Physical and functional interactions between Smad proteins and Phox2 homeodomain transcription factors

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Abstract

Dopamine β-hydroxylase (DBH), the key enzyme for norepinephrine synthesis is exclusively expressed in noradrenergic and adrenergic neurons. DBH expression is regulated by a class of homeodomain transcription factors known as Phox2a (ARIX) and Phox2b (PmxB). Phox2 factors bind to at least four high-affinity binding sites in the upstream region of the DBH gene promoter. During noradrenergic neuron development, Phox2 expression is induced by bone morphogenetic proteins (BMPs), a subfamily of secreted growth factors belonging to the transforming growth factor-β (TGFβ) ligand superfamily. The observation that continuous presence of BMPs is required for the maintenance of DBH expression, led us to investigate possible cooperative mechanisms between BMP signaling and Phox2 function. We found that Smad proteins, intracellular signaling mediators of TGF-β family ligands, cooperated synergistically with Phox2a and Phox2b in the induction of DBH expression, even in the absence of *de novo* protein synthesis. Smads and Phox2 proteins cooperated in the activation of the DBH gene promoter and the initiation of transcription from multimerized Phox2 DNA binding sites. Phox2 proteins and Smads were shown to interact by direct protein-protein association. The ability of Smads to interact directly and cooperate synergistically with Phox2 proteins in the regulation of the noradrenergic neurotransmitter phenotype suggests a novel role for Smad signaling in coordinating genetic interactions by forming higher order complexes with factors at different levels in regulatory gene networks.

Introduction

Metazoan development is orchestrated by the coordinated deployment of multiple and diverse programs of cell differentiation. Cell fate decisions and phenotypic differentiation result from the integration of specific sets of cell-autonomous (genetic) transcriptional programs with signaling pathways activated by extracellular (epigenetic) cues provided by local organizing centers. One of the fundamental questions in this process is how a discrete number of transcriptional programs and extracellular cues combine to produce the enormous cellular complexity that characterizes highly evolved metazoan organisms.

The TGF-β superfamily is a large family of cytokines that includes TGF-βs, bone-morphogenetic proteins (BMPs), activins, nodals, and growth and differentiation factors (GDFs). TGF-β family members are expressed in complex spatio-temporal patterns and play important roles in development, homeostasis and repair in practically all tissues in all metazoans (1-4). The signal transduction mechanism of TGF-β proteins is based on the cooperation between two receptor serine-threonine kinases (i.e. type I and type II receptors). TGF-β ligands typically bind first to type II receptors and only then are the type I receptors recruited to the complex and activated. Activation of type I receptors allows docking and subsequent phosphorylation of a subgroup of receptor-associated Smads (R-Smads). Only a few R- Smads –Smads 2 and 3 for TGF-bs, activins and nodals, and Smads 1, 5 and 8 for BMPs and certain GDFs– are in charge of mediating the activities of all TGF-β proteins known (4-6). Smad proteins are characterized by two conserved domains (MH1 and MH2) separated by a linker sequence. Phosphorylation of R-Smads by type I receptors in the C-terminal tail of the MH2 domain leads to their dissociation from the receptor complex, homotrimerization via their MH2 domains, and their translocation into the nucleus (4-6). A distinct kind of Smad protein common to all R-Smad pathways (Co-Smad or Smad4) can also associate with phosphorylated R-Smads in a heteromeric complex and translocate to the nucleus (7,8). Although some Smads (i.e. Smad3 and Smad4) have intrinsic DNA-binding activities, all Smads have been found to require other factors to bind efficiently to the promoters of responsive genes (4-6). Smad signaling is negatively regulated by several mechanisms, one of which is through the action of the inhibitory Smads (I-Smads) 6 and 7 (4-6). I-Smads can function by antagonizing the interactions of R-Smads with

the activated receptors and with Smad4, and by recruitment of ubiquitin ligases (Smurf1 and 2) to the receptor complex (9).

Once in the nucleus, multimeric Smad complexes serve as platforms for the recruitment of general transcription factors, co-activators, co-repressors and tissue-restricted factors. The formation of this multimeric complex is of crucial importance in TGF-β signaling, as this is the step in which DNA binding specificity as well as the sign and strength of the response are determined. This is also where genetic and epigenetic signals are integrated to produce a distinct response. Multiple nuclear factors are known to interact with either the MH1 or MH2 domains of Smads (10,11). These include the co-activator p300/CBP (12-14) and the negative regulators c-Ski and c-SnoN (15,16). General transcription factors known to associate with R-Smads include c-Fos, c-Jun and Sp1 (17,18). Finally, and of crucial importance for the biological specificity of TGF-β responses, are a growing number of tissue-restricted factors carrying DNA binding specificity. An example of this category is the zinc-finger transcription factor GATA-3, an important regulator of T helper cell and noradrenergic neuron development (19,20).

Noradrenergic transmitter identity is controlled by a network of genes that crossregulate each other, including Mash1, Phox2b, Phox2a, dHand, and GATA-3 (21). Activation of this genetic program leads to induction of expression of tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH), the two critical enzymes for the synthesis of the neurotransmitter noradrenaline (Fig. 1A). During sympathetic neuron development, BMP signals derived from the dorsal aorta are responsible for the initial induction of Mash1 and Phox2b in neuronal precursors. Phox2a (also known as ARIX) and Phox2b (also known as PmxB) are homeodomain transcription factors essential for DBH expression in both the peripheral and central nervous systems (22-26). Several Phox2 binding sites have been identified in the upstream region of the DBH gene (27). Although in the current model of noradrenergic neuron development induction of TH and DBH expression by BMPs is thought to occur indirectly through the action of Mash1 and Phox2 proteins, efficient induction of TH and DBH expression in sympathetic neuron progenitors requires continuous presence of BMPs (28,29), suggesting regulatory roles for Smad proteins at multiple levels in the network.

In the present work, we have investigated the mechanisms by which BMPs and other members of the TGF-β superfamily regulate *DBH* expression. Our results

indicate that DBH is a direct target of BMP and TGF-β signaling, and suggest that Smad proteins cooperate with Phox2 factors in the induction of DBH expression by direct protein-protein interactions.

Results

We first looked at the ability of TGF-β superfamily ligands to induce DBH expression in the Neuro2A cell line, a neuroblastoma derived from the neural crest that expresses endogenous Phox2 proteins (30). Neuro2A cells were treated with BMP-4 for 60 min and DBH mRNA levels were analyzed by real time PCR. BMP-4 treatment increased DBH expression in Neuro2A cells (Fig. 1B). Cells were then pre-treated with cycloheximide (Chx), a protein synthesis blocker, prior to stimulation with BMP-4 to find out whether DBH expression could still be induced by this factor independently of new protein translation. BMP-4 was still able to induce DBH expression in Chx-treated cells (Fig. 1B), indicating that DBH is a direct target gene of BMP signaling in Neuro2A cells. The low responsiveness of these cells to TGF-β prevented us from assessing the effects of this factor on DBH expression. To circumvent this problem, and to provide *in vivo* evidence of the effects of TGF-β ligands on DBH expression, we introduced a constitutively active construct of the TGF-β receptor (CA-ALK5) in chick neural tube using *in ovo* electroporation. Electroporated tissue, identified by expression of a green fluorescent protein (GFP) reporter, was dissected and evaluated for DBH mRNA expression by real time PCR. CA-ALK5 induced an increase in DBH mRNA levels in chick neural tube (Fig. 1C), indicating that DBH may also be a target of TGF-β signaling.

We next examined the effects of BMP and TGF-β on the activity of a 394 bp fragment of the proximal region of the DBH gene promoter containing Phox2 binding sites (27). Stimulation with BMP-4 increased the activity of this promoter in Neuro2A cells, and this effect could be potentiated by overexpression of Phox2b (Fig. 2A). Transfection of Smad1 or Smad4 further increased the activity of this promoter construct both in the presence and absence of BMP-4 (Fig. 2A). Also transfection of CA-ALK5 upregulated DBH promoter activity in Neuro2A cells, and this effect could also be enhanced in the presence of exogenous Phox2b (Fig. 2B). Overexpression of Smad2 and Smad3 greatly increased promoter activity in the presence of exogenous Phox2b (Fig. 2B). These results indicated that both BMP and

TGF-β signaling can induce the activity of a proximal fragment of the DBH gene promoter containing Phox2 binding sites.

In order to further explore the requirement of Phox2 proteins for the effects of TGF-β ligands on DBH expression, we used C2C12 myoblasts, lacking endogenous expression of Phox2 transcription factors but highly responsive to TGF-β ligands. In the absence of exogenous Phox2 proteins, however, neither BMP-4 or TGF-β had any effects on the activity of the proximal DBH gene promoter in these cells (Figs. 3A and B). Overexpression of Phox2a augmented promoter activity by 20- to 30-fold over baseline (Figs. 3A and B), indicating that the effects of Phox2 proteins on DBH gene activity are not restricted to neuronal cells and can be reproduced in other cellular environments. In the presence of Phox2a, both BMP-4 and TGF-β were able to potentiate DBH promoter activity in C2C12 cells (Figs. 3A and B). Overexpression of Smad1 could further enhance the effects of BMP-4, (Fig. 3A), while exogenous Smad3 was able to significantly boost promoter activity in response to TGF-β (Fig. 3B). The requirement of Phox2 protein expression for the effects of BMP-4 and TGF-β on DBH promoter activity was also confirmed using a longer construct of the DBH promoter encompassing 1648 bp of upstream sequence (Fig. 3C). Although inactive in the absence of exogenous Phox2 proteins, this promoter could be induced by 20-fold upon overexpression of Phox2a, and by additionally 2-fold upon treatment with either BMP-4 or TGF-β (Fig. 3C). To further test the functional synergy between Phox2 proteins and TGF-β signaling, we constructed an artificial promoter based on multimerized Phox2 DNA binding sites placed in front of the adenovirus major late promoter (4xPhox2-luc) and examined its activity in C2C12 cells. As with the promoter constructs derived from the DBH gene, the synthetic construct was only active in the presence of exogenous Phox2 protein (Fig. 3D). Under these conditions, the reporter became responsive to TGF-β (Fig. 3D), in agreement with a synergistic interaction between Phox2 activity and TGF-β signaling.

The above results suggested that Smad proteins may be able to interact directly with Phox2 transcription factors. In order to test this possibility, we prepared different Smad proteins as fusions to glutathione-S-transferase (GST) in bacteria and used them in pull-down assays together with *in vitro* translated, ^{35}S-labeled Phox2a, Phox2b and different deletion constructs (Fig. 4A). These experiments revealed that

Smad1, Smad3 and Smad4 were all able to bind to radiolabeled Phox2a when allowed to interact *in vitro* (Fig. 4B). A control GST protein produced in the same conditions did not interact with Phox2a. Deletion of the MH1 domain abolished the ability of Smad3 to interact with Phox2a, while a similar deletion in the MH2 domain had no effect (Fig. 4B), suggesting that Phox2 proteins interact with the N-terminal MH1 domain of Smads. Similarly, a GST fusion of the MH1 domain of Smad4, but not of its MH2 domain, was able to bind *in vitro* translated Phox2b (Fig. 4C). In contrast, a GST fusion of Smad7 did not interact with Phox2a (data not shown), suggesting specificity in the interaction of different Smad proteins with Phox2 factors. The N-terminal region of Phox2a contains the homeodomain and a short motif with homology to the T-box protein Brachyury (31), and has been shown to be sufficient for transactivation of the DBH promoter (31,32). On the other hand, the C-terminal region downstream of the homeodomain has been shown to have negative regulatory functions on Phox2a activity (31). In order to identify the region in Phox2a mediating binding to Smads, we deleted the C-terminal or N-terminal halves of Phox2a and tested their ability (as GST fusion proteins) to pull-down deletion constructs of Smad3 from lysates of transfected COS cells. We found that the N-terminal region of Phox2a (Phox2a-ΔC) was capable of binding a truncated Smad3 lacking the MH2 domain (Fig. 4D). In contrast, the C-terminal domain of Phox2a (Phox2a-ΔN) was unable to interact with Smad3 (Fig. 4D). (A weak binding could be observed to Smad3-ΔMH2 but this was comparable to that of GST alone.) In agreement with previous observations (31), the N-terminal region of Phox2a was still able to activate the synthetic promoter carrying multimerized Phox2 DNA binding sites (Fig. 4E). In addition, this construct was sufficient to mediate enhancement of promoter activity by TGF-β (Fig. 4E), supporting the ability of the N-terminal region of Phox2 proteins to functionally interact with Smad3. Together, these results indicate that R-Smads as well as Smad4, but not the inhibitory Smad7, have the ability to directly interact with Phox2 proteins. This interaction is mediated by the MH1 domain of Smads and the N-terminal region of Phox2 proteins containing the homeodomain.

Discussion

Although the role of BMP signaling in the induction of DBH expression has been

well established, the underlying molecular mechanisms had not been clarified. The ability of BMP proteins to induce expression of Phox2 transcription factors, essential regulators of the noradrenergic neurotransmitter phenotype, had suggested an indirect mechanism for the actions of BMPs on DBH expression. As shown in the present study, however, the ability of BMP-4 to induce DBH expression in the presence of cycloheximide indicates that some of the effects of BMPs on DBH expression are direct and do not require *de novo* synthesis of intermediate components. In addition, our results in Neuro2A and C2C12 cells highlight the importance of cellular context for the ability of BMPs to activate the DBH promoter, and confirm the essential role of Phox2 proteins in the induction and maintenance of DBH expression.

Although we are not able at present to determine whether TGF-β is also able to directly regulate DBH expression independently of new protein synthesis, our results from reporter and protein-protein interaction assays would appear to suggest a mode of action similar to that of BMP-4. It remains to be determined, however, whether TGF-βs, like BMPs, are also able to induce expression of Phox2 proteins.

Our findings highlight what appears to be a recurrent theme in Smad signaling, namely their ability to induce the expression of factors with which they subsequently interact and cooperate to regulate expression of common downstream target genes. In addition to provide tissue specificity to the actions of TGF-β proteins, this type of feed-forward mechanism may be essential for their ability to give rise to switch type behavior, by which distinct developmental phenotypes arise at different signal strength levels (i.e. morphogenetic effects).

The ability of Smad proteins to interact directly with Phox2 factors and to cooperate synergistically in the activation of a synthetic promoter carrying Phox2 DNA binding sites suggest that functional cooperation between Smads and Phox2 proteins may not be limited to the DBH gene and could also underlie their regulatory effects on other genes, including several in the gene network controlling the noradrenergic neurotransmitter phenotype such as TH and the norepinephrine transporter NET. The ability of Smads to interact directly and cooperate synergistically with several factors in this network, including GATA-3 (20) and Phox2a and b, suggests a novel role for these proteins in coordinating genetic interactions by forming higher order complexes with factors at different levels in

the network. Thus, signaling by Smads may not only help to initiate genetic programs but also keep the function of gene networks in "sync" with environmental (epigenetic) signals by forming multimeric complexes with different network factors and thereby regulating their activity.

Materials and Methods

Real-time PCR

Transfected (i.e. GFP-expressing) neural tube tissue was manually cleaned from the surrounding mesoderm. One day after transfection, Neuro2A cells were treated with TGF-β for 60 min before RNA isolation and cDNA synthesis. RNA was isolated using the RNeasy kit (Qiagen). Single stranded cDNA was synthesized using the ProStar RT kit (Stratagene). Real-time PCR was performed using a LightCycler rapid thermal cycler system (PE) according to the manufacturer's instructions. Reactions were performed in 25 µl volume. Nucleotides, Taq DNA polymerase, and buffer were included in the LightCycler–DNA Master SYBR Green I mix (PE). All results are expressed relative to GAPDH values obtained in parallel reactions.

In ovo electroporation of chicken embryos

Fertilized white leghorn eggs obtained from commercial sources were incubated at 37°C in a standard humidified avian incubator. Embryos were staged according to the developmental table of Hamburger and Hamilton (HH) (33). Electroporation was essentially performed as previously described (34,35) using constructs at a final concentration of 0.4 µg/ml in presence of 0.4 µg/ml EGFP (Clontech) in 1x PBS; 0.2% Fast Green. Embryos were injected with a glass microneedle at HH stages 8-10 (≈E2). Stage 10 embryos were injected into the lumen of the trunk neural tube. Electroporation was performed essentially using an Electro-Square Porator ECM830 (BTX, Genetronics, Inc.) at 20 Volt with 5 pulses of 15 msec delivered via a platinum electrode. 48 hours after electroporation (HH stages 21-23), surviving embryos were evaluated for transfection efficiency by visualizing GFP expression using a fluorescent lamp (BLS, Budapest, Hungary) mounted onto a dissection microscope (Stemi 2000, Zeiss). Tissues with the appropriate targeting of GFP expression were dissected and processed for real-time PCR as described.

Plasmid constructs, cell transfection and reporter assays

DBH reporter constructs contained either 394 or 1648 bp of upstream sequence of the DBH gene followed by a luciferase reporter. The 4xPhox2-luc reporter contains four tandem copies of consensus Phox2 DNA binding sites in the sense orientation in front of the adenovirus major late promoter driving the luciferase gene. All Smad expression plasmids used the pCDNA3 backbone and have been described elsewhere (20,36). The constitutively activated TGF-β receptor (CA-ALK5) carries a mutation in the intracellular juxtamembrane domain (T204D) and has been described previously (37,38).

Mouse neural crest-derived neuroblastoma Neuro2A, mouse myoblast C2C12 and mink lung epithelial Mv1Lu cells were transfected in complete medium with FuGene6 according to the manufacturer's instructions (Roche). After a 24 h incubation, cell monolayers were washed with serum-free medium and incubated for a further 16 h in 0.1% serum-containing medium. Where indicated medium was supplemented with 40 ng/ml BMP-4 or 10 ng/ml TGF-β1 (R&D). Cycloheximide (Sigma) was used at 25 μg/ml. Reporter assays were performed and analyzed as previously described (20). All treatments and transfection conditions were analyzed in triplicate.

Pull-down and co-immunoprecipitation assays

GST fusions were produced in *E. coli* and purified by chromatography on glutathione-conjugated agarose beads (Pharmacia). *In vitro* translated products were produced using a kit from Promega. *In vitro* pull-down assays were done as previously described (20).

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Figure legends

Figure 1. Regulation of DBH expression by TGF-β superfamily ligands

(A) Regulatory network controlling the development of the noradrenergic neurotransmitter phenotype in sympathetic neurons. BMPs are required for the expression of Mash1 and Phox2b. Mash1 and Phox2b precede dHand, Gata3 and Phox2a; Mash1 and Phox2b are also genetically upstream of Phox2a and dHand, and Phox2b is genetically upstream of Gata3. Expression of the noradrenaline-synthesizing enzymes tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) has been shown to depend on Mash1, Phox2b and Gata3. Promoter studies indicate that the regulation of the DBH gene by Phox2b, Phox2a and dHand is direct. At present, it is not known whether Mash1 acts on TH and DBH expression only through dHand, or whether Gata3 expression requires Mash1 activity. Phox2b is also required for the maintenance of Mash1 expression. Solid arrows indicate regulatory interactions shown by loss- and gain-of-function experiments; dashed arrows denote an interaction deduced only from gain-of-function experiments. Note that most interactions are reciprocal. Figure and legend text adapted from ref. (21).

(B) Real-time PCR analysis of DBH mRNA expression in Neuro2A cells. C2C12 cells were treated with 40 ng/ml BMP-4 for 70 min prior to RNA extraction. Cycloheximide treatment (+Chx) was initiated 10 min prior to stimulation with BMP-4 and maintained thereafter. Results are presented as average ± SD of triplicate determinations.

(C) Real-time PCR analysis of DBH mRNA expression in electroporated embryonic chick neural tube. Only neural tubes showing correct targeting of the GFP marker were used for expression analysis. The histogram shows averages of 2 determinations.

Figure 2. Activation of the DBH promoter by TGF-β ligands in neural crest-derived Neuro2A cells.

(A) Activation of the DBH promoter in Neuro2A cells by BMP-4. A 394 bp construct of the DBH promoter coupled to a luciferase reporter was introduced into Neuro2A cells along with full length Phox2a, Smad1 and Smad4 as indicated. Results are

presented as average ± SD of triplicate determinations.

(B) Activation of the DBH promoter in Neuro2A cells by TGF-β. A 394 bp construct of the DBH promoter coupled to a luciferase reporter was introduced into Neuro2A cells along with full length Phox2a, Smad2 and Smad3 as indicated.

Figure 3. Activation of the DBH promoter by TGF-β ligands requires expression of Phox2 proteins

(A) Activation of the DBH promoter in C2C12 myoblasts by Phox2a and BMP-4. A 394 bp construct of the DBH promoter coupled to a luciferase reporter was introduced into C2C12 cells along with full length Phox2a and Smad1 as indicated. Results are presented as average ± SD of triplicate determinations.

(B) Activation of the DBH promoter in C2C12 myoblasts by Phox2a and TGF-β. A 394 bp construct of the DBH promoter coupled to a luciferase reporter was introduced into C2C12 cells along with full length Phox2a, Smad2, Smad3 and Smad4 as indicated.

(C) A 1648 bp construct of the DBH promoter coupled to a luciferase reporter was introduced into C2C12 cells along with full length Phox2a as indicated.

(D) Activation of a multimerized Phox2 promoter construct (4xPhox2-luc) in epithelial Mv1Lu cells by Phox2a and TGF-β.

Figure 4. Physical interaction between Phox2 proteins and Smads

(A) Domain organization of Smad3 and Phox2a. Deletion constructs of Phox2a used in *in vitro* co-precipitation assays are indicated. MH1 and MH2 denote Mad homology domains 1 and 2, respectively. The location of Brachyury-like motif and homeodomain in Phox2a are indicated.

(B) *In vitro* pull-down of *in vitro* translated ^{35}S-Phox2a by GST-Smad fusion proteins. Full length [^{35}S]-labeled Phox2a produced by *in vitro* translation was used in precipitation assays together with equal amounts of the indicated GST-Smad fusion proteins. Note that deletion of MH1 or MH2 domains (ΔMH1 or ΔMH2, respectively) results in constructs that retain the linker between the two domains. As control, 25% of the [^{35}S]-labeled Phox2a used for the precipitations was also run

in the gel.

(C) *In vitro* pull-down of *in vitro* translated ^{35}S-Phox2b by GST fusion proteins containing either the MH1 or MH2 domains of Smad4 as indicated.

(D) The indicated Smad3 deletion constructs were produced in transfected COS cells as N-terminally myc-tagged proteins and used in co-precipitation assays together with the indicated GST-Phox2a fusion proteins lacking C-terminal (Phox2a-ΔC) or N-terminal (Phox2a-ΔN) domains.

(E) Activation of the 4xPhox2-luc reporter in Mv1Lu cells by Phox2a-ΔC and TGF-β.

Figure 1

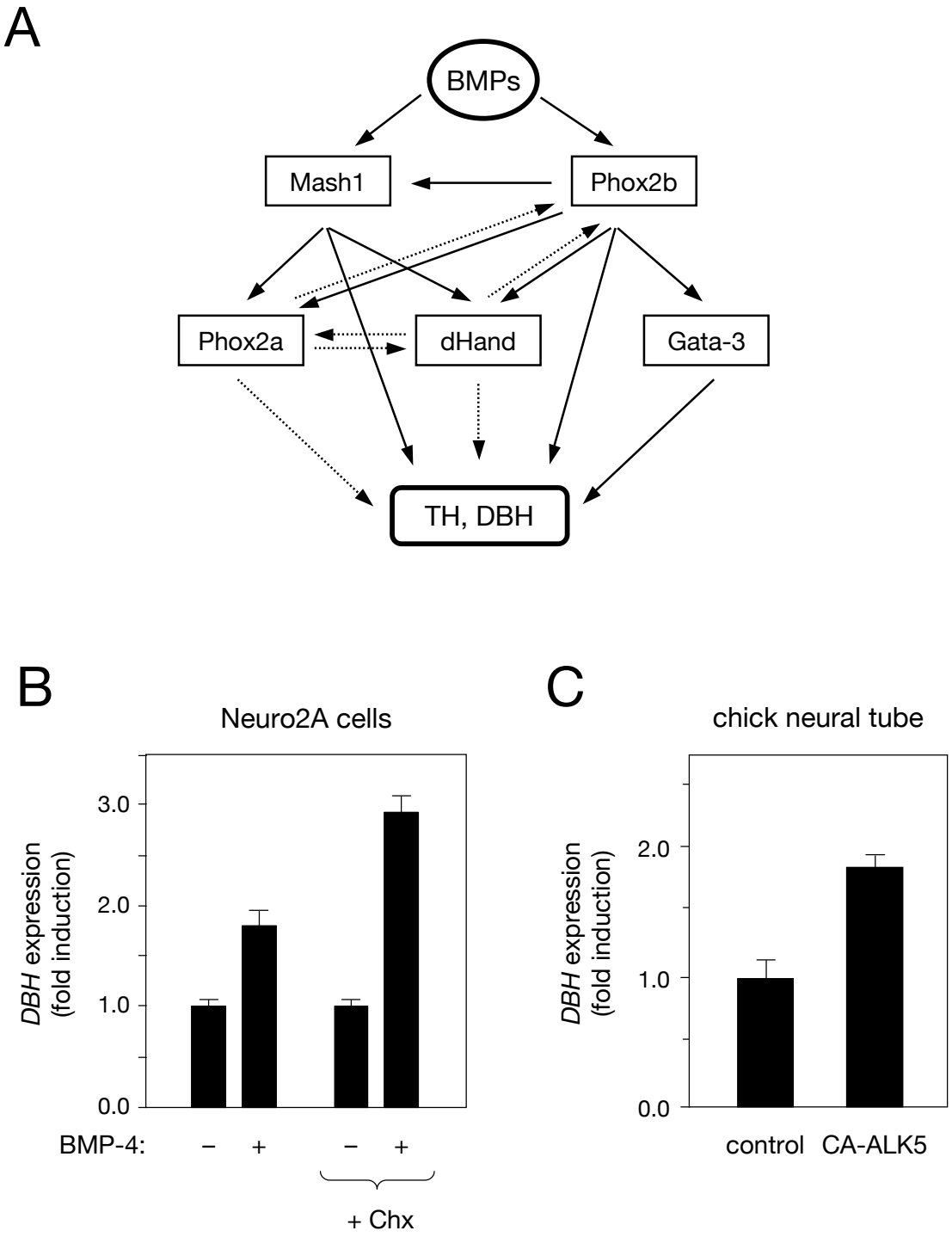
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Figure 2

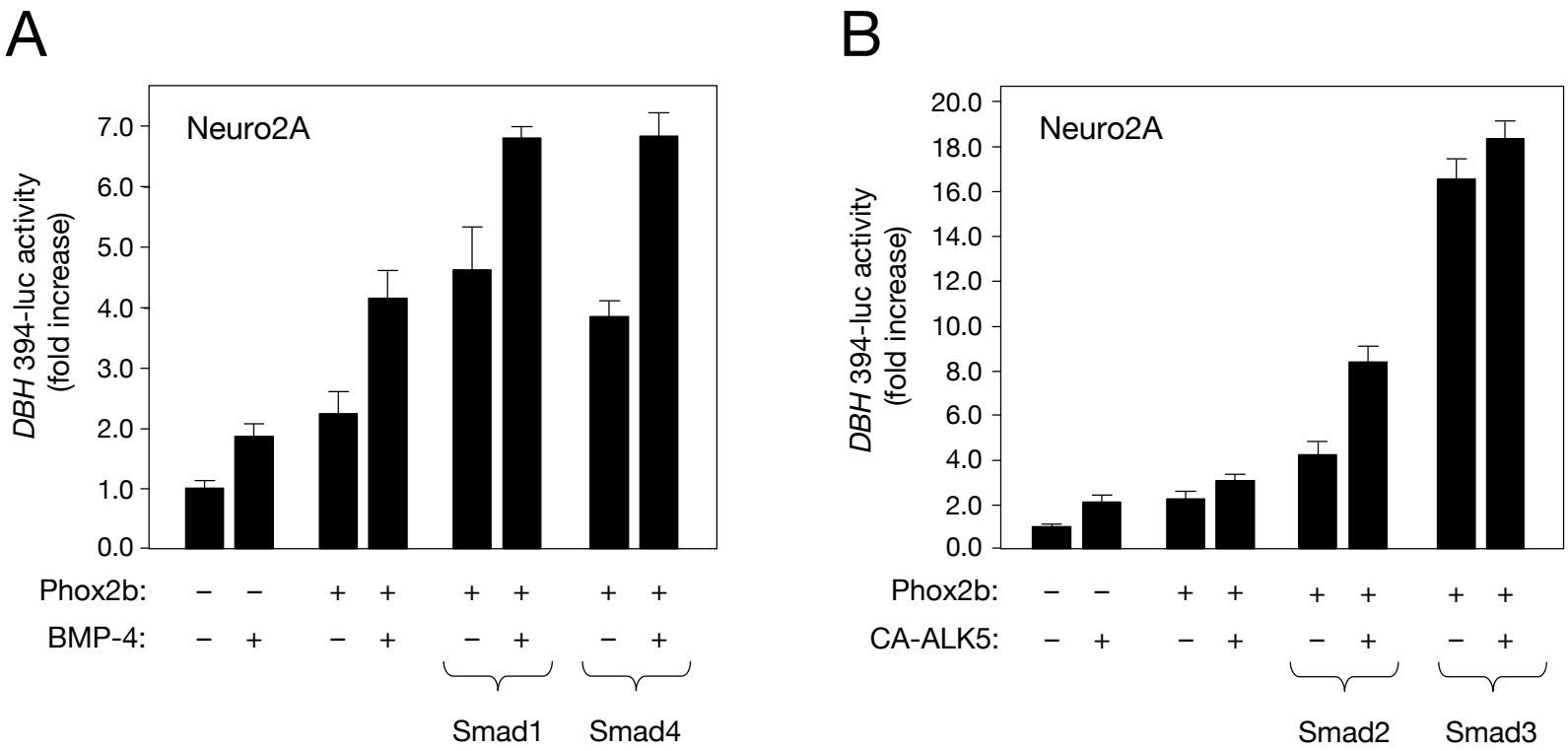

A
Neuro2A
DBH 394-luc activity (fold increase)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Phox2b: – – + + + + + +
BMP-4: – + – + – + – +
Smad1
Smad4
B
Neuro2A
DBH 394-luc activity (fold increase)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Phox2b: – – + + + + + +
CA-ALK5: – + – + – + – +
Smad2
Smad3

Figure 3

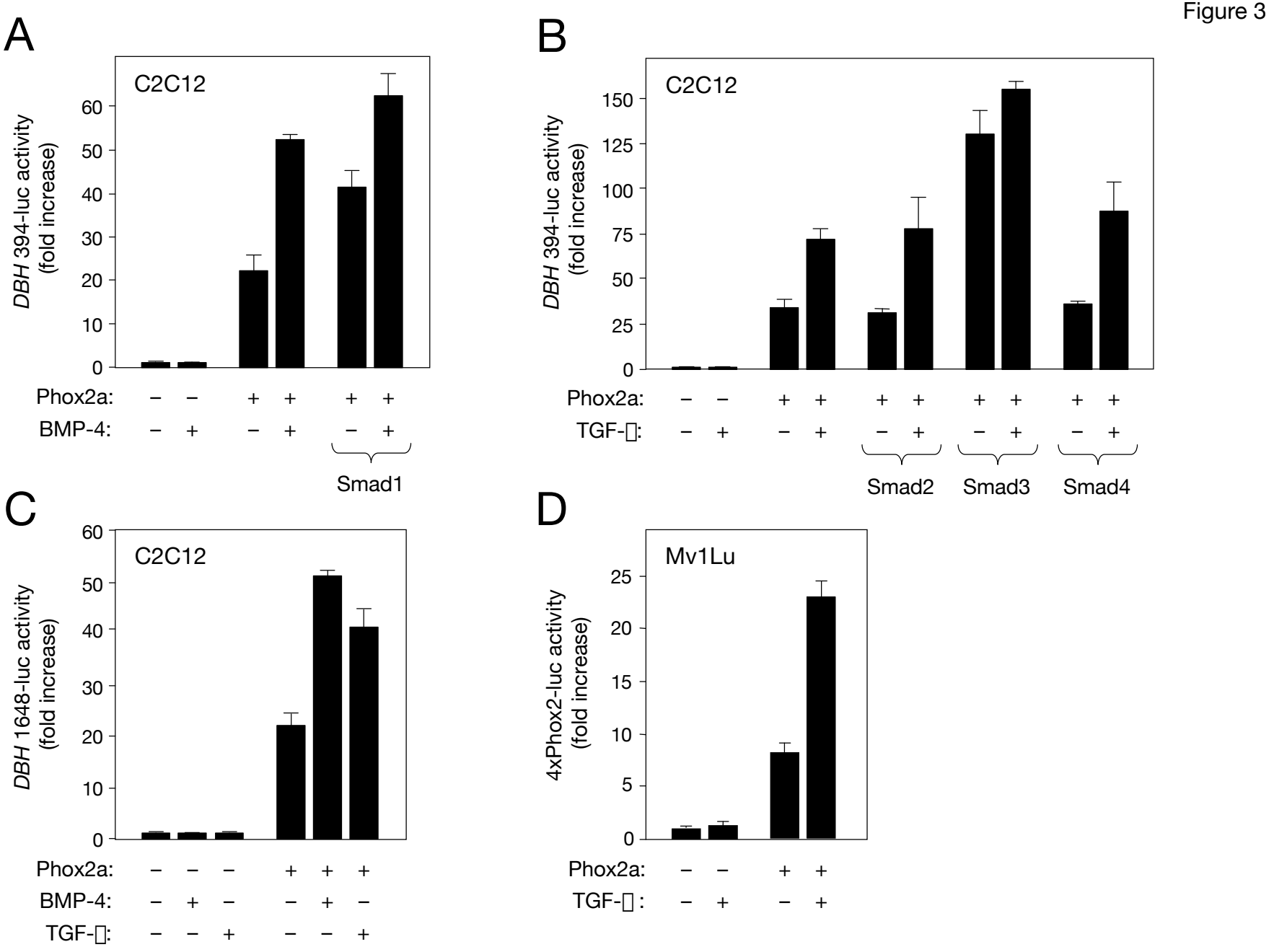
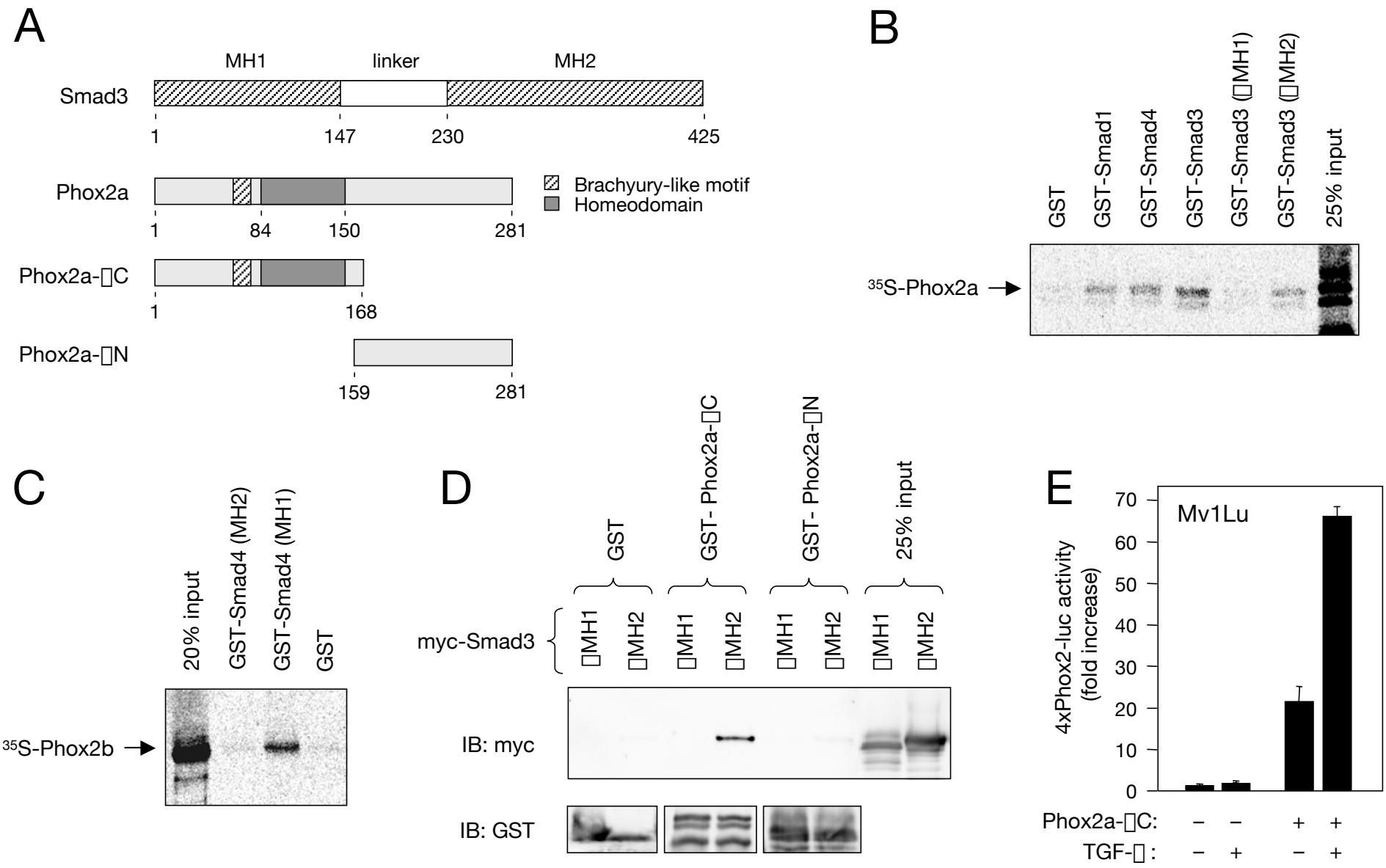

A
C2C12
DBH 394-luc activity (fold increase)
0
10
20
30
40
50
60
Phox2a: – – + + + +
BMP-4: – + – + – +
Smad1
B
C2C12
DBH 394-luc activity (fold increase)
0
25
50
75
100
125
150
Phox2a: – – + + + + + + + +
TGF-β: – + – + – + – + – +
Smad2
Smad3
Smad4
C
C2C12
DBH 1648-luc activity (fold increase)
0
10
20
30
40
50
60
Phox2a: – – – + + +
BMP-4: – + – – + –
TGF-β: – – + – – +
D
Mv1Lu
4xPhox2-luc activity (fold increase)
0
5
10
15
20
25
Phox2a: – – + +
TGF-β: – + – +

Figure 4


A
MH1
linker
MH2
Smad3
1
147
230
425
Phox2a
Brachyury-like motif
Homeodomain
1
84
150
281
Phox2a-ΔC
1
168
Phox2a-ΔN
159
281
B
GST
GST-Smad1
GST-Smad4
GST-Smad3
GST-Smad3 (ΔMH1)
GST-Smad3 (ΔMH2)
25% input
35S-Phox2a
C
20% input
GST-Smad4 (MH2)
GST-Smad4 (MH1)
GST
35S-Phox2b
D
GST
GST- Phox2a-ΔC
GST- Phox2a-ΔN
25% input
myc-Smad3
ΔMH1
ΔMH2
IB: myc
IB: GST
E
Mv1Lu
4xPhox2-luc activity
(fold increase)
70
60
50
40
30
20
10
0
Phox2a-ΔC: − − + +
TGF-β: − + − +