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Trim11 increases expression of dopamine β-hydroxylase gene by interacting with Phox2b

Seok Jong Hong, **Han Chae**, **Thomas Lardaro**, **Sunghoi Hong**, and **Kwang-Soo Kim***

Molecular Neurobiology Laboratory, McLean Hospital, Harvard Medical School, 115 Mill Street, Belmont, MA 02478

Abstract

The homeodomain transcription factor Phox2b is one of the key determinants involved in the development of noradrenergic (NA) neurons in both the central nervous system (CNS) and the peripheral nervous system (PNS). Using yeast two-hybrid screening, we isolated a Phox2b interacting protein, Trim11, which belongs to TRIM (Tripartite motif) or RBCC proteins family, and contains a RING domain, B-boxes, a coiled-coil domain, and the B30.2/SPRY domain. Protein-protein interaction assays showed that Phox2b was able to physically interact with Trim11. The B30.2/SPRY domain of Trim11 was required for the interaction with Phox2b. Expression of Phox2b and Trim11 was detected in the sympathetic ganglia (SG) of mouse embryos. Forced expression of Trim11 with Phox2b further increased mRNA levels of dopamine β-hydroxylase (DBH) gene in primary avian neural crest stem cell (NCSC) culture. This study suggests a potential role for Trim11 in the specification of NA phenotype by interaction with Phox2b.

Keywords

Phox2b; Trim11; yeast two-hybrid screening; protein-protein interaction; noradrenergic neuron; neural crest stem cell

Introduction

A regulatory network of extracellular signals and nuclear transcription factors has been demonstrated to be critical for specification of individual neurons [1-4]. Among the various phenotypes of a particular neuron, neurotransmitter identity is an important feature because it determines the nature of the chemical neurotransmission a given neuron will mediate, and influences the specific connectivity with target cells. NA neurons are found in both the CNS and the PNS. The regulatory mechanism determining the NA neurotransmitter phenotype has been extensively studied, leading to the identification and functional characterization of critical signaling molecules and transcription factors [2,5]. Development of NA neurons in SG of the PNS and locus coeruleus (LC) of the CNS depends on bone morphogenetic proteins (BMPs) that are expressed in dorsal aorta and dorsal neural tube, respectively [2]. cAMP signaling pathway is also necessary for the induction of NA neurons in rat and avian NCSC culture. Both loss of function and gain of function studies showed that transcription factors, such as Mash1, Phox2b, Phox2a, dHand, and GATA-2/3, work cooperatively to specify NA neuron phenotype [2,5-8].

*To whom proofs and reprint requests should be addressed, Phone: (617) 855-2024, Fax: (617) 855-3479, email: kskim@mclean.harvard.edu, Molecular Neurobiology Laboratory, MRC215, McLean Hospital, Harvard Medical School, Belmont, MA 02478

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The two closely related transcription factors Phox2a and Phox2b belong to paired-like homeodomain transcription factors family. They showed overlapping expression pattern in the location of NA neurons of PNS and CNS suggesting functional redundancy. They have similar biochemical activity *in vitro* - i.e., transactivation of DBH promoter and binding activity [9, 10]. Overexpression of Phox2a/2b can induce expression of TH and DBH *in vivo* and *in vitro* [2]. However, their knockout mice showed distinct phenotypes. Development of LC is absent in Phox2a knockout mice, while other (nor)adrenergic centers such as A1/C1, A2/C2, A5, and A7 in the CNS and SG in the PNS developed normally [11]. In contrast, Phox2b knockout mice showed developmental defects in all NA neurons of the CNS and PNS. Phox2b is required for brachial and visceral motoneurons in CNS and autonomic neurons (sympathetic, parasympathetic, and enteric) in PNS development [12,13], while Phox2a is required for cranial Oculomotor and Trochlear motor neurons [11]. In addition, reciprocal gene replacements in mice showed that Phox2a and Phox2b are not functionally equivalent [14]. These *in vitro* and *in vivo* studies of Phox2a and Phox2b suggest the presence of specific interacting protein(s). Here, we isolated and characterized a Phox2b interacting protein, Trim11, using yeast two-hybrid screening.

Materials and methods

Yeast two-hybrid screening

We cloned the full length mouse Phox2b gene in pGBKT7 bait vector containing GAL4 DNA-binding domain (Clontech) to make pGBKT7-Phox2b. Yeast two-hybrid screening was performed by mating yeast strain Y187, which was transformed with mouse brain cDNA library (Clontech), with yeast strain AH109 that was transformed with pGBKT7-Phox2b. The mated cell were plated on selection medium lacking Leu, Trp, His in the presence of 4 mM 3-AT (3-amino-1,2,4-triazole). DNAs from growing cells were isolated and retransformed to AH109 with pGBKT7-Phox2b. Isolated colonies were cultured in the same liquid medium and β-galactosidase activity was assayed according to the manufacturer's protocol (Clontech).

Plasmid construction

Mouse brain mRNA was used to amplify full length Trim11 by RT-PCR followed by cloning into pcDNA3.1/Zeo. Phox2b and Trim11 cDNAs were cloned into pEGFP-C2 and pDsRed2-C1 (BD Biosciences) to make living color fusion proteins, eGFP-Phox2b and DsRed2-Trim11, respectively.

In vitro and in vivo co-immunoprecipitation (Co-IP)

In vitro Co-IP was performed using the Matchmaker Co-IP kit (Clontech) according to the manufacturer's protocol. Briefly, *in vitro* [^{35}S]-methionine labeled myc tagged proteins were obtained using the TNT-coupled wheat germ extract system (Promega). The myc tagged C-terminal half of the protein Nurr1 was similarly synthesized and used as negative control. *In vitro* translated myc and HA-tagged proteins were incubated for 1 h at RT followed by addition of c-myc monoclonal antibody or HA polyclonal antibody. After adding protein A beads, complexes were washed, the liquid removed and the beads were resuspended in SDS loading buffer (60 mM Tris-HCl (pH 7.0), 2% SDS, 6% glycerol, 0.1M dithiothreitol, 0.01% bromophenol blue). The products were subjected to SDS-polyacrylamide gel electrophoresis, fluorographic reagent (Amersham), and autoradiography.

For *in vivo* Co-IP, 293FT cells were harvested 48 h after transfection with CMV promoter driven Flag-tagged Trim11 and GFP-tagged Phox2b. Cell extracts were treated with 1 μg anti-GFP (Sigma) and Protein A-Sepharose. Bound proteins were analyzed by Western blotting. Detections were performed with anti-GFP (1:5,000), and M2 anti-FLAG (1:5,000) antibodies using Enhanced Chemiluminescence (ECL) western blotting system (Amersham).

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Northern blot analysis

Using ^{32}P labeled Trim11 as a probe, a nitrocellulose membrane containing murine mRNA from different tissues (Origene technologies) was hybridized (6 X SSC, 5 X Denhardt's solution, 0.5% SDS, 100 μg/ml salmon sperm DNA, 50% formamide) at 42°C. The membrane was washed in 0.25X SSC, 0.1% SDS at 65°C and subjected to radiography. The membrane was later stripped and reprobed with the β-actin probe.

Cell culture and transient transfection assays

293FT cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Hyclone), 100 μg/ml of streptomycin, and 100 units/ml of penicillin in a CO_2 incubator. Transfection was performed using lipofectamine (Invitrogen).

Immunohistochemistry (IHC) and in situ hybridization (ISH)

Mouse embryos were fixed overnight in 4% paraformaldehyde in PBS at 4°C. Embryos were treated with 30% sucrose overnight at 4°C prior to embedding in OCT compound and stored at -70°C. 16 μm embryos sections were subjected to IHC or ISH. Antibodies were detected using the Vectastain kit (Vector Labs) and the signal was visualized using 3,3′-diaminobenzidine (DAB). Rabbit anti-Phox2b (1:10,000 dilution, gift from Dr. Brunet) was used. Antisense Trim11 RNA probes were made from pGEM-Easy T vector (Promega) in which PCR product were cloned using the following primer set 5′-TTCACCGACCCGGTGATGACC-3′ and 5′-GTTATGTCCCCTCGGAACCTCC-3′. Hybrids with digoxigenin (DIG)-labeled probes were visualized by treatment with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

Construction of RCAS-cGATA-3 retrovirus and NCSC culture

Mouse Phox2b and Trim11 were cloned at the *Cla*I site of RCASBP(A) and RCASBP(B), respectively. The resulting RCASBP(A)-Phox2b and RCASBP(B)-Trim11were used to transfect DF1 chicken fibroblast cells (ATCC) for viral production. Viruses were harvested and concentrated, and primary cultures of the trunk region of quail eggs were performed as described [15]. NCSCs were plated at a density of 1.8×10^4 cells in 24 well plates and allowed to settle for 5 h. Cells were infected with 9×10^4 infectable units (IU) of RCAS viruses and harvested after seven days in culture.

Real-time PCR analysis

Total RNA was isolated using Trizol Reagent (Sigma) and treated with DNase I according to the manufacturer's protocol. cDNA was made from five μg of total RNA using superscript II (Invitrogen) with oligo $(dT)_{12\text{-}18}$ as a primer. For quantitative analysis of the expression level of mRNAs, real-time PCR analyses using SYBR green I were performed using DNA engine Opticon™ (MJ Research). Oligonucleotides 5′- TACACACGGACACTTCAAGGGC - 3′ and 5′- ATGATGTTCTGGGCAGCACCTC - 3′, 5′- ATGGACACCTCGAGCCTGGCTTCA - 3′ and 5′ - CTCGGTGAGGTCGATCTTGAGCGC - 3′, 5′ - CACCACATAATCATGTATGAGCCA - 3′ and 5′-GTGTGGAGCTGGGAGGCGAAGATG - 3′, 5′- CCACGAGGAGAACCCCTACTTC- 3′ and 5′- CTTATCGCTGCTGCTAACTGTGC-3′ were used to detect glyceraldehyde- 3-phosphate dehydrogenase (GAPDH), Phox2b, DBH, and dHand mRNA with amplicon size of 468 bp, 357 bp, 509 bp, and 490 bp, respectively.

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Results

Phox2b interacts with Trim11

We used mouse Phox2b as a bait molecule and screened a mouse brain cDNA library using the yeast two-hybrid system to isolate protein(s) interacting with Phox2b. Plasmid DNAs were isolated from growing colonies on the selection medium. Each candidate plasmid was used to retransform yeast strain AH109 containing the bait. The interaction was verified by plating the transformants on the selection medium. As shown in Fig. 1A, transformation of AH109 containing the bait gene, Phox2b, with candidate gene, #2b-1, resulted in growth on selection medium but not when pACT2, which does not have inserts, was used. The interaction was further confirmed by β-galactosidase activity assay (data not shown). The candidate clone contains 181 amino acids from the C-terminal of Trim11 which is one of TRIM family proteins [16]. Trim11 contains several subdomains such as RING, B2-box, coiled-coil, and B30.2/SPRY in its full length 467 amino acids (Fig. 1B).

We performed *in vitro* Co-IP to confirm protein-protein interaction between Phox2b and Trim11. [^{35}S]-methionine labeled myc or HA tagged proteins were immunoprecipitated. Immunoprecipitation with HA antibody brought down myc-Phox2b (Fig. 1C, lane 2, asterisk) but not myc-Nurr1C terminal (Fig. 1C, lane 4, asterisk) suggesting that interaction between Phox2b and #2b-1 is specific. The known interaction between p53 and large T antigen was used as positive control (Fig. 1C, lane 5, 6). Furthermore, we did *in vivo* Co-IP to confirm the physical interaction between Phox2b and Trim11. We co-transfected 293FT cells with GFP-tagged Phox2b and Flag-tagged Trim11. Expression of tagged transcription factors was confirmed by Western blot analysis. Cell lysates were immunoprecipitated with GFP antibody and the Western blot analysis was performed with α-Flag antibody. As shown in Fig. 1D, we observed *in vivo* interaction between Phox2b and Trim11.

Trim11 is expressed in the SG

To examine the expression pattern of Trim11 in various mouse tissues, we performed Northern blot analysis. Our result shows that Trim11 mRNA is ubiquitously expressed in all tissues tested although its relative expression level was different (Fig. 2A). The 2.4 kb transcript was the major form found in most tissues. Two larger transcripts, 3.5 kb and 10 kb, were also detected (Fig. 2A). We next fused Phox2b and Trim11 cDNA to living color proteins, and expressed them in 293FT cells to study subcellular localization. Both GFP-fused Phox2b and DsRed2-fused Trim11 are localized in the nucleus, suggesting that the interaction between Phox2b and Trim11 may occur in the nucleus (Fig. 2B).

Phox2b is one of key transcription factors involved in NA neuron development. SG are the major NA neuron centers in the PNS. We analyzed the expression of Trim11 and other NA-specific marker genes, such as Phox2b and TH, in the SG of developing embryos. Our IHC analyses showed that Trim11 is prominently expressed in SG of e14.5 mouse embryos (Fig. 3C). This expression pattern of Trim11 in the SG of developing embryos further suggests the interaction between Phox2b and Trim11 *in vivo*.

Forced expression of Trim11 and Phox2b increases the expression of DBH transcript

NCSCs originate from the dorsal neural tube, migrate to target sites, and become PNS including SG in developing embryos. Previous studies demonstrated that NCSC is a useful system to study potential functional role of transcription factors because they give rise to diverse cell types [17]. To explore potential *in vivo* function of Trim11, we infected primary NCSCs isolated from the trunk region of E2 quail embryos with replication competent avian specific retroviruses, RCAS. Forced expression of Trim11 alone did not change the expression levels of NA specific genes, such as dHand and Phox2b, when compared to NCSCs infected with

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virus alone as a control (Fig. 4). Forced expression of Phox2b alone increases expression of most of NA specific genes, such as DBH and Phox2b, but not dHand (Fig. 4). When Phox2b and Trim11 were forced expressed together in NCSCs, expression of DBH was specifically increased compared to cultures where only Phox2b was forced expressed.

Discussion

Phox2a and Phox2b knock out mice of harbor distinct phenotypes, though these two genes showed largely overlapping expression patterns in the PNS and CNS. Previous studies indicated that Phox2a/2b directly activate DBH gene expression [9,18-20]. To delineate of the differential effects of Phox2a and Phox2b during NA neuron specification, we previously characterized the transcriptional function of Phox2a and Phox2b [21-23]. Though Phox2a and Phox2b proteins share high homology (53% identity and 100% identity in the DNA binding homeodomain), little DNA sequence homology was found in their promoters. Based on these observations, we searched for Phox2b interacting protein(s) using yeast two-hybrid screening. After screening 1.54×10^5 colonies in the presence of 4 mM 3-AT, we isolated a candidate, #2b-1, encoding C-terminal Trim11 protein. Trim11 belongs to the TRIM family of proteins also known as RBCC proteins. These proteins contain a RING domain, one or two zinc-binding motifs called B-boxes, a coiled-coil domain, and the B30.2 or SPRY domain [24-26]. The RING domain contains a specialized zinc finger that binds two zinc atoms and has ubiquitin E3 ligase activity. B-boxes are distinct feature of TRIM proteins, though no specific function has been assigned. The coiled-coil domain is involved in homo- or hetero-oligomerization and specification of cellular compartment. Though its function is not clearly attributed, protein-protein interaction activity has been suggested for B30.2 domain [27]. Members of the TRIM family are implicated in various cellular processes, such as cell proliferation, differentiation, oncogenesis, apoptosis, viral response, and development [24,25]. Recent bioinformatics approaches have been identified new TRIM proteins [16,25] - 68 genes and one pseudogene have been identified in human. However, only a small number of TRIM proteins have been characterized at the biological and molecular levels.

Trim11 contains the RING finger, B2-box, coiled-coil domain, and B30.2/SPRY domain. The clone that was isolated by yeast two-hybrid screening with Phox2b contains 181 amino acid of the C-terminal of Trim11 containing B30.2/SPRY domain, which has protein-protein interaction activity. So far, three proteins which interact with Trim11 have been reported. Trim11 interacts with Humanin, a neuroprotective peptide that specifically suppresses Alzheimer's disease related neurotoxicity [28]. Trim11 interacts with Pax6, a member of the PAX family of transcription regulators and essential for ocular and neural development [29]. Trim11 also interacts with activator-recruited cofactor 105-kDa component (ARC105) which mediates chromatin-directed transcription activation [30]. The B30.2/SPRY domain is required for interaction with Humanin while the coiled-coiled and B30.2/SPRY domains of Trim11 are required for interaction with ARC105. In line with these, our study showed that the B30.2/SPRY domain of Trim11 is responsible for the interaction with Phox2b. This further support the role of the B30.2/SPRY domain in protein-protein interaction [27].

High sequence homology between mouse (Genbank access number NM_053168) and human (NM_145214) Trim11 proteins (89% identity) suggests that they have distinct functional roles. Phox2b is critical for the development of NA neuron both in the PNS and CNS [12,13]. We addressed whether Trim11 cooperates with Phox2b to transactivate NA neuron specific promoters. However, we could not detect synergistic effect of Trim11 and Phox2b on the promoter activity of DBH in transient transfection assay (data not shown). The role of Trim11 in development of the PNS was further addressed in primary NCSC culture. Forced expression of mouse Phox2b lead to increased mRNA expression of Phox2a, Phox2b, Cash1, SCG10, cRET, TH, and DBH but no significant difference was found in the expression of dHand and

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Sox10 (Fig. 4 & data not shown). Coexpression of Trim11 with Phox2b further increased mRNA levels of DBH compared to expression of Phox2b alone. Recent studies identified new members of the TRIM/RBCC family. However, little is known about their biological function. Our study suggests a potential role for Trim11, a member of the TRIM/RBCC family, in the specification and/or maintenance of NA phenotype.

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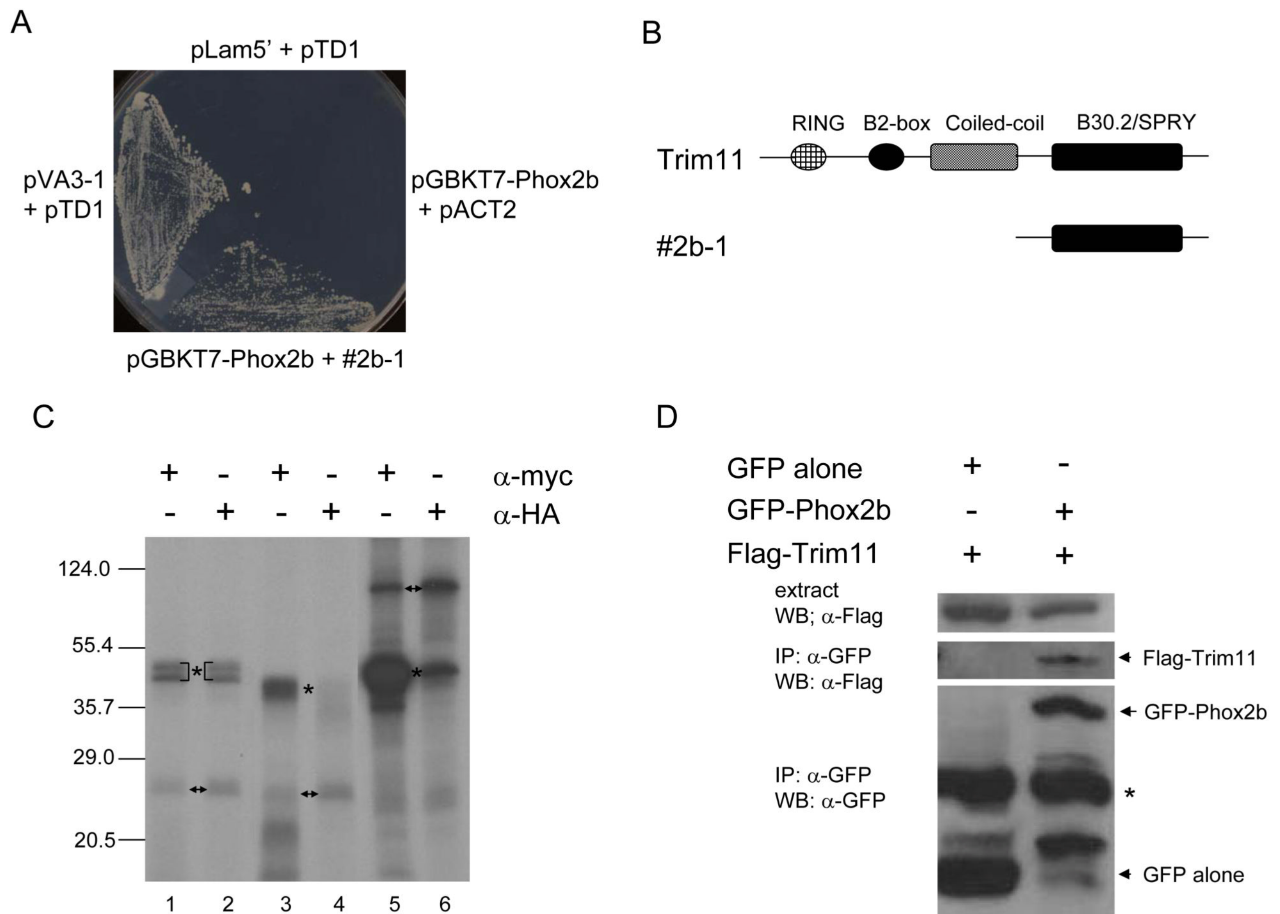

Fig. 1.

Specific interaction between Phox2b and Trim11. (A) AH109 containing pGBKT7-Phox2b and its interacting clone (#2b-1) were grown on the selection medium lacking Leu, Trp, and His. Positive control contains both pVA3-1 (encodes p53 fused to GAL4 DNA binding domain) and pTD1 (encodes SV40 large T antigen fused to GAL4 activation domain), and negative control containing both pLam5′ (encodes human lamin C fused to GAL4 DNA binding domain) and pTD1. AH109 containing pGBKT7-Phox2b and pACT2 was plated to exclude self-activation. (B) Structure of Trim11. Full length of mouse Trim11 gene and the clone (#2b-1), which is isolated by yeast two-hybrid screening, are drawn. RING zinc finger domain, B box type zinc finger, coiled-coil domain, and B30.2/SPRY region are shown. (C) *In vitro* Co-IP was performed with [^{35}S]-methionine labeled *in vitro* translated myc or HA tagged proteins. The reaction was immunoprecipitated with either myc antibody or HA antibody as indicated. Lane 1, 2: myc-Phox2b (asterisk) + HA-#2b-1 (arrow); lane 3, 4: myc-Nurr1Cterminal (asterisk) + HA-#2b-1 (arrow); lane 5, 6: myc-p53 (asterisk) + HA-SV40 large T antigen (arrow). Protein size markers are shown on the left. (D) *In vivo* Co-IP was performed in 293FT cells which were cotransfected with the expression vectors as indicated on the top. The cell lysates were precipitated with an anti-GFP. Monoclonal anti-FLAG was used to detect the FLAG-tagged Trim11 protein. The blot was stripped and re-probed with the anti-GFP to detect the precipitated GFP or GFP-Phox2b (bottom). Top, crude cell extracts; middle, immunoprecipitates; bottom, GFP-Phox2b. Asterisk indicates non-specific bands containing the heavy chain of IgG.

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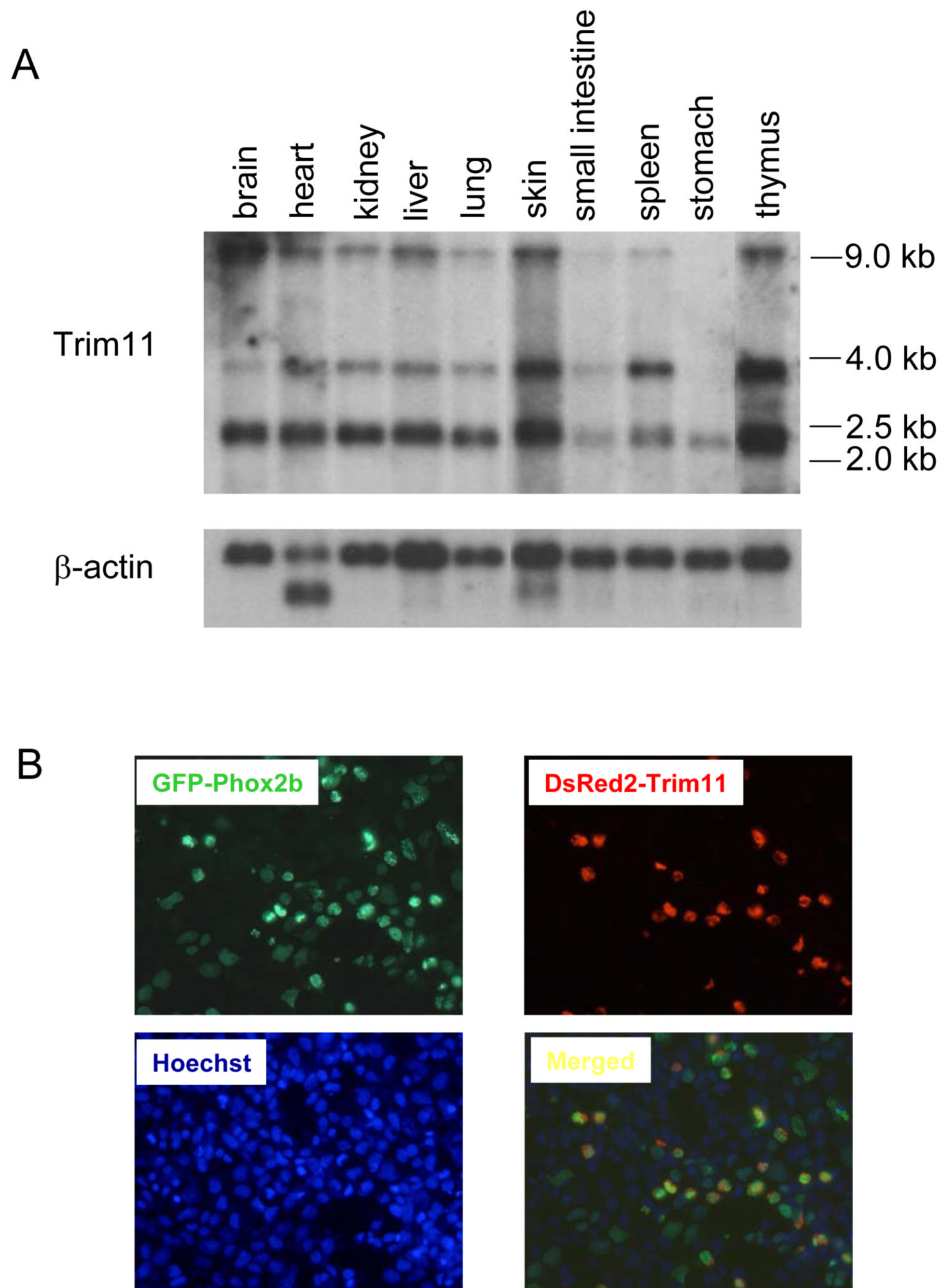

Fig. 2.
Expression and subcellular localization of Trim11. (A) Expression of Trim11 in mouse tissues. Northern blot analysis was done with Trim11 gene as a probe in the indicated tissues on the top (upper panel). β-actin was detected as a loading control (lower panel). mRNA size markers are shown on the right. (B) Subcellular localization of Trim11. GFP-fused Phox2b and DsRed2-fused Trim11, which are under CMV promoter, are co-transfected 293FT cells. Nuclei are stained using Hoechst dye.

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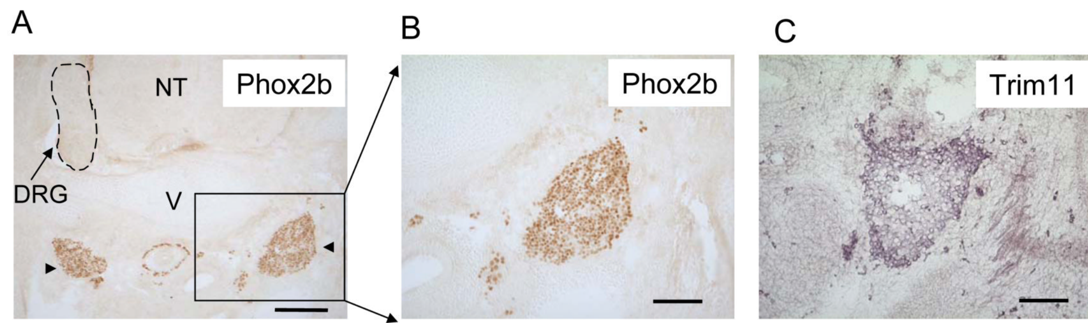

Fig. 3.

Trim11 is expressed in the SG. Trunk region of e14.5 mouse embryos was analyzed for the expression of Phox2b (A, B) and Trim11 (C). Phox2b and Trim11 were detected by IHC and ISH, respectively. Scale bar, 200 μm (A), 100 μm (B, C). Dorsal root ganglia (DRG), vertebrae (V), and neural tube (NT) are shown. Sympathetic ganglia (SG) are indicated by arrowheads (A).

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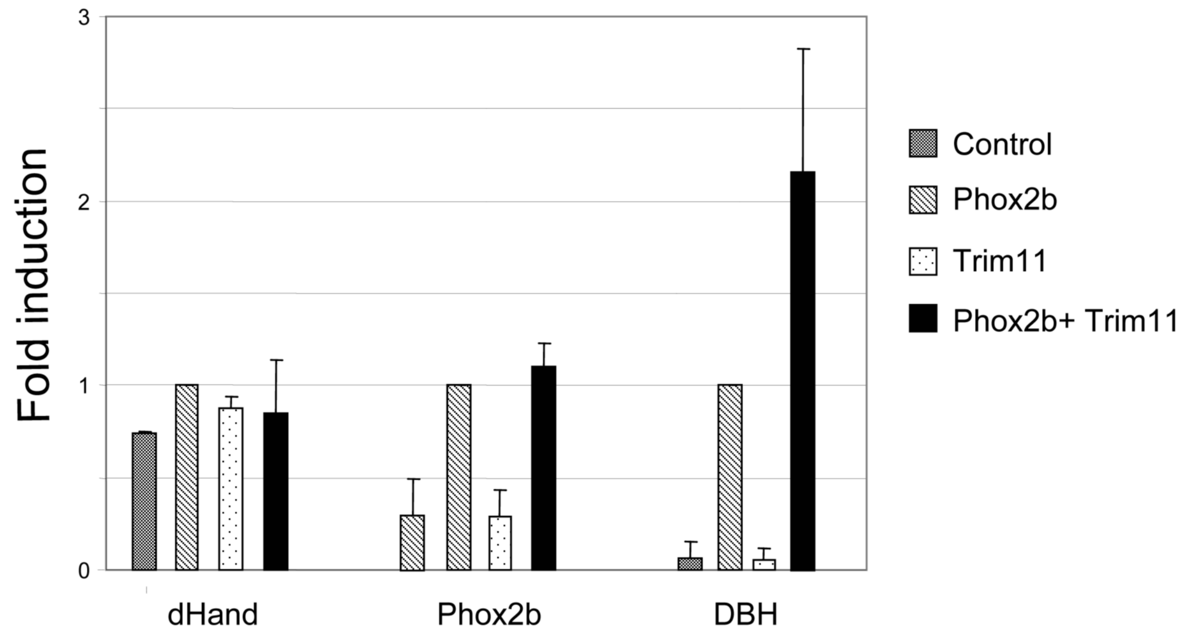

Fig. 4.
Coexpression of Phox2b and Trim11 increases expression of DBH mRNA in NCSCs culture. Expression NA specific transcription factors in the avian NCSCs culture. Avian NCSCs cells were infected with i) RCAS virus alone, ii) RCASBP(A)-Phox2b, iii) RCASBP(B)-Trim11, iv) RCASBP(A)-Phox2b and RCASBP(B)-Trim11. Expression of mRNA was analyzed by real time PCR and normalized by expression of GAPDH. Expression level of genes by forced expression of Phox2b (ii) is set as 1.