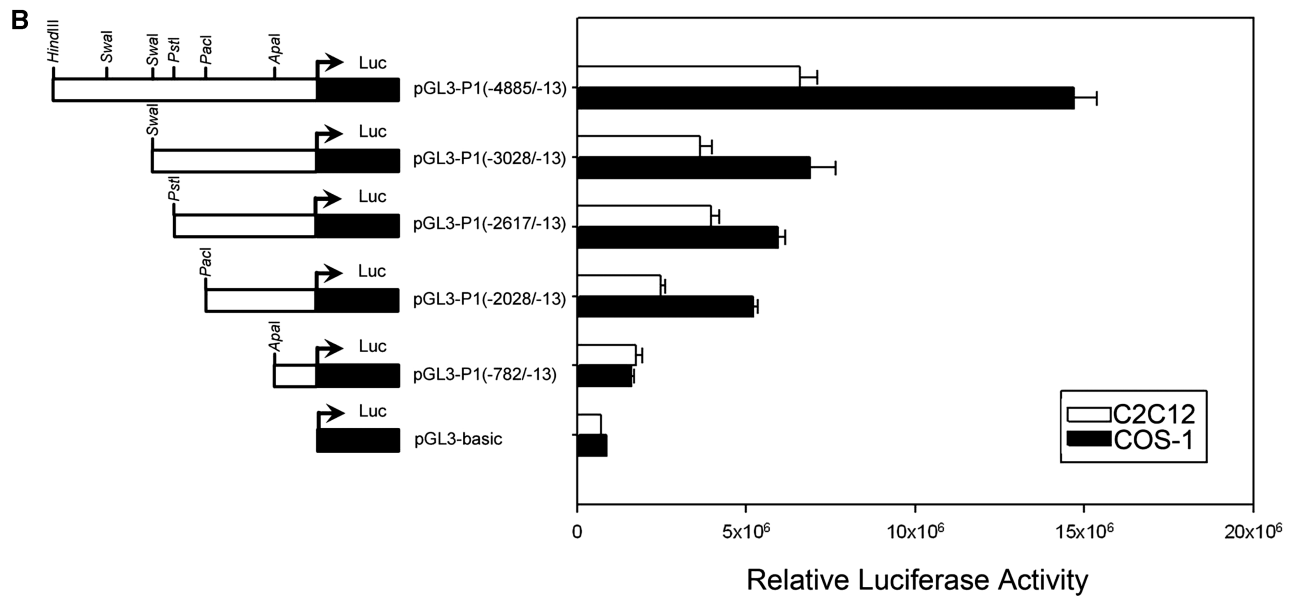
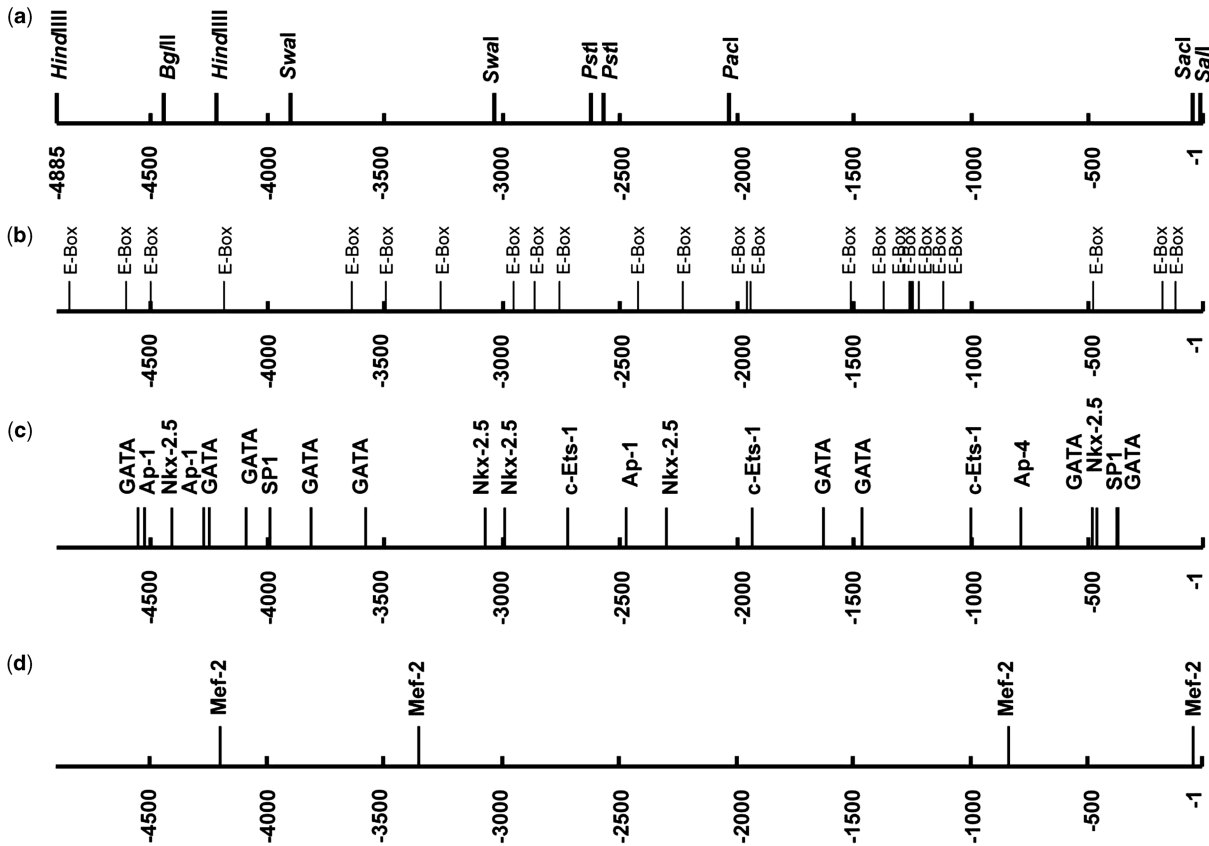
#### Expression pattern of Tg(*Nogo-B: GFP*) transgenic zebrafish recapitulates the *Rtn4-l/Nogo-B* expression pattern in various organs including the liver and intestines

In order to validate the results of the *in vitro* analysis of the P1 promoter in cultured cells and zebrafish embryos, we generated Tg(*Nogo-B:GFP*) transgenic zebrafish by the method of Tol2-mediated transgenesis (21,23). Only the large fragment (-4885 to -13) was cloned into a Tol2 expression vector containing the GFP reporter transgene (17) and co-injected with Tol2 *transposase* mRNA into one-cell zygotes. Embryos that exhibited mosaic GFP expression were raised to adulthood and out crossed to the AB wild-type strain. GFP-positive F1 embryos were identified, again were raised to adulthood, and used to generate several stable transgenic lines. Among the three transgenic fish lines generated, only the line possessing the highest expression level, designated Tg(*Nogo-B:GFP*), was used for further analysis.

GFP distribution patterns in a live Tg(*Nogo-B:GFP*) F3 embryo at different developmental stages are shown in Figure 4A. In 1- and 2-day post-fertilization (dpf) embryos, the GFP signal was observed in the brain, eye and muscle (panels a, a', b and b'). Starting at 3-dpf, high levels of GFP expression were detected in the liver and intestines which were sustained to 6-dpf (panels c, c', d, d', e, e', f and f'). The GFP signal was also detected in the brachial arches (panels b, c, d, e and f).

We further performed *in situ* hybridization to investigate whether the endogenous *Rtn4-l/Nogo- $\alpha$ /Nogo-B* mRNA was colocalized to the GFP expression site in the corresponding organs. In 1-, 2-, 3-, 4-, 5- and 6-dpf embryos (Figure 4B), *Rtn4-l/Nogo-B* mRNA was expressed in eye, midbrain, hindbrain, midbrain-hindbrain boundary, brachial arch, muscle, liver and intestine. Altogether GFP expression in the transgenic Tg(*Nogo-B:GFP*) line recapitulated the endogenous expression pattern of *Rtn4-l/Nogo- $\alpha$ /Nogo-B* mRNA (14).

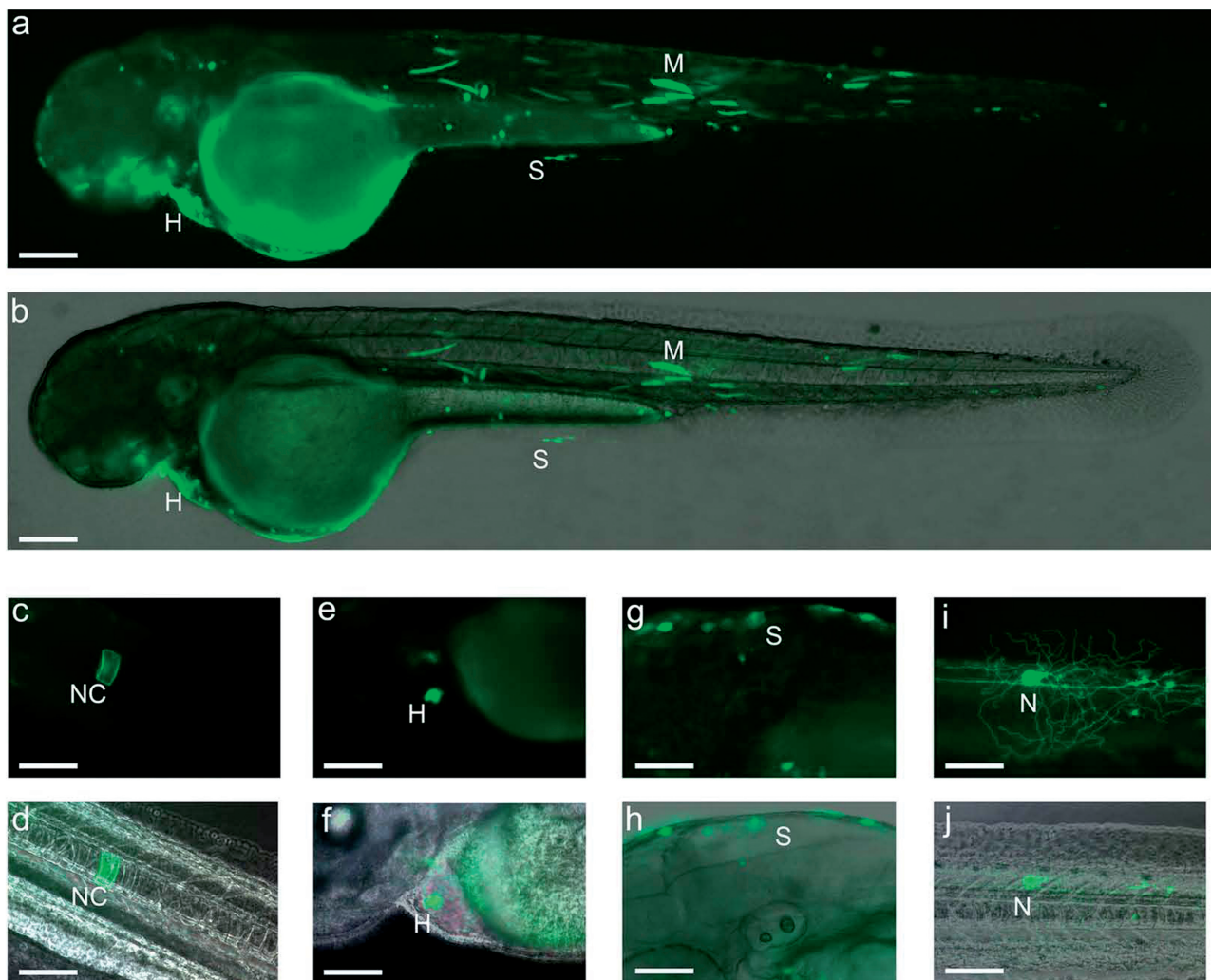
**A** P1 promoter



**Figure 2.** Activity of the 5'-upstream region of the P1 promoter in cultured cells. (A) Restriction map and possible transcription factor-binding motifs in the P1 promoter (−4885/−13). (B) COS-1 and C2C12 cells were cotransfected with 1 μg of each reporter construct and pSV-β-galactosidase, respectively. Cell lysates were prepared at 48 h after transfection and subjected to a luciferase activity assay. pGL3-Basic was used as the negative control.

**Table 1.** Expression of GFP in zebrafish embryos at 48-hpf injected with expression constructs containing different regions of the Nogo-P1 promoter

Construct	No. of embryos observed	No. of embryos with GFP expression	No. of embryos with GFP expression in					
			circulating blood cells (%)	heart (%)	muscle (%)	neurons (%)	notochord (%)	skin (%)
P1(-4885/-13)-GFP	162	115	15 (13.0)	14 (12.2)	94 (81.7)	29 (25.2)	5 (4.4)	56 (48.7)
P1(-3028/-13)-GFP	154	124	9 (7.3)	11 (8.9)	97 (78.2)	22 (17.7)	13 (10.5)	60 (48.4)
P1(-2028/-13)-GFP	144	95	10 (10.5)	10 (10.5)	68 (71.6)	12 (12.6)	6 (6.3)	42 (44.2)

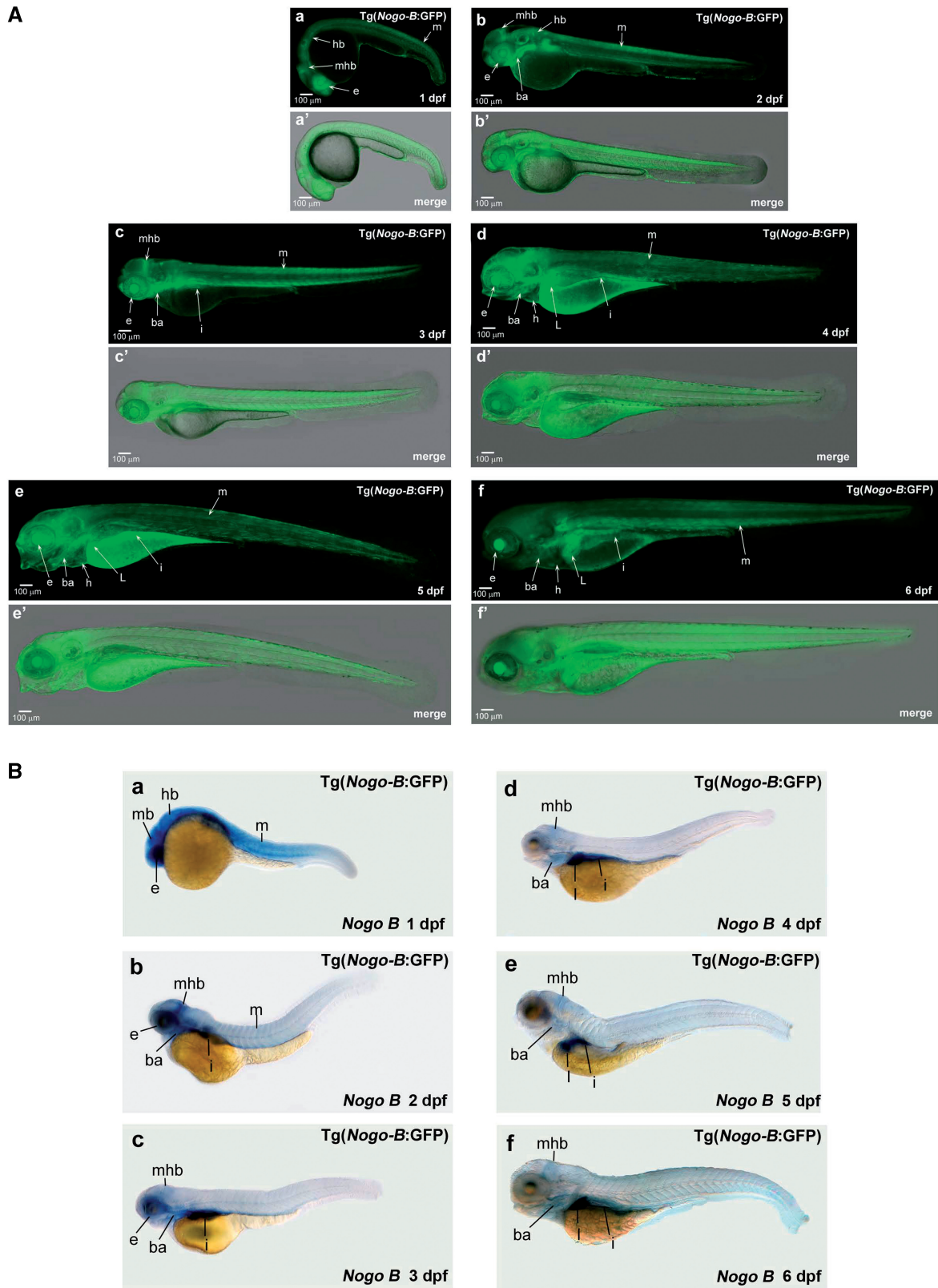
**Figure 3.** Activity of the 5'-upstream region of the P1 promoter in zebrafish embryos. P1(-4885/-13)-GFP was microinjected into zebrafish embryos at the one-cell stage. Zebrafish embryos at 48-h post-fertilization (hpf) with GFP signals were selected for image analysis. Embryos are shown in lateral view with the anterior to the left and dorsal to the top. H, heart; S, skin; M, muscle; N, neuron; NC, notochord. Scale bars represent 100 (panels a and b) and 20  $\mu$ m (panels c-j).

### Analysis of P2 and P3 promoter activities in cultured cells and zebrafish embryos

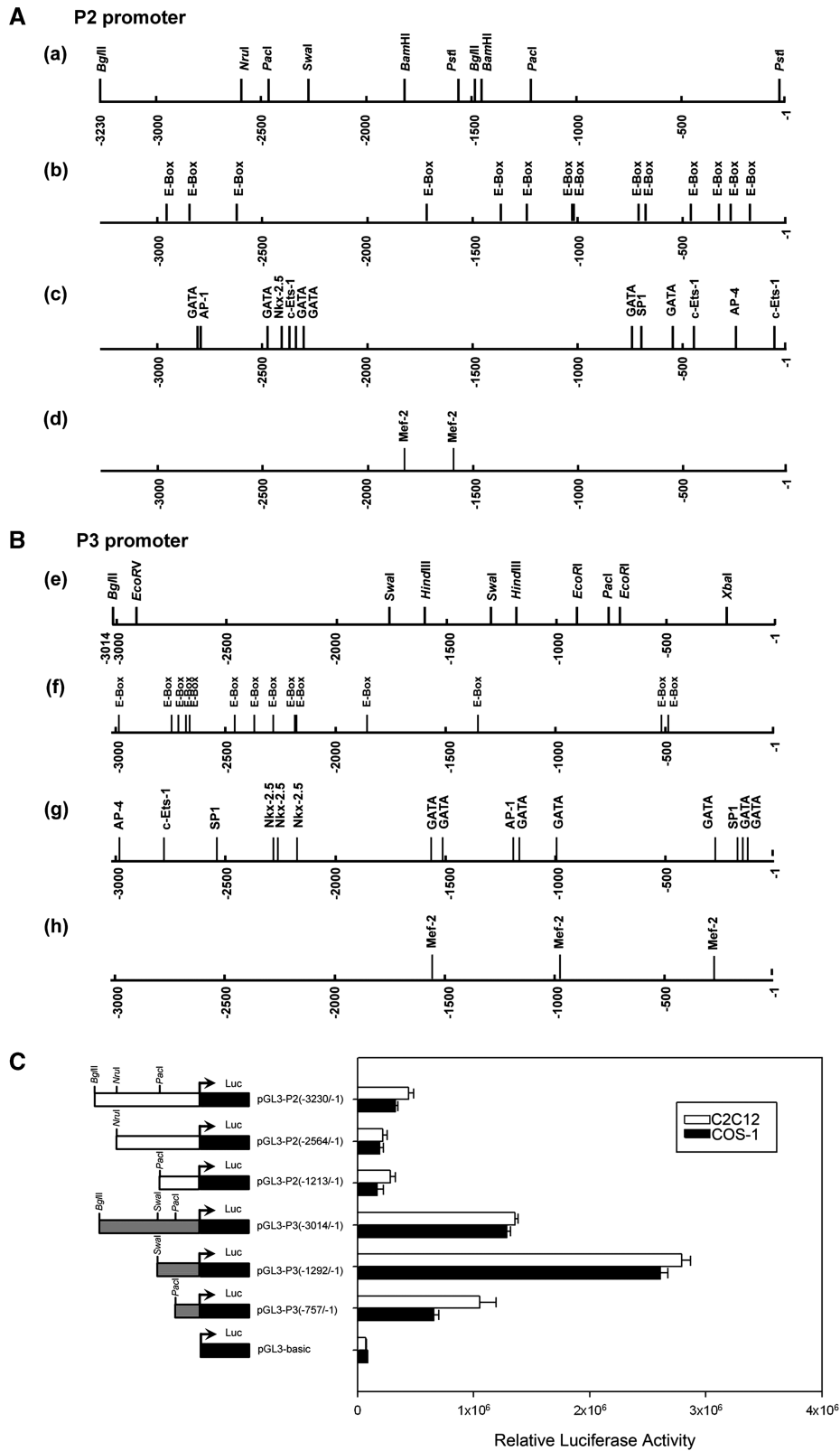
We also analysed the P2 (-3230 to -1) and the P3 promoter regions (-3014 to -1). There were several putative transcription factor binding sites, such as Nkx2.5, c-Ets-1, SP1, AP1, AP4 and GATA and 14 E-boxes present in each region (Figure 5A, B). In addition, there are two and three MEF-2 binding motifs

in the P2 and P3 promoters (Figure 5A, panel d and Figure 5B, panel h). The P2 promoter region (-3230 to -1) corresponded to nucleotides 77844-81073 of the BAC clone, DKEY-103K8, while the P3 promoter region (-3014 to -1) corresponded to nucleotides 83588-86601 of the same BAC clone.

To test the activity of the P2 promoter *in vitro*, a large fragment (-3230 to -1) and two deletions, P2(-2564/-1)



**Figure 4.** Expression patterns of the GFP in the transgenic zebrafish *Tg(Nogo-B:GFP)* line. Microinjection of the expression construct, P1(-4885/-13)-GFP, into zebrafish embryos at the one-cell stage and generation of a transgenic GFP line via Tol2-mediated transgenesis are described in the text. (A) contains images from the *Tg(Nogo-B:GFP)* transgenic line at different developmental stages. Merged bright field and fluorescence images are shown in panels a'-d', while fluorescence images are shown in panels a-d. (B) Localization of *Rtn4-1/Nogo-B* mRNA in *Tg(Nogo-B:GFP)* fish at different developmental stages. ba, brachial arches; e, eyes; hb, hindbrain; i, intestine; L, liver; m, muscle; mhb, midbrain-hindbrain boundary.



**Figure 5.** Activity of the 5'-upstream regions of the P2 and P3 promoters in cultured cells. Restriction map and putative transcription factor-binding motifs of the P2 (-3230/-1) (A) and P3 promoters (-3014/-1) (B) are shown. (C) Transfection of expression constructs into COS-1 and C2C12 cells and luciferase activity were assayed in the same way as described in Figure 3.



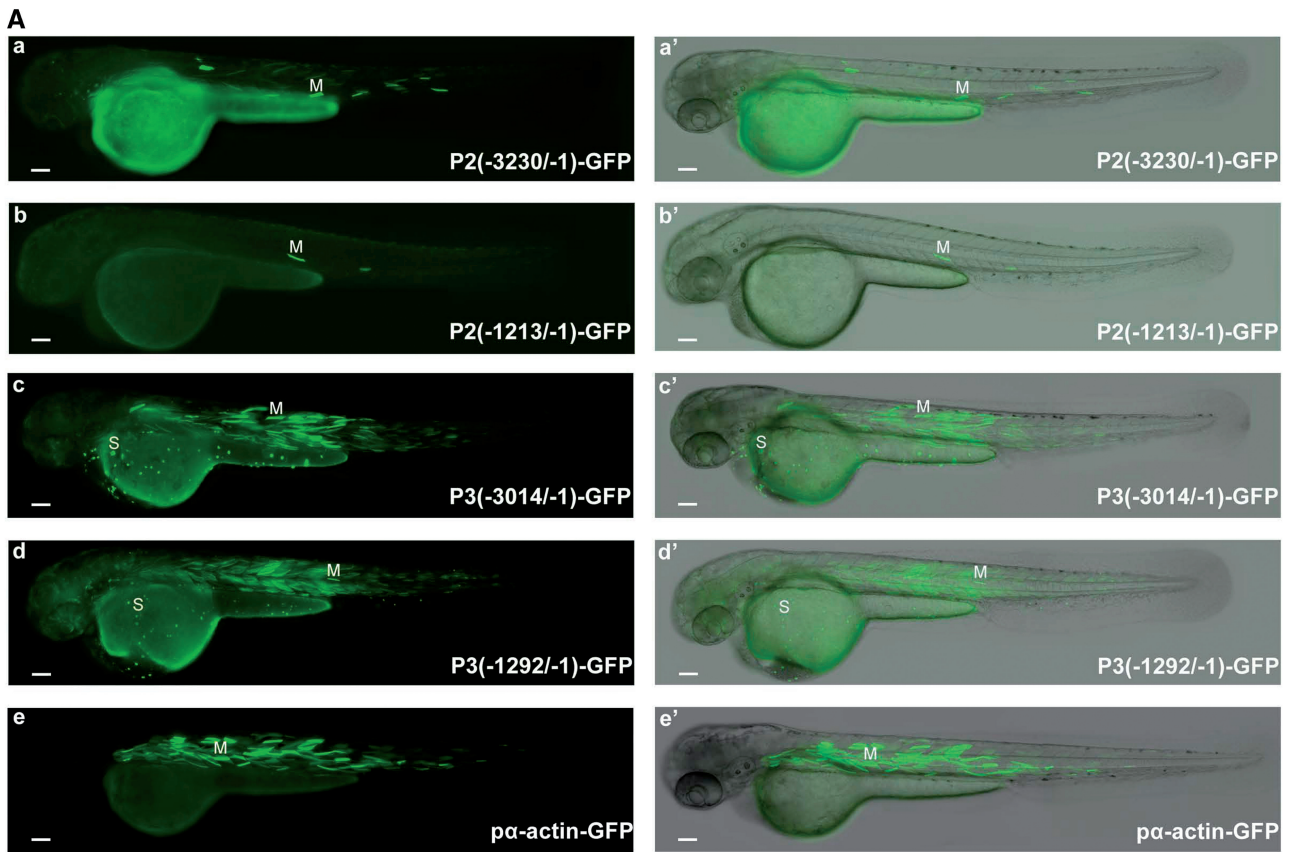
and P2(-1213/-1), were fused to the luciferase reporter gene in the pGL3-Basic vector. Similarly, three expression constructs, pGL3-P3(-3014/-1), pGL3-P3(-1292/-1) and pGL3-P3(-757/-1), were generated. As shown in Figure 6A, the luciferase activities of pGL3-P2(-3230/-1), pGL3-P2(-2564/-1) and pGL3-P2(-1213/-1) were low in both COS-1 and C2C12 cells, but were significant compared to that of the control pGL3-Basic construct. On the other hand, the luciferase activity of pGL3-P3(-1292/-1) was ~3- and 2-fold higher compared to those of pGL3-P3(-3014/-1) and pGL3-P3(-757/-1) in COS-1 and C2C12 cells, respectively. These data suggest that there are negative (-3014 to -1292) and positive regulatory sequences (-1292 to -757) present in the P3 promoter region.

We also tested the promoter activities of the P2 and P3 regions in zebrafish. Embryos injected with P2(-3230/-1)-GFP (Figure 6, panel a), P2(-1213/-1)-GFP (panel b), P3(-3014/-1)-GFP (panel c) and P3(-1292/-1)-GFP (panel d) all mainly displayed GFP expression in muscles. P3(-1292/-1)-GFP-injected embryos had much stronger

GFP signals than those in P3(-3014/-1)-GFP-injected embryos, which is consistent with the luciferase activity assay in cultured cells as shown in Figure 5C. However, some GFP signals were observed in the skin. On the other hand, the activity of the P2 promoter region was still very weak in zebrafish embryos, which was similar to that in culture cells. For comparison, embryos injected with the  $\alpha$ -actin promoter (22) displayed strong GFP expression specifically in muscles (panel e).

To further clarify which muscle type expressing GFP, zebrafish embryos were injected with P1(-4885/-13)-GFP, P3(-3014/-1)-GFP and P3(-1292/-1)-GFP, respectively, and then analysed by using different antibodies against slow muscle (F-59), fast muscle (EB-165) and GFP protein. As shown in Figure 6 B, the expression patterns of GFP and fast muscle are similar, but are different from that of slow muscle, suggesting that the muscle type with GFP belongs to fast muscle.

We also generated Tg(*Nogo-C1:GFP*) transgenic zebrafish by the method of Tol2-mediated transgenesis (21,23). Only the large fragment (-3014 to -1) was



**Figure 6.** Activity of the 5'-upstream regions of the P2 and P3 promoters in zebrafish embryos. (A) P2(-3230/-1)-GFP, P2(-1213/-1)-GFP, P3(-3014/-1)-GFP and P3(-1292/-1)-GFP were separately microinjected into zebrafish embryos at the one-cell stage. Zebrafish embryos at 48-hpf with GFP signals were selected for image analysis. For comparison, embryos injected with the  $\alpha$ -actin promoter (panel e) displayed strong GFP expression specifically in muscles. Merged bright-field and fluorescence images are shown in panels a'-e', while fluorescence images are shown in panels a-e. Scale bars indicate 100  $\mu$ m. (B) Zebrafish embryos at 4 dpf mentioned above were subjected to cryosection and labelled with different antibodies as follow. The primary antibodies were mAb F59 (anti-MyHC, slow muscle) at 1: 20, mAb EB165 (anti-MyLC, fast muscle) at 1: 200 and rabbit anti-GFP at 1: 200 dilution. After washing, slides were incubated with peroxidase-tagged secondary antirabbit antibodies at 1: 200 dilution and stained with Fast DAB. (C) Expression patterns of GFP and *Rtn4-n/Nogo-C1* in the transgenic zebrafish Tg(*Nogo-C1:GFP*) line. Images were taken from the Tg(*Nogo-C1:GFP*) transgenic line at 3 dpf. Merged bright-field and fluorescence images are shown in panel (a), while fluorescence images are shown in panel (a'). Expression of *Rtn4-n/Nogo-C1* (panel b) and GFP (panel c) mRNA in the transgenic zebrafish Tg(*Nogo-C1:GFP*) line was analysed by whole-mount *in situ* hybridization. ba, brachial arches; e, eyes; i, intestine; m, muscle; mhb, midbrain-hindbrain boundary.

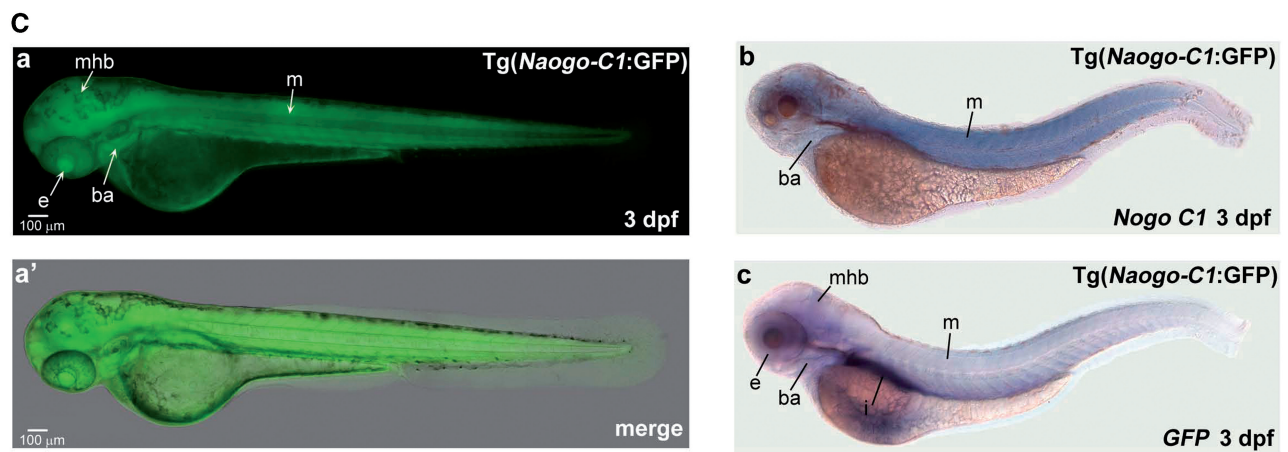
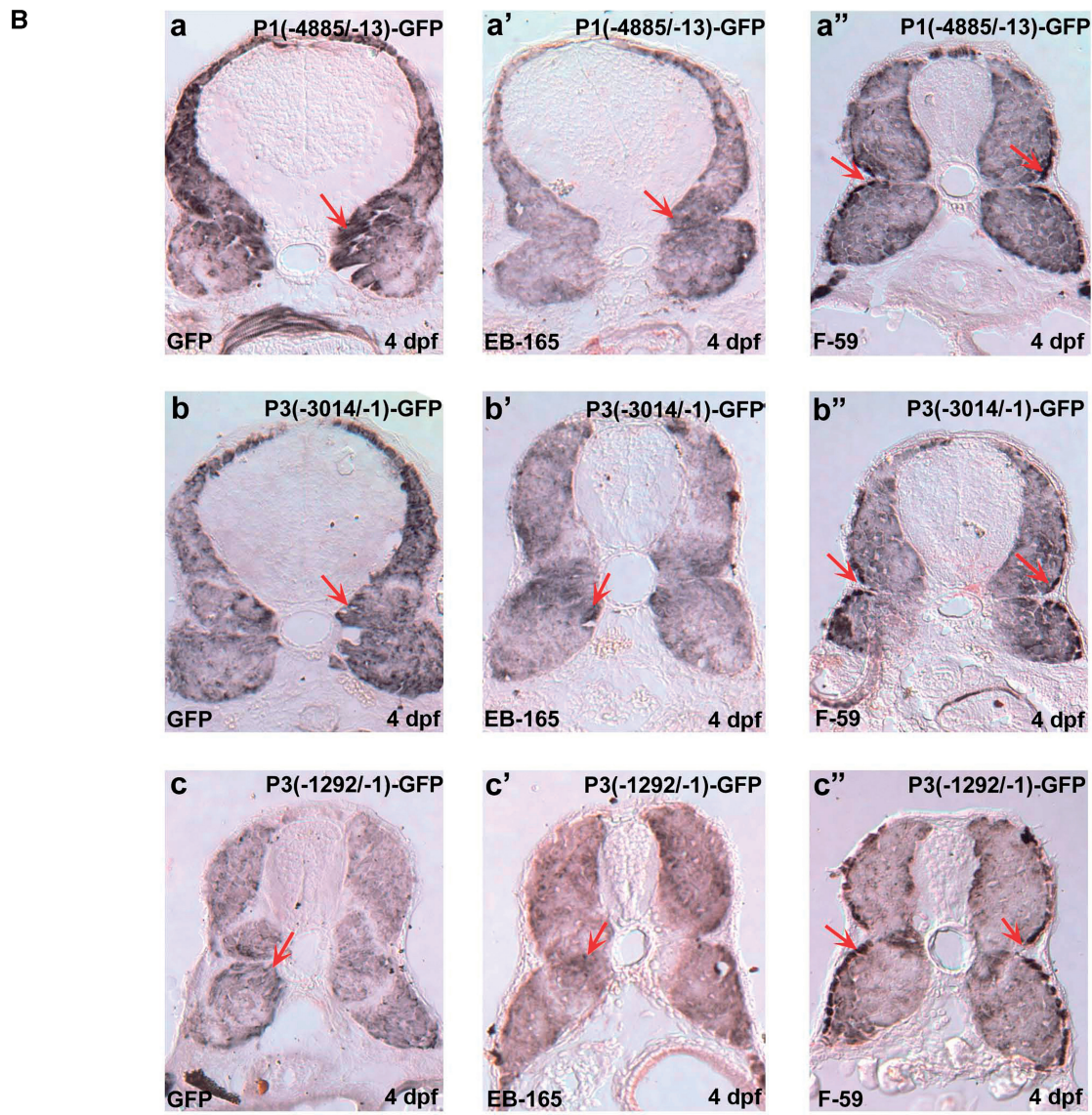


Figure 6. Continued.

cloned into a Tol2 expression vector containing the GFP reporter transgene (17) and co-injected with Tol2 *transposase* mRNA into one-cell zygotes. Embryos that exhibited mosaic GFP expression were raised to adulthood and out-crossed to the AB wild-type strain. The GFP signal in F1 embryo at 3-dpf was observed in muscle, midbrain–hindbrain boundary, eye and brachial arch (Figure 6C, panel a). In addition, the expression of GFP in Tg(Nogo-C1:GFP) was compared with the endogenous Rtn4-n/Nogo-C1 expression by whole-mount *in situ* hybridization (panels b and c). Their expression patterns are similar in muscle, eye and brachial arch. However, only GFP signal was detected in intestine and midbrain–hindbrain boundary. No *Rtn4-n/Nogo-C1* expression was observed in these two regions. This difference may be attributed to the short length of specific *Rtn4-n/Nogo-C1* probe, which is only 120 bp.

**GATA4 and GATA6 are required for P1 promoter activities in the liver and intestine, while Myf5 and MyoD are important for P1 and P3 promoter activities in muscles**

In zebrafish embryo, *myod* and *myf5* are required for induction of myogenesis. It has been reported that either *myf5* or *myod* is sufficient to promote slow muscle formation from adaxial cells, and that *myod* is required for fast muscle differentiation (24,25). Recently, at least three groups used *myf5/myod* double-morphant embryos to study muscle development (26–28). As shown in Figure 7A, we found that *myf5/myod* double morphants abolished the GFP expression in muscles driven by either P1 or P3 promoters (panels b, d, f and h). Moreover, the expression of GFP in the muscle of wild-type or *myf5/myod* double morphants was further analysed by whole-mount *in situ* hybridization (Figure 7B). There is no GFP signal in *myf5/myod* double morphants (panels a', b' and c'), suggesting that Myf5 and MyoD are important for the P1 and P3 promoter activities.

As shown in Figure 2A, panel c, there are nine GATA binding motifs in the P1 promoter region. In order to investigate whether GATA factors can regulate the P1 promoter activities, we injected both *GATA4*-MO and *GATA6*-MO into zebrafish embryos of transgenic Tg(*Nogo-B:GFP*) line at one- to two-cell stage. The *GATA4/GATA6* double morphants showed very low GFP expression in the liver and intestine (Figure 7C). Taken together, *GATA4* and *GATA6* are involved in the control of P1 promoter activity in the liver and intestine, while Myf5 and MyoD are important for the control of P1 and P3 promoter activities in muscles.

## DISCUSSION

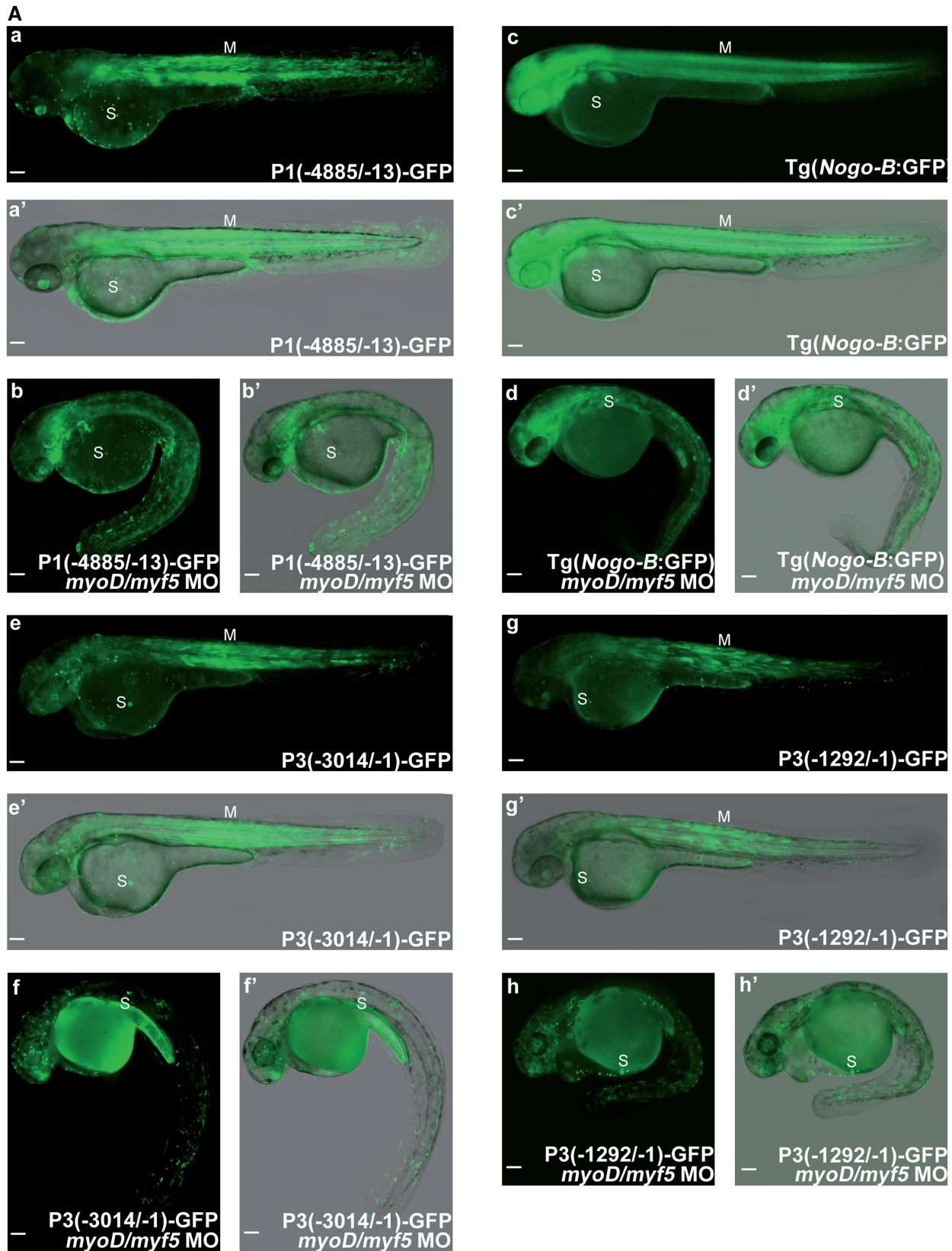
In this study, we demonstrate that those three Rtn-4/Nogo transcripts may be generated by alternative promoter usage and alternative RNA splicing. These data are consistent with the mammalian counterparts, which consist of Nogo-A, Nogo-B and Nogo-C.

In humans, the Nogo-A transcript was shown to preferentially be expressed in oligodendrocytes and some CNS

neuronal cells as studied by *in situ* hybridization using a Nogo-A-specific probe. However, the Nogo-C transcript is also expressed at a high level in skeletal muscles, and small amounts of Nogo-B and -C transcripts were detected in the kidneys and lungs (29). Similarly, the zebrafish Rtn-4l/Nogo-B transcript is ubiquitously expressed in many tissues including the brain, liver, intestines, eyes and muscles (14), while Rtn-4n/Nogo-C2 and Rtn-4m/Nogo-C1 are predominantly expressed in muscles (13).

In mammals, the 5'-upstream 2.8- and 3.3-kb portions of the human Nogo P1 and P2 promoter regions were characterized in a variety of cultured cells (15). The Nogo P1 promoter is used for the transcription of human Nogo-A and Nogo-B, while the Nogo P2 promoter is used for the transcription of human Nogo-C. The promoter activity of P1 was strong in all transfected cells including COS-7, 3T3 and C2C12 cells. However, the activity of the P2 promoter was weak in both Nogo-C-expressing and Nogo-C-non-expressing cells. Similarly, the promoter activity of zebrafish P1 was stronger than those of P2 and P3 in both COS-1 and C2C12 cells (Figures 4C and 6C). Moreover, when P1(–4885/–13)-GFP was injected into zebrafish embryos at the one-cell stage, GFP signals were observed in a variety of tissues in embryos at 72 hpf (Figure 5). In contrast, embryos injected with P2(–3230/–1)-GFP or P2(–1213/–1)-GFP displayed weak GFP signals, but they were specific in skeletal muscles (Figure 6). Similarly, embryos injected with either P3(–3014/–1)-GFP or P3(–1292/–1)-GFP also showed strong GFP signals in muscles (Figure 6).

The consensus sequence of the E-box is CANNTG. This binding motif is recognized by myogenic regulatory factors (30). Multiple E-boxes were found in promoters of muscle-specific genes including creatine kinase, myosin light chain and myogenin (31,32). In this study, we found that there were 24, 14 and 14 E-boxes present in the 5'-upstream regions of the P1, P2 and P3 promoters, respectively. In the case of the P1 promoter, zebrafish embryos injected with P1(–4885/–13)-GFP predominantly displayed GFP expression in muscle fibers (81.7%) (Table 1). However, embryos injected with either P2(–3230/–1)-GFP, P2(–1213/–1)-GFP, P3(–3014/–1)-GFP or P3(–12920/–1)-GFP all mainly displayed GFP expression in muscles (Figure 6). Analysis of the P3 promoter region (–3014 to –1) indicated that two E-boxes are located in the proximal 550-bp region, while 10 sites are in the distal 2464-bp region. As shown in Figure 6, P3(–1292/–1)-GFP had stronger GFP expression in muscles, while P3(–3014/–1)-GFP had lower GFP expression in muscles. Determining the relevance of promoter activity to the presence of potential regulatory E-box elements in the 5'-flanking region of the P3 promoter requires further mutation analysis. On the other hand, the GFP signal in muscles either driven by P1 or P3 promoters in *myf5/myod* double-morphants could not be detected (Figure 7A and B). These data suggest that the E-box binding transcription factors, Myf5 and MyoD, are important for the regulation of P1 and P3 promoter activities.



**Figure 7.** Loss of MyoD and Myf5 ablates somatic fast muscle and knockdown of *GATA4* and *GATA6* results in loss of GFP signal in the liver and intestine. (A) P1(-4885/-13)-GFP, P3(-3014/-1)-GFP and P3(-1292/-1)-GFP were separately injected or each coinjected with *myod/myf5* double MOs into zebrafish embryos at the one-cell stage. Alternatively, *myod/myf5* double MOs were injected to the transgenic Tg(*Nogo-B*:GFP) line at the one-cell stage. Zebrafish embryos at 48 hpf with GFP signals were selected for image analysis. Merged bright-field and fluorescence images are shown in panels (a'-h'), while fluorescence images are shown in panels (a-h). Scale bars indicate 100  $\mu$ m. (B) Those embryos mentioned above at 3 dpf were subjected to whole-mount *in situ* hybridization using GFP as probe. All *myod/myf5* double morphants did not show GFP signal. (C) *GATA4*/*GATA6* double MOs were injected into zebrafish embryos of transgenic Tg(*Nogo-B*:GFP) line at one- to two-cell stage. The *GATA4*/*GATA6* double-morphants and the parental transgenic line at 4 dpf were subjected to whole-mount *in situ* hybridization using GFP (panels a and b) and LFABP/iFABP (panels c and d) as probe. The liver and intestine were enlarged in panels (a'-d').

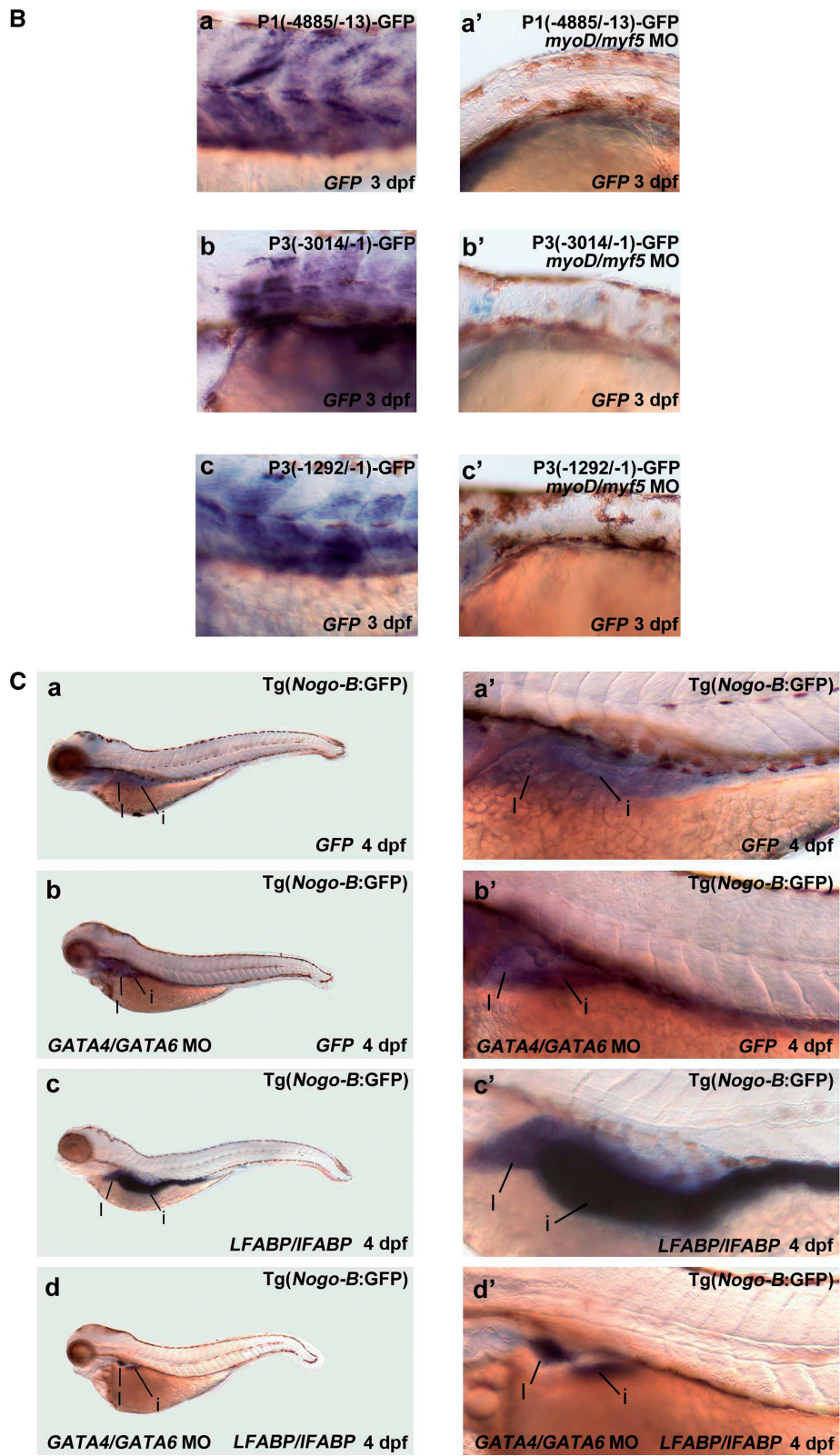


Figure 7. Continued.

The MEF2 family of transcription factors plays important roles in linking calcium-dependent signaling pathways to the genes responsible for cell division and differentiation. The MADS domain in MEF2 family proteins mediates dimerization and binding to the DNA sequence CTA(A/T)<sub>4</sub>TAG/A (33). MEF2 factors associate with a variety of transcriptional cofactors to control specific sets of downstream target genes. The interaction between MEF2 and MyoD is involved in the regulation of the skeletal muscle differentiation. Four MEF2 isoforms (A–D) have been identified in mammals, and all except MEF2B are expressed in skeletal muscle (34). In zebrafish, there are three members in MEF2 family (35). In this study, there are four, two and three MEF-2 binding motifs in the P1, P2 and P3 promoters, respectively. The functional roles of these MEF-2 binding motifs in the regulation of the P1, P2 and P3 promoters need further investigation.

Although there is only one Nogo-C protein in mammals, an artificial fusion protein of the human Amino-NogoA-24 domain and Nogo-66 was generated which had higher Nogo receptor binding (36). Structurally, this fusion protein is similar to zebrafish Rtn4-n/Nogo-C1 and Rtn4-m/Nogo-C2, with 67 and 64% identities, at the level of aa sequences. The Nogo-A-24 domain was mapped to the region of 995–1018 aa of Nogo-A. The fusion proteins of human Nogo-A-24 and Nogo-66 contain 198 aa, while zebrafish Rtn4-m/Nogo-C1 contains 196 aa. Both human Nogo-A-24 and Nogo-66 domains can bind to the Nogo receptor, NgR. Whether zebrafish Rtn4-n/Nogo-C1 and Rtn4-m/Nogo-C2 are functionally related to this fusion protein remains to be further investigated by ligand- and receptor-binding assays. In zebrafish, there are four Nogo receptors, NgR, NgRH1a, NgRH1b and NgRH2 (37). By *in situ* hybridization, all Nogo receptor transcripts were expressed in the brain including forebrain, midbrain and hindbrain, but there was no signal present in the muscle of zebrafish embryos (unpublished data). It seems that Rtn4-n/Nogo-C1 and Rtn4-m/Nogo-C2 may not interact with any of those four Nogo receptors in the muscle.

Analysis of the upstream 4.8-kb promoter region of the zebrafish Rtn4-l/Nogo-B gene revealed the existence of many binding motifs for transcription factors such as Nkx2.5, c-Ets-1, SP1, AP1, AP4, GATA and E-box (Figure 2A). Interestingly, a group of GATA, MyoD/E-box and Nkx motifs has been reported to involve in the regulation of mouse fetoprotein transcription factor gene promoter (38). In addition, the promoter activity of the mouse alpha3 integrin gene was regulated by a group of c-Ets-1, GATA and Myo-D/E-box motifs (39). Such a group could be also found in P1 promoter. As shown in Figure 7, GATA4, GATA6, Myf5 and MyoD play important roles in the regulation of P1 promoter activities. The functional role of c-Ets-1 in the regulation of P1 promoter awaits further investigation.

The presence of nine GATA-binding sites in the zebrafish Rtn4-l/Nogo-B gene promoter suggests that the Gata4/Gata5/Gata6 transcription factor possibly regulates Rtn4-l/Nogo-B gene expression in the liver and intestines. In zebrafish, Gata4 regulates the formation of

multiple organs including the liver (40), while Gata5 is also essential for gut morphogenesis and liver development (41,42). In the mouse, Gata6 is essential for embryonic development of the liver (43). In this study, we injected both GATA4-MO and GATA6-MO into zebrafish embryos of transgenic Tg(Nogo-B:GFP) line and found that the GATA4/GATA6 double morphants displayed very low GFP expression in the liver and intestine (Figure 7C), suggesting that GATA4 and GATA6 are involved in the control of P1 promoter activity in the liver and intestine.

In mammals, Nogo-A and Nogo-C are highly expressed in the CNS, while Nogo-B is ubiquitously expressed in many tissues, including the central and peripheral nervous systems, spleen, skeletal muscles, heart and vessel walls (15,44). A previous study indicated that zebrafish Rtn4-l/Nogo-B is also expressed in many tissues including the brain, eyes, muscles, branchial arches, liver and intestines during different developmental stages (14). It is interesting to note that the 4.8-kb promoter of Rtn4-l/Nogo-B has the ability to drive GFP expression in these organs (Figure 4). In addition to this Tg(Nogo-B:GFP) line, other transgenic zebrafish lines exhibited GFP expression in the liver or intestines or both. The 2.8-kb 5'-flanking sequence of the zebrafish L-FABP gene is sufficient to direct GFP expression in the liver (45), while the 4.5-kb promoter region of zebrafish I-FABP gene can drive GFP expression in the intestines (46). Morphogenesis of the liver and intestines can be visualized in the Tg(GATA6:GFP) transgenic line, which expresses GFP in both organs (40). Gata6 gene expression was previously associated with endoderm-derived organs, and a mouse knockout model showed specific functions for GATA6 in the visceral endoderm (47). On the other hand, mammalian fatty acid-binding proteins are small cytosolic proteins present in various tissues including the liver, intestines, brain and heart, which play important roles in intracellular fatty acid trafficking and metabolism (48–52). In this study, we demonstrated that the expression of zebrafish Rtn4-l/Nogo-B in the liver and intestine was mediated by GATA proteins to regulate its promoter activity and specificity (Figure 7). So far, there is no evidence to indicate that Rtn4-l/Nogo-B plays any role in the morphogenesis of the liver and intestine.

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