Nucleic Acids Research, 2005, Vol. 33, No. 20 **6555–6565**
doi:10.1093/nar/gki964

# Human *Bex2* interacts with *LMO2* and regulates the transcriptional activity of a novel DNA-binding complex

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Received as resubmission September 20, 2005; Revised and Accepted October 31, 2005

## ABSTRACT

**Human *Bex2* (brain expressed X-linked, *hBex2*) is highly expressed in the embryonic brain, but its function remains unknown. We have identified that *LMO2*, a LIM-domain containing transcriptional factor, specifically interacts with *hBex2* but not with mouse *Bex1* and *Bex2*. The interaction was confirmed both by pull-down with GST-*hBex2* and by coimmunoprecipitation assays *in vivo*. Using electrophoretic mobility shift assay, we have demonstrated the physical interaction of *hBex2* and *LMO2* as part of a DNA-binding protein complex. We have also shown that *hBex2* can enhance the transcriptional activity of *LMO2 in vivo*. Furthermore, using mammalian two-hybrid analysis, we have identified a neuronal bHLH protein, *NSCL2*, as a novel binding partner for *LMO2*. We then showed that *LMO2* could up-regulate *NSCL2*-dependent transcriptional activity, and *hBex2* augmented this effect. Thus, *hBex2* may act as a specific regulator during embryonic development by modulating the transcriptional activity of a novel E-box sequence-binding complex that contains *hBex2*, *LMO2*, *NSCL2* and *LDB1*.**

## INTRODUCTION

Interactions among transcriptional factors provide molecular mechanisms for the highly regulated temporal and spatial gene expression during embryonic development. Importantly, unveiling the function of a novel gene often relies on identifying its interactions with proteins of known function. We have identified a group of genes specifically expressed in human fetal brains (1) including human *Bex2* (previously reported as human *Bex1*), a previously reported brain-expressed, X-linked protein of unknown function (2). The *Bex* gene family is composed of at least six members: *Bex1/Rex3/EG2RVC/NADE4*, *Bex2*, *Bex3/NADE/HGR74* and three new *Bexs*, *Bex4/5/6* (2–6). Two additional *Bex* orthologs, *NADE2* and *NADE3*, were reported to be only expressed in human (7). Recently, the nomenclature for human *Bex1* and *Bex2* was revised since the previously designated human *Bex1* has higher sequence identity with mouse *Bex2* than with mouse *Bex1* (74 and 68% identity, respectively) (8). With this revision in the nomenclature, the localization of *Bex1–4* on the X chromosome from three species—human, rat and mouse—matches within the cluster (2,8). It has been shown that mouse *Bex1* is transiently expressed during early development at the 2–8 cell stage, and then again expressed at the blastocyst stage (9). Murine *Bex1* has also been suggested to play a role in neural development. For example, the expression of *Bex1* was suppressed in F9 cells and PC12 cells upon retinoic acid or NGF treatment, respectively (4,10). Rat *Bex1* was reported as one of the most commonly identified genes in a subtractive screen for ventral mesencephalic genes expressed at E10 (8). Additionally, the high expression profile of *Bex1/2* in the adult brain supports a role of both proteins in the neural activity in mature neurons (2,6,8). However, the molecular mechanisms by which Bex proteins are involved in neurodevelopment remain elusive. Interestingly, both *Bex1* and *Bex2* were recently identified as binding partners for olfactory marker protein (OMP), a soluble protein possibly involved in olfactory signal transduction (11,12). Despite its interaction with OMP, *Bex2*'s broader expression in the brain

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other than that restricted for OMP suggests that it may have a broader functional role in the brain (6). Moreover, *mBex3*, which has a relatively weaker sequence similarity with *Bex1/2*, was observed to interact with p75NTR in inducing apoptotic cell death (NADE) (5,7). Thus, the *Bex* family may play a dual role at the transcriptional and cytoplasmic signaling levels during embryonic development.

To determine the molecular and cellular mechanisms underlying the function of human *Bex2* (previously named *Bex1*) (13), we have used a yeast two-hybrid system to screen for interacting proteins of *hBex2*, and identified *LMO2* as a binding protein. *LMO2*, a member of the LIM-only class proteins and formerly named rhombotin-2 (*rbtn-2* or *Ttg-2)*, has been extensively reported to play a role in hematopoietic stem cell differentiation (14,15). The *Drosophila* homolog of *LMO*, *dLMO*, was also indicated in an evolutionarily conserved mechanism involved in patterning appendages (16). Interestingly, recent evidence indicates that *dLMO* controls the responsiveness of PDF-expressing ventral lateral neurons (principle circadian pacemaker cells) to cocaine, which in turn regulate the behavioral sensitivity of the flies to the drug (17). Furthermore, one of its distant relatives, *LMO4*, has been suggested to play a role in neurogenesis and neurodegenerative disease (18–20). As a highly expressed protein in the brain (21,22), *LMO2* has been reported to be expressed in the hippocampus during development (23). This expression is up-regulated during seizure-induced responses, suggesting a role in neuronal regeneration after epileptic pathogenesis (24).

We next examined whether *NSCL2*, a neuronal form of Class II bHLH protein SCL, can interact with *LMO2*. It has been reported that *LMO2* may interact with *NSCL1* as an adaptor in addition to other transcription factors (25–27). However, this interaction failed to show direct regulation of transcriptional activity in a previous report (25). Here we have shown the functional interaction between *LMO2* and NSCL2, suggesting the existence of a novel complex that is composed of *hBex2*, *LMO2*, *NSCL2* and *LDB1*. The unveiling of this complex may help to understand the molecular mechanisms by which *hBex2* plays a role in transcriptional regulation in the brain during neurodevelopment.

## MATERIALS AND METHODS

### cDNA plasmid construction

The full-length cDNAs for *hBex1, hBex2, hBex3(NADE), mBex1, mBex2, mBex3* and various deletion mutants of *hBex2* used in the yeast two-hybrid assay were cloned in frame with the GAL4 DNA-binding domain into vector pAS2-1 (BD_Clontech, CA). The Genbank accession numbers for the various Bex genes are: *hBex1, AF251053* (for nomenclature change see Introduction); *hBex2, AF237783; hBex3(NADE), AF187064; mBex1, AF097438; mBex2, AF097439; mBex3* and *AF097440*. The GAL4 AD fused *LMO2* expressing plasmid was generated by inserting the LMO2 cDNA into pACT2 (BD Clontech, CA). The GST fusion protein constructs (*pGEX-hBex2* and pGEX-*LMO2*) of *hBex2* and LMO2 were constructed by in-frame insertion of *hBex2* and LMO2 cDNAs into pGEX4T-1 and pGEX6P-1 (Amersham Pharmacia Biotech, Piscataway, NJ). *hBex2*-EGFP was constructed by in-frame insertion of the *hBex2* cDNA with leading Kozak sequence into pEGFP-N1 vector (BD Clontech, CA). The chimeric constructs between *hBex2* (H) and *mBex1* (m) (*mHm* and *HmH*) were subcloned into pMRFP containing a monomer red fluorescent protein (R. Tsien, UCSD). pcDNA-LMO2 was constructed by inserting the LMO2 cDNA with leading Kozak sequence into pcDNA 3.1(+) vector. The same cDNA was used in constructing EGFP fused *LMO2* expression plasmid pEGFP-*LMO2*. The PM3 (BD Clontech), PM3-*LMO2*, PM3-*NSCL1* and PM3-*NSCL2* are SV40 promoter-driven vectors expressing Gal4BD fused proteins. VP16 (BD Clontech), *VP16-NSCL1* and *VP16-NSCL2* are plasmids expressing VP16 fused proteins. The wild-type E-box-luciferase and the mutant form of E-box-luciferase were generated from pGAL-luc and were obtained as kind gifts from Dr Hakan Axelson.

### Yeast two-hybrid screen

The full-length *Bex2* was used as bait to screen a human fetal brain cDNA library as described in the user manual for the MATCHMAKER yeast two-hybrid system 2 (BD Clontech, CA). Yeast colonies with potential positive interactions were further tested for *LacZ* activity and survival on selective media. Plasmids containing cDNA for interacting proteins were extracted from the positive yeast clones and further sequenced with pACT2 specific primers.

### Cell culture and transfection

All cells were maintained in 5% $CO_2$ at 37°C in medium containing penicillin and streptomycin unless otherwise noted. HeLa and COS7 cells were maintained in DMEM (Invitrogen) with 10% cosmic calf serum (Hyclone, Logan, UT) and 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM nonessential amino acids. For transfection, cells were first plated on poly-D-lysine and Matrigel double-coated glass coverslips in a 24-well dish in culture medium without antibiotics the day before transfection. Cells on coverslips were then transfected by using LipofectAMINE 2000 according to the manufacturer's instructions (Invitrogen, San Diego, CA). After 12 h, transfected cells were fixed for immunofluorescence.

### GST fusion protein and antiserum generation

pGEX-*hBex2* and pGEX-*LMO2* plasmids were transformed into *Escherichia coli* strain BL21(DE3). The protein expression and purification were performed according to the manufacturer's user manual (Amersham Pharmacia Biotech, Piscataway, NJ). Two New Zealand white female rabbits were immunized with 600 μg purified protein in complete Freund's adjuvant (Sigma, St Louis, MO) and were boosted three times with 300 μg protein in incomplete Freund's adjuvant at 3 week intervals. The titer and specificity of antisera were then determined by ELISA after each boost until the titer reached $1:1 \times 10^6$. The antisera were further tested by western blot against either the recombinant or expressed proteins.

### GST fusion protein mediated pull-down assay

GST or GST-hBex2 fusion protein was expressed in *E.coli* strain BL21(DE3) and purified with Sepharose CL-4B resin (Amersham Pharmacia Biotech, Piscataway, NJ). The cell lysates from either pEGFP- or pEGFP-*LMO2*-transfected

HeLa cells were then incubated with *GST* or *GST-hBex2* bound resin at 4°C for 4 h. The pellets were washed three times with phosphate-buffered saline (PBS), separated by 10% SDS–PAGE and analyzed with western blot.

### Immunofluorescent microscopy

HeLa cells transfected with the FLAG-tagged *hBex2* and pEGFP-*LMO2* were grown on 25 mm glass coverslips. The cells were fixed with 4% PFA and permeabilized with 0.1% Triton X-100 in PBS (pH 7.4). Cells were then incubated with anti-FLAG antibody (M2, Sigma, St Louis, MO), followed by incubation with TRITC conjugated secondary antibody. Confocal microscopic analysis was performed as described previously (28).

### Electrophoretic mobility shift assay (EMSA)

EMSAs were performed with $^{32}$P-labeled double-strand oligonucleotides as described previously (29). Oligonucleotides containing the E-box motif (underlined sequences) were synthesized as follows: 5′-CTGGCTAGCGGGTCG-CAGCTGCGTCCCGGGTCGCAGCTGCGTCCCGGGTCG-CAGCTGCGTCCCCTCGAGATC-3′ and 5′-GATCTCGAG-GGGACGCAGCTGCGACCCGG GACGCAGCTGCGACC-CGGGACGCAGCTGCGACCCGCTAGCCAG-3′ (underlined is the hexameric sequence of E box). Nuclear extracts from M17 cells were prepared and incubated with labeled oligonucleotide for 20 min at room temperature, followed by incubation with pre-immune serum, anti-LMO2 anti-serum or anti-*hBex2* anti-serum. The gel was dried, exposed to Kodak film overnight and then processed.

### Transfection of cDNA plasmid into mammalian cells and luciferase assay

Cos-7 cells growing in 12-well plates with 90% confluence were transfected with 1 μg cDNA plasmid per well using LipofectAMINE 2000 as described in the manufacturer's user manual (Invitrogen, Carlsbad, CA). After 24 h, cell extracts were prepared and luciferase reporter assays were performed using the Dual-Luciferase Reporter Assay System (Catalog no. E1910, Promega, Madison, WI). The luciferase activity was measured using the LB9507 Luminometer (EG&G Berthold, Wellesley, MA). The transcriptional activity of the reporter gene was measured by relative luciferase activity value, which is the value of firefly luciferase activity divided by the value of renilla luciferase activity.

### Coimmunoprecipitation

Fetal brain tissue (50 g) was homogenized in 250 ml of buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.5 mM phenylmethlysulfonyl fluoride (PMSF), 1 mM DTT, and 10 mg/ml for each of the following protease inhibitors: leupeptin, aprotinin and pepstatin]. The homogenate was then treated with NP-40 (0.2% final) for 15 min on ice and centrifuged for 15 min at 15 000 *g*, 4°C. The nuclear pellet was resuspended in 20 ml of buffer B (10 mM HEPES, pH 7.9, 0.1 mM EGTA, 0.4 M NaCl, 0.5 mM PMSF, 1 mM DTT and protease inhibitors) and incubated with rotation at 4°C for 15 min. The extract was then centrifuged, and the supernatant was dialyzed against 500 ml of buffer C (10 mM Tris–HCl, pH 7.9, 120 mM NaCl, 0.3% NP-40, 0.25% BSA and 1 mM DTT) at 4°C. Nuclear extracts (∼100 mg protein) were incubated with polyclonal anti-*hBex2*, anti-LDB1 or control antisera for 6 h at 4°C. The complexes were precipitated with protein A or G agarose, washed four times in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1% deoxycholate and 0.1% SDS), and then detected by western blot using antisera α-*LMO2* (Santa Cruz Biotechnology, Santa Cruz, CA) or α-*NSCL2*, (CeMines, Inc., Golden, CO), as indicated.

## RESULTS

### Identification of *LMO2* as an interacting partner for *hBex2*

To gain insight into the function of *hBex2* in development, we used a human fetal brain cDNA library in a Gal4 yeast two-hybrid system (BD Clontech, CA) to identify interacting proteins. Using the full-length *hBex2* as bait, we screened $\sim 1 \times 10^7$ transformants and acquired ∼59 His and β-galactosidase positive yeast colonies. Following cDNA plasmid extraction and sequencing analysis, we chose to focus on a candidate cDNA clone corresponding to the complete coding sequence of *LMO2*, a LIM containing a transcriptional regulator involved in neurogenesis (24). The interaction between two proteins was further verified by retransformation into yeast strain SFY-526 to exclude the possibility of a false positive as a result of self-activation.

To determine the specificity of the interaction between *LMO2* and *hBex2* in the yeast two-hybrid system, we tested whether *LMO2* could interact with other members of the *Bex* family, including *hBex3, mBex1, mBex2* and *mBex3*. For a positive control, we also used the TAX protein of human T-cell leukemia virus type1 because of its self-association (28) (Table 1). Among the five *Bex* homolog genes tested in the yeast Gal4-BD construct, only *hBex2* interacted with *LMO2* (Table 1). More specifically, although it shares 74% amino acid sequence identity with *hBex2* (Figure 1), *mBex2* failed to show its interaction with *LMO2* in our assay. Similarly, *mBex1* also showed no interaction with *LMO2* although it also shares high sequence identity (68%) with *hBex2*. Thus, the interaction of human *Bex2* and *LMO2* might be a human species-specific event.

To identify the binding domain within *hBex2* responsible for interaction between *hBex2* and *LMO2*, we generated

**Table 1.** Identification of *LMO2* as an interaction partner for *hBex2*

| AD plasmid | BD plasmid | Result |
|---|---|---|
| pAS2-1-TAX | pACT2-TAX | + |
| pAS2-1 | pACT2 | − |
| pAS2-1 | pACT2-LMO2 | − |
| pAS2-1-hBex2 | pACT2 | − |
| pAS2-1-hBex2 | pACT2-LMO2 | + |
| pAS2-1-hBex3 | pACT2-LMO2 | − |
| pAS2-1-mBex1 | pACT2-LMO2 | − |
| pAS2-1-mBex2 | pACT2-LMO2 | − |
| pAS2-1-mBex3 | pACT2-LMO2 | − |

GAL4 yeast two-hybrid system was used to evaluate the interaction of genes in the *Bex* family with *LMO2*. TAX protein of human T-cell leukemia virus type 1 was used as a positive control. The interactions of listed GAL4–BD and GAL4–AD fusion pairs were indicated only as positive or negative for β-galactosidase activity tested in the yeast strain SFY526.

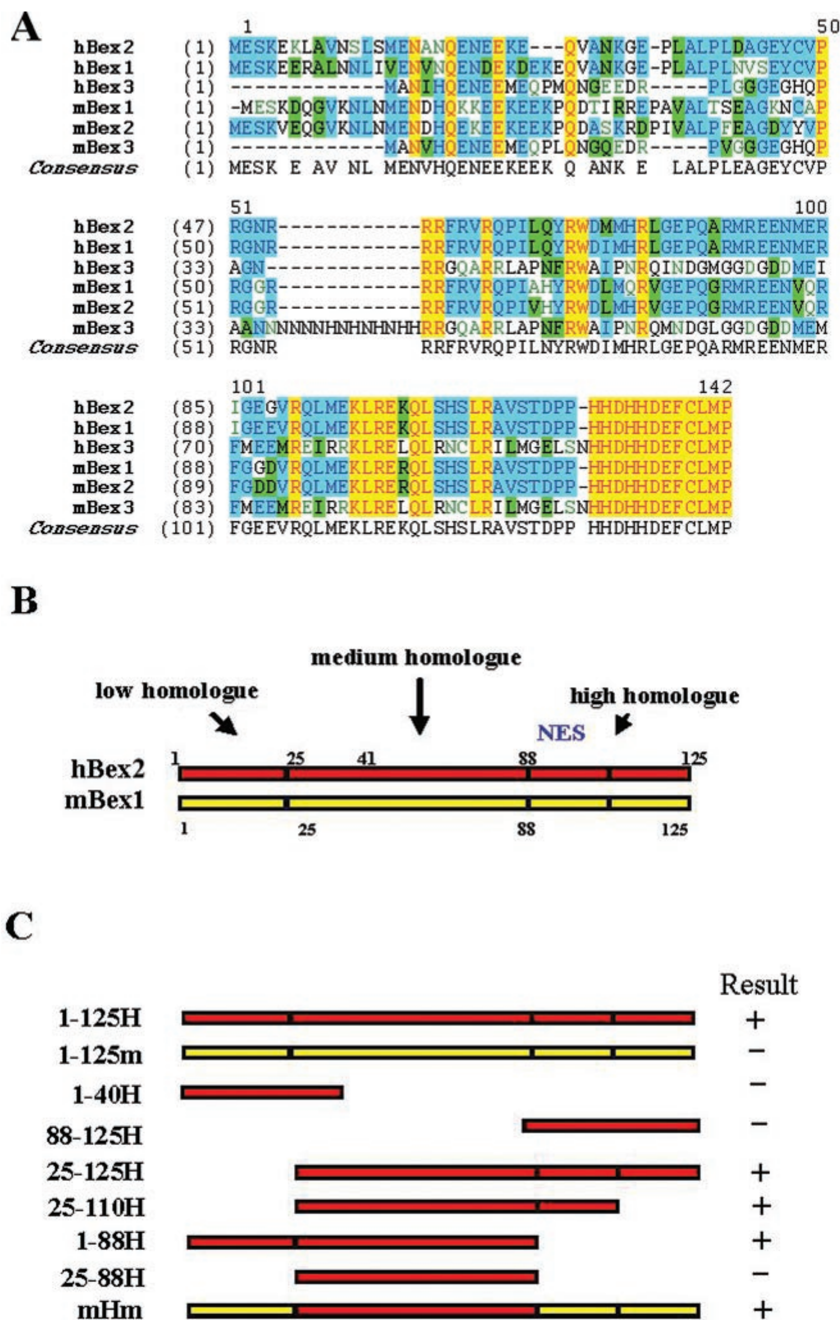
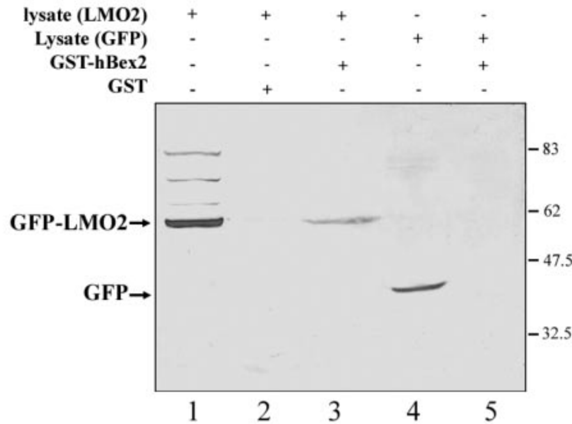
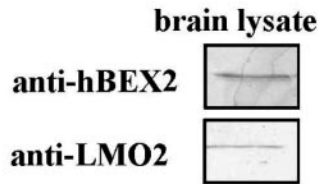



**Figure 1.** (**A**) Multiple alignment of members of the *Bex* family. The C-terminals of the *Bexs* (*hBex2* 88–125) are conserved throughout the family, and the last 12 amino acids (114–125) are identical. *hBex2* shares 74% amino acid sequence identity with *mBex2* and 68% identity with *mBex1*. (**B**) Schematic diagram of *hBex2* deletion constructs that were tested for interaction with *LMO2* in yeast two-hybrid assay. *hBex2* can be divided into three regions according to similarity analysis among members of the *Bex* family: the N-terminal low homolog region (1–25), the middle medium homolog region (25–88) and the C-terminal high homolog region (88–125). Deletion mutations were constructed accordingly. (**C**) Mapping of the *LMO2*-binding domain in *hBex2*. The interactions of the truncation mutation of *hBex2* with full-length *LMO2* were examined by a β-galactosidase activity assay in the yeast two-hybrid system. *mHm* indicates the chimeric construct containing the middle part of human *Bex2* flanked by N- and C-termini of mouse *Bex1*. The TAX gene was also included as a positive control. The plus symbol indicates the positive binding between the transformed constructs.

several deletion and chimeric *hBex2* mutants for yeast two-hybrid analysis. As shown in Figure 1A, members of the *Bex* family share sequence similarity mainly in the middle (58% identity) and C-terminal (95% identity) regions. Indicated by the yeast two-hybrid assay, the N-terminal region with relatively lower sequence similarity between *hBex2* and *mBex1* (1–26 amino acid sequence, 48% identity) was not required for binding to *LMO2*, nor was the C-terminal region (88–125), which shares high sequence homology among *Bex* family members (Figure 1C). In contrast, all constructs

containing the middle region of *hBex2* (26–88) expressed strong interaction (Figure 1C). However, the middle-region fragment alone does not bind to *LMO2*, suggesting that the sequence itself is insufficient for the binding. Interestingly, the chimeric *mBex1* containing a swapped middle fragment (26–88) of *hBex2* (*mHm*) showed positive interaction with *LMO2* (Figure 1C), further supporting the specific requirement of this fragment within the *hBex2* sequence for protein–protein interaction.



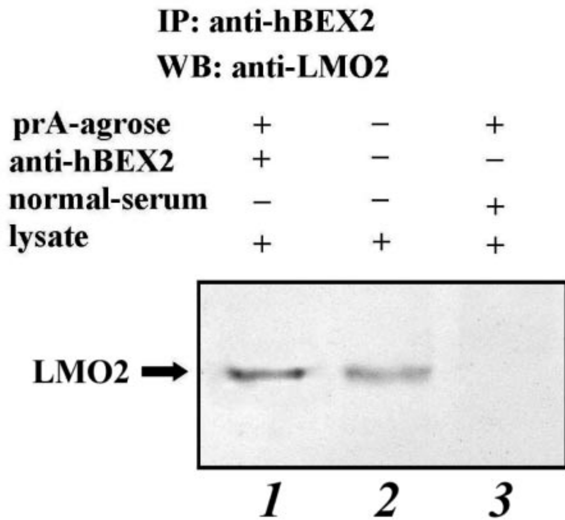




**Figure 2.** Interaction between *hBex2* and *LMO2 in vitro* and *in vivo*. (**A**) pEGFP-*LMO2* or pEGFP (short for pEGFP–*LMO2* lysate or pEGFP lysate) were transiently expressed in HeLa cells. Cell lysates were used to perform pull-down assay by incubating with GST-*hBex2* or GST bound to glutathione beads. Western analysis was performed with anti-GFP antibody. Lane 1, pEGFP-*LMO2* cell lysate only; lane 2, GST pull-down from pEGFP-LMO2 lysate; lane 3, GST-hBex2 pull-down of pEGFP-*LMO2* lysate; lane 4, pEGFP cell lysate only; lane 5, GST-hBex2 pull-down of pEGFP lysate. (**B**) Endogenous expression of *hBex2* and *LMO2* in human fetal brain to confirm the specificity of the antibodies. (**C**) Interaction of *hBex2* and *LMO2* in human fetal brain tissue. The fetal brain tissue was used to immunoprecipitate the human *Bex2* with a polyclonal Ab. Precipitate was blotted with an anti-*LMO2* Ab.

### Protein–protein interaction between *hBex2* and *LMO2*

To further characterize the interaction between *hBex2* and *LMO2*, we performed GST pull-down assays. *GST* and *GST-hBex2* fusion proteins were used to test their interaction with EGFP-tagged *LMO2* from cell extracts from transiently transfected HeLa cells. As shown in Figure 2A, EGFP-LMO2 bound to *GST-hBex2* (lanes 3) but not to GST alone, as indicated by the antibody to the *LMO2* fusion protein (lanes 2). We further tested whether human Bex2 interacts with *LMO2 in vivo*. As shown in Figure 2B, both proteins were expressed in human fetal brain extracts. Coimmunoprecipitation of *hBex2* and *LMO2* from these extracts using anti-*hBex2* Ab indicates that their interaction may occur during development (Figure 2C).

### Nuclear colocalization of *hBex2* and *LMO2*

*LMO2* is localized to the nucleus to play a role in transcriptional regulation (22). The interaction of *hBex2* with *LMO2* suggests their subcellular colocalization in the nucleus. Consistent with this prediction, evidence suggests that *hBex2*'s nuclear export sequence (NES) may be insufficient for transporting the protein out of the nucleus (7). However, it has been suggested that *hBex2* has a cytoplasmic localization pattern when it is overexpressed in HEK293 cells (8). To clarify this issue, we examined the subcellular localization of both endogenous and overexpressed *hBex2* and *LMO2* in cell lines. As

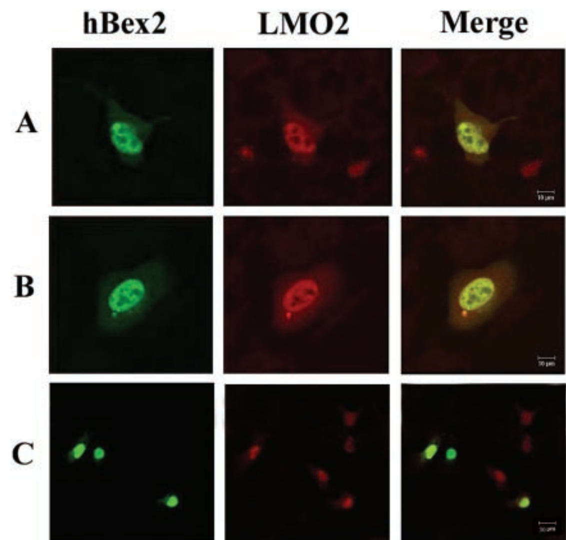



**Figure 3.** Nuclear colocalization of *hBex2* and *LMO2*. (**A**) Endogenous *Bex2* and *LMO2* were colocalized in the nuclei of M17 cells using specific antibodies for immunofluorescent staining. (**B**) FLAG-tagged *hBex2* and dsRed-*LMO2* were colocalized in the transient transfected HeLa cells. Anti-FLAG mAb and TRITC-conjugated secondary anti-mouse antibody were used sequentially to detect the expression of *hBex2*. The staining image was collected with a 60× objective. (**C**) Same as (B) with a 20× objective. Bars indicate 10 μm.

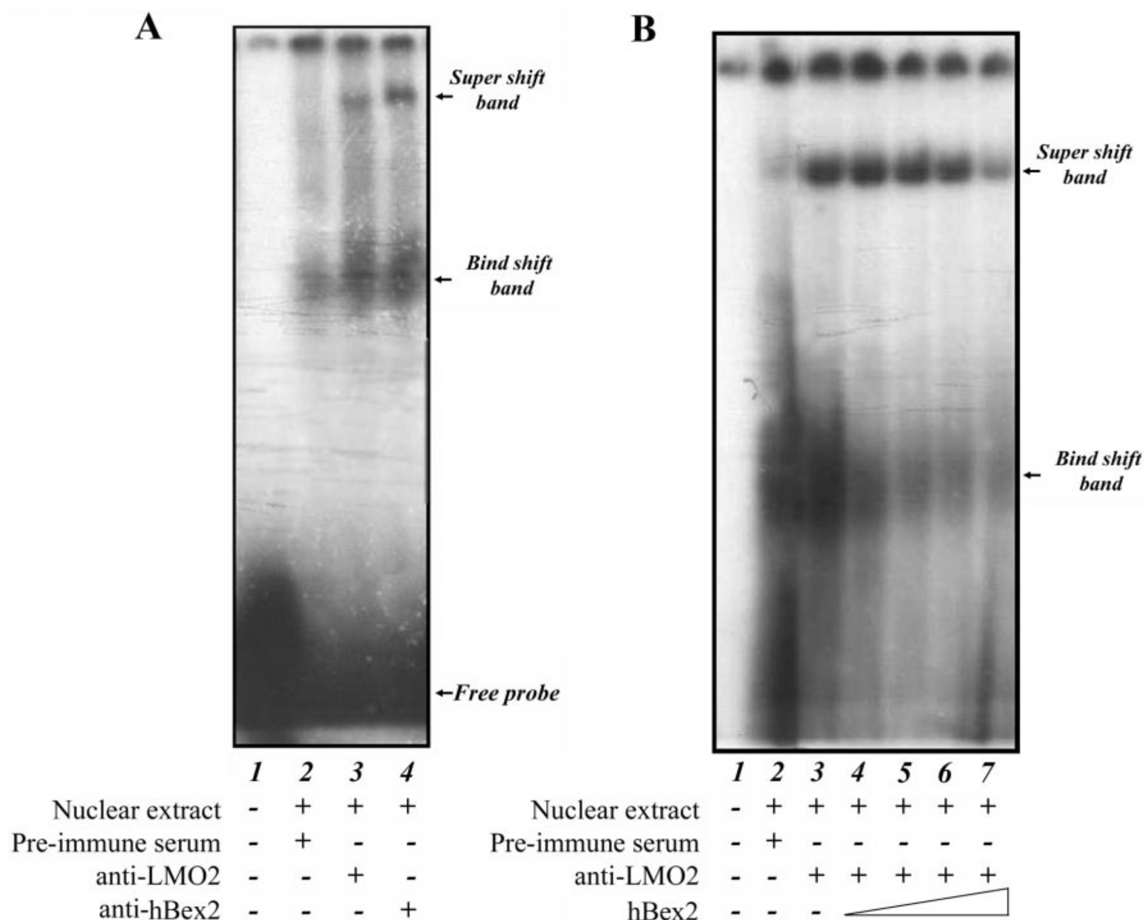



**Figure 4.** *hBex2* is part of a complex with *LMO2* that recognizes the E-box element. (**A**) EMSAs were performed with the $\gamma^{32}$-ATP-labeled oligonucleotides of the E-box sequence using the nuclear extract from M17 cells. Labeled probe was incubated with nuclear extract for 20 min at room temperature (lanes 2–4), then further incubated with pre-immune serum (lane 2), anti-*LMO2* anti-serum (lane 3) or anti-*hBex2* anti-serum (lane 4). The super-shift band and bind-shift band are indicated by arrows. (**B**) The specificity of the formation of bind-shift band and super-shift band by interaction between *hBex2* protein and its antibody. Purified *hBex2* protein was added into the binding assay to compete with the anti-*hBex2* Abs as follows: 0 µg (lane 3), 10 µg (lane 4), 20 µg (lane 5), 50 µg (lane 6) and 100 µg (lane 7). The competitive binding of the recombinant *hBex2* reduced the intensity of both the bind-shift band and the super-shift band.

shown in Figure 3, the endogenous *Bex2* and *LMO2* were primarily colocalized in the nuclei of human neuroblastoma M17 cells by double immunofluorescent staining. Similarly, the heterogeneously expressed FLAG-*hBex2* and dsRed-*LMO2* were also colocalized in the nuclei of transient transfected HeLa cells (Figure 3B). The nuclear localization of either protein does not seem to be facilitated by the overexpression of their binding partners (Figure 3C).

## *hBex2* increases the transcriptional activity of *LMO2*

Previous studies have suggested that *LMO2* can form a DNA-binding complex mediated by the E-box sequence within Class II bHLH transcription factors (29,30). We speculated that *hBex2* might be involved in the complex formation and play a role in transcriptional regulation. To test this hypothesis, we performed EMSAs using a polyclonal antiserum against *LMO2* and *hBex2*. As shown in Figure 4A, we found that anti-*hBex2* antibody could generate a super-shift band similar to that seen in the lane with anti-*LMO2* antibody. These data indicate that *hBex2* participates in the formation of a *LMO2*/E-box complex without interfering in the interaction of *LMO2* with other members of the complex that bind to the E-box motif. Furthermore, when the recombinant *hBex2* was used in the assay, it clearly blocked the shift in a concentration-dependent manner (Figure 4B), supporting the specificity of the antibodies for the interactions.

The transcriptional regulation activity of *LMO2* has been detected by using reporter genes containing either the E-box motif (31,32) or Gal4-binding sequences in which *LMO2* was a fusion protein with Gal4-BD domain (33). In order to examine whether the interaction between *LMO2* and *hBex2* affects the transcriptional activity of *LMO2*, we performed the luciferase reporter gene assay using the Gal4 system (25,33). cDNA plasmids for *GFP-hBex2* and Gal4-BD-*LMO2* were cotransfected into Cos-7 cells together with a plasmid for luciferase reporter gene driven by Gal4-binding enhancer. *GFP-hBex2* alone had no effect on the luciferase activity (Figure 5A, column 3), while *LMO2* activated the expression of the reporter gene (Figure 5A, column 4). When *hBex2* and *LMO2* were coexpressed, the luciferase activity was significantly increased compared with that in *LMO2* expression alone (Figure 5A, column 6). The *hBex2*-mediated up-regulation

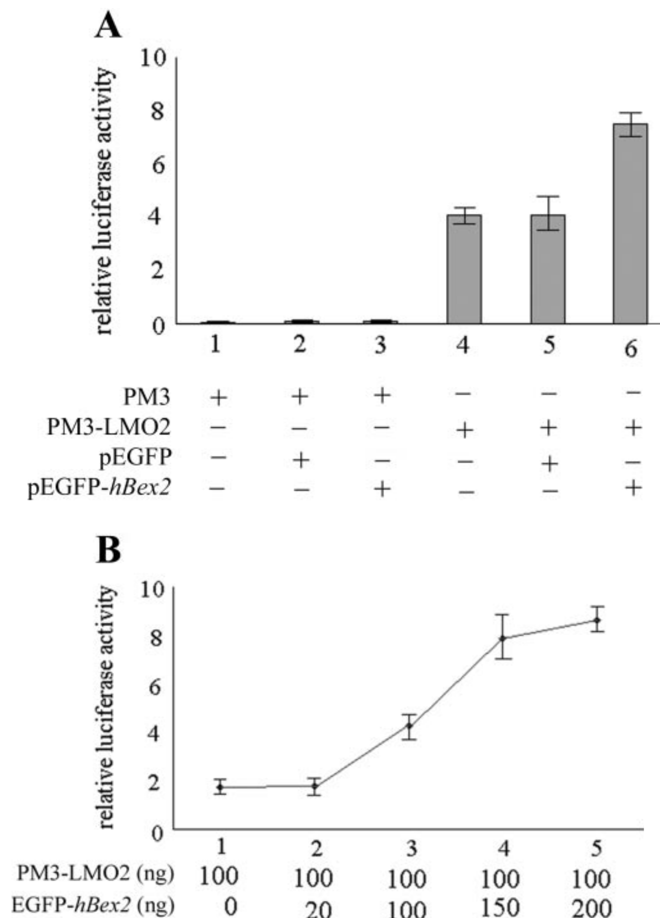



**Figure 5.** *hBex2* enhances the transcriptional activity of *LMO2*. (**A**) pEGFP-*hBex2* increases *LMO2*-induced luciferase activity in a mammalian GAL4 system. The reporter plasmid contains a GAL4-BD-binding motif within the promoter sequence upstream of the luciferase gene, allowing the binding of the *LMO2*-GAL4-BD chimeric protein. Cos-7 cells were transiently transfected with pGAL-LUC (firefly luciferase reporter gene plasmid) and TK (renilla luciferase expression plasmid) as reporter gene plasmids and expression gene plasmids including pM3-*LMO2* and pEGFP-*hBex2*. pM3 and pEGPF were used as control vector DNAs. The transcriptional activity of the reporter gene was measured by relative luciferase activity value, which is the value of firefly luciferase activity divided by the value of renilla luciferase activity. All indicated relative luciferase activity values represent the means of three independent transfections, and error bars indicate the standard deviations. (**B**) The effect of *hBex2* on *LMO2* activity is correlated with the amount of *hBex2* cDNA plasmid. Increased amounts of cDNA plasmids were used in the luciferase system as indicated from 0 to 200 ng.

of GAL4-BD-*LMO2*-dependent luciferase activity also correlates with the amount of *hBex2* cDNA used in transfection (Figure 5B).

### *LMO2* interacts with *NSCL1* and *NSCL2*

To further elucidate the mechanisms by which *hBex2* regulates *LMO2* function at the transcriptional level, we examined the interaction of *LMO2* with class II bHLH proteins during the formation of a DNA-binding complex. There are two class II bHLH proteins, SCL (also known as TAL-1) and *NSCL1*, which are known to interact with *LMO2* (25,29). Interestingly, *LMO2* interacts with the brain specific *SCL* homologue *NSCL1*, but showed no effect on their transcriptional activity

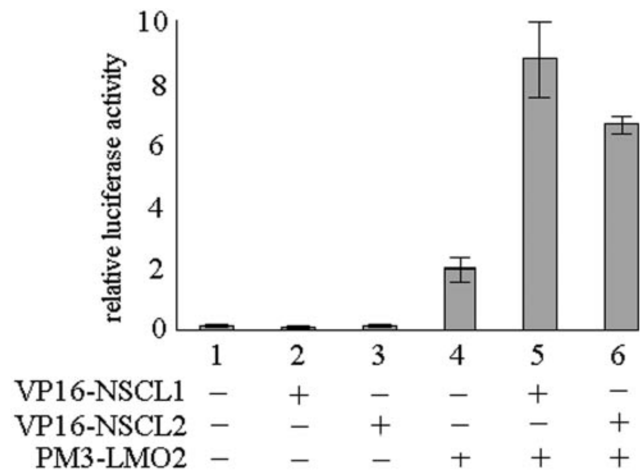



**Figure 6.** Functional analysis of interaction between *LMO2* and *NSCL1* or *NSCL2*. A mammalian two-hybrid system was used to detect the protein–protein interaction as indicated by luciferase activity assay. Cos-7 cells were transiently transfected with pGALLUC and TK as reporter plasmids along with expression plasmids as indicated. The interactions were measured by relative luciferase activity. All experiments were repeated more than three times with triplication for each experiment. Error bars indicate standard deviations.

(25). To test whether *LMO2* interacts with *NSCL2*, a *NSCL1* homolog with high sequence similarity (34,35) that regulates its transcriptional function, we performed a mammalian two-hybrid assay. Both pGAL4-BD-*LMO2* and pV16-NSCL2 fusion proteins were expressed in HeLa cells to examine their interaction as indicated by luciferase activity. As a control, pPM3-*LMO2* and pVP16-*NSCL1* were coexpressed and were found to induce luciferase activity, consistent with the previous report of their interaction (25) (Figure 6, column 5). Coexpression of GAL4-BD-*LMO2* and VP16-*NSCL2* significantly activated transcription of the reporter gene. This indicates that *NSCL2* is also a binding partner for *LMO2* (Figure 6, column 6). To test if *NSCL2* is part of the complex of *hBex2* and *LMO2 in vivo*, we performed coimmunoprecipitation with human fetal brain tissue. As shown in Figure 7A–C, *NSCL2* was coexpressed and coimmunoprecipitated with *hBex2* and *LMO2* in human fetal brain extracts.

### *hBex2* influences the transcriptional activity of *LMO2* and *NSCL2*

To further explore the physiological relevance of the interaction between *LMO2* and *NSCL2*, and the potential regulatory role of *hBex2* on the *LMO2*–*NSCL2* complex, we generated a reporter construct containing three tandem repeated E-boxes adjacent to the firefly luciferase gene. In the absence of *NSCL2*, neither *LMO2* alone, nor the coexpression of *hBex2* and *LMO2*, had any effect on the basal luciferase activity (Figure 7D, columns 3–4). *NSCL2*, but not *NSCL1*, was able to activate transcription of the reporter gene (Figure 7D, columns 5 and 8), consistent with the previous report that *NSCL1* was a relatively weak transcriptional activator (25). It has been reported that the enhancement or repression of class II bHLH proteins on transcriptional activity depends on both the target genes and the cellular context (32). Furthermore, *LMO2* was able to up-regulate *NSCL2*'s transcriptional activity (Figure 7D, column 10), but had no

A brain lysate
anti-NSCL2

B IP: anti-*hBex2* WB: anti-NSCL2

C IP: anti-LMO2 WB: anti-NSCL2

D

**Figure 7.** *hBex2* regulates *NSCL2* activity through formation of transcriptional complex. (**A**) Endogenous expression of *NSCL2* in human fetal brain to confirm the specificity of its polyclonal antibody. (**B** and **C**) Interaction of *hBex2* and *LMO2* with *NSCL2*, respectively, in human fetal brain tissue. The fetal brain tissue was used to immunoprecipitate the *hBex2* (B) or *LMO2* (C) with specific polyclonal Abs. Precipitate was blotted with anti-*NSCL2* Ab. (**D**) *hBex2* regulates E-box-dependent gene expression through *LMO2* and *NSCL2*. The reporter system used here contains three tandem repeat sequences of wild-type E-box motif adjacent to the firefly luciferase. The mutant reporter gene contains an inactive form of E-box motif as a control. Cos-7 cells were transiently transfected with reporter gene plasmids along with expression plasmids as indicated. A renilla luciferase plasmid was cotransfected in each transfection to evaluate transfection efficiency. All experiments were repeated more than three times with triplication for each experiment. Error bars indicate standard deviations.

effect on *NSCL1* (column 6). Importantly, coexpression of *hBex2* augmented *LMO2*'s effect on *NSCL2* (column 11). In contrast, expression of *hBex2* alone (data not shown) or together with *LMO2* did not affect *NSCL1*-dependent transcription (Figure 7D, column 7). This result suggests that the ability of *hBex2* to up-regulate the transcriptional activity of *NSCL2* may be mediated by *LMO2* within the complex.

### *hBex2* complex contains NL1/CLIM2/LDB1

*LDB1* is an important cofactor that is involved in the assembly of multiprotein complexes through interacting with the LIM-domain (hence *LDB1*—LIM domain-binding protein 1) (36). To examine if *LDB1* is part of the *hBex2* complex *in vivo*, we again performed coimmunoprecipitation with human fetal brain tissue. As shown in Figure 8A and B, both *LDB1* and *hBex2* were coexpressed in human fetal brain extracts and coimmunoprecipitated by either anti-*hBex2* or anti-*LDB1* antibody. Furthermore, we used antibodies to *LDB1* and *hBex2* for immunoprecipitation and confirmed that both *LMO2* and *NSCL2* are part of the complex now composed of at least *hBex2*, *LMO2, NSCL2* and *LDB1* (Figure 8C and D).

## DISCUSSION

In this report, we have presented evidence supporting the notion that human *Bex2* (previously named human *Bex1*) can specifically bind to *LMO2* and regulate the transcriptional activity of the E-box sequence-binding complex containing *LMO2, NSCL2* and *LDB1*. Using a yeast two-hybrid system, we first identified *LMO2* as a binding partner of *hBex2*. The interaction was characterized by both protein–protein interaction *in vitro* and nuclear colocalization. Deletion mutation analysis revealed that the interaction is a human *Bex2* specific regulatory mechanism. Immunoprecipitation experiments with human fetal brain tissue further showed the interactions among the three proteins. Using EMSA and luciferase activity assay, we have demonstrated that *hBex2* can physically interact with *LMO2* and enhances its transcriptional activity through the formation of an E-box-binding complex. We further examined the interaction of *LMO2* with the neuronal bHLH protein, *NSCL2*, and first showed that *NSCL2* serves as functional binding partner for *LMO2*. Our work also suggests that *hBex2* is able to augment the transcriptional activity of the target gene upon expression of all three proteins *in vitro*. Thus, *hBex2* may act as a transcriptional regulator to modulate the activity of *LMO2* and *NSCL2*, which play an important role in embryonic development.

### Functional interaction between *hBex2* and *LMO2*

The interaction of *hBex2* with LIM domain-only protein, *LMO2*, was demonstrated in four independent assays: yeast two-hybrid analysis, *in vitro* affinity chromatography, nuclear colocalization and immunoprecipitation *in vivo*. As a 'bridge molecule', *LMO2* plays a role in transcriptional regulation through its interaction with other proteins as a DNA-binding complex. There are two possible consequences of a direct interaction between *hBex2* and *LMO2*. One is that *hBex2* inhibits *LMO2* from binding to other proteins. The second is that *hBex2* facilitates the formation of an LMO2-containing protein complex. Our EMSA experiment supports the latter

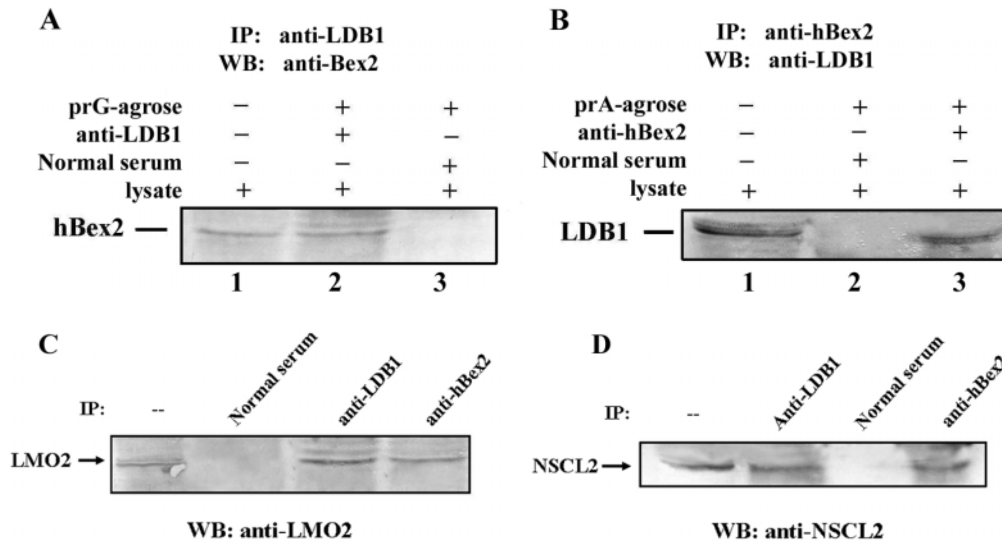



**Figure 8.** Interaction of *LDB1* with *hBex2* mediated complex *in vivo*. Coimmunoprecipitation was performed using nuclear extracts from 4-month-old human fetal brain. Antibodies against either *LDB1* (**A**) or *hBex2* (**B**) were used to precipitate the protein complex, followed by western blotting conversely with two antibodies. Similar coimmunoprecipitation was performed to confirm the interaction of *LMO2* (**C**) or *NSCL2* (**D**) with *LDB1* and *hBex2*.

possibility (Figure 4). Furthermore, *hBex2* up-regulates transcriptional activity of *LMO2* through protein–protein interaction (Figure 5). Our work has further unveiled a novel binding partner for LMO2, NSCL2. *hBex2* augmented *NSCL2*-dependent transcriptional activity, presumably through interacting with *LMO2* (Figure 7). *LMO2* contains two transactive domains in both the N- and C-terminus as well as two repressive LIM domains. The mechanisms by which *hBex2* up-regulates *LMO2* transcriptional activity may be a result of inhibiting the transcriptional activity of the complex through blocking both repressive LIM domains of *LMO2*, although our attempt in mapping the binding domain within *LMO2* did not yield any conclusive information (data not shown).

## Relevance of the novel *LMO2* complex in neuronal function

Studies on the interaction between *LMO2* and *SCL* in hematopoiesis has implicated a functional role of bHLH proteins in the LIM-modulation system (32,37). Since both *hBex2* and *LMO2* are expressed in areas of the brain where *SCL* is not detected, *NSCL1* and *NSCL2* became natural candidate bHLH proteins to examine their participation in the binding complex for the E-box motif (34,38,39). We have shown that *LMO2* does not affect *NSCL1*-dependent transcription, consistent with a previous finding (25). However, we cannot exclude a potential regulatory role of NSCL1 in an *LMO2* complex. The interaction between *LMO2* and *NSCL2* reported here suggests a neuronal-specific regulatory mechanism mediated by *LMO2* and its binding partners. Furthermore, our work and that of others also provide evidence indicating a potential role for *hBex2* complex in neurodevelopment. This evidence includes the high expression of *hBex2, LMO2* and *NSCL2* in brain; fetal brain tissue-derived yeast two-hybrid system used for detecting their interaction; and demonstration of interaction *in vivo* with human fetal brain tissue. However, further investigation should be performed to determine whether these proteins are colocalized in the same region or are differentiating neurons in embryonic brain tissue.

## Nuclear translocation of *hBex2* and its function

Consistent with bioinformatics predictions and physiological function, our study suggests that *hBex2* is localized to the nucleus in order to play a role in transcriptional regulation. Interestingly, previous work has suggested that members of *Bex* family may differ in their translocation between the cytoplasm and nucleus. For example, the interaction between *Bex* and OMP may induce the nuclear localization of *Bex* (11). The overexpression of human Bex2 in HEK293 cells has shown a cytoplasmic localization pattern (8). The discrepancy between our work and this reported result may be due to the use of different cell lines. Future work on subcellular localization of *hBex2* in neuronal tissue would be helpful to clarify this issue. In the case of *hBex3* or NADE, it is predominantly distributed in the cytoplasm for its role in the P75NTR signaling pathway (5). It has been suggested that the protein translocation of *Bex3* from the nucleus to the cytosol is required for this function (5). This translocation is determined by a NES motif, although mechanisms of regulating the subcellular trafficking remain elusive (5).

Finally, our study supports a working model in which *hBex2* regulates the transcriptional activity of oligomeric DNA-binding complex containing *LMO2* and *NSCL2* (Figure 9). We suggest that human *Bex2* binds to *LMO2* as part of the oligomeric complex that binds to the E-box motif-containing sequence. This interaction may regulate the transcriptional activity of *LMO2* through its binding to *NSCL2* within the DNA-binding complex. The stoichiometry of the complex is unknown, but the E-box-binding modules of the oligomeric complex are provided by at least one *NSCL2* molecule. Another half of an E-motif-binding protein could be either *NSCL2* or another closely related bHLH protein.

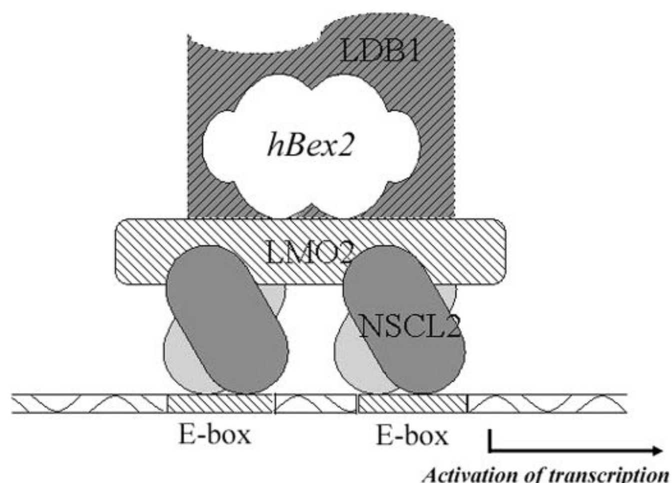



**Figure 9.** *hBex2* regulates the transcriptional activity of oligomeric DNA-binding complex containing *LMO2* and *NSCL2*. Human *Bex2* binds to *LMO2* as part of the oligomeric complex that binds to the E-box motif-containing sequence. This interaction may regulate the transcriptional activity of *LMO2* through its binding to *NSCL2* and *LDB1* during the formation of DNA-binding complex. The stoichiometry of the complex is unknown, but the E-box-binding modules of the oligomeric complex are provided by at least one molecule of *NSCL2*. The other half-E-motif-binding protein may also be *NSCL2*, or other bHLH proteins. *hBex2, LMO2, NSCL2* and *LDB1* form a complex at the E-box site and regulate specific gene expression.

Therefore, the complex containing *hBex2, LMO2, NSCL2* and *LDB1* will be formed at the E-box site to regulate specific gene expression.

## ACKNOWLEDGEMENTS

We thank Ms. Lesley Colgan for critical review of this manuscript. This work was supported by grants from the National Program for the Key Basic Research Project ('973'-No. 2001CB510206, 2005CB522507, 2004CB518604, 2004CB518604 and 2005CB522400), the National High Technology Research and Development Program (2004AA221060) and the National Sciences Foundation of China (No. 30421003 and 30430200). Part of the work was also supported by grant from NIMH to YL (R01 MH62092-01A1). Funding to pay the Open Access publication charges for this article was provided by National Program for the Key Basic Research Project, China.

*Conflict of interest statement*. None declared.

## REFERENCES

1. Pan,M., Yuan,J., Chen,B., Zhou,Y. and Qiang,B. (1997) [Differential display analysis of human fetal brain mRNAs and isolation of brain-specific novel ESTs]. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao*, **19**, 93–99.
2. Brown,A.L. and Kay,G.F. (1999) Bex1, a gene with increased expression in parthenogenetic embryos, is a member of a novel gene family on the mouse X chromosome. *Hum. Mol. Genet*., **8**, 611–619.
3. Rapp,G., Freudenstein,J., Klaudiny,J., Mucha,J., Wempe,F., Zimmer,M. and Scheit,K.H. (1990) Characterization of three abundant mRNAs from human ovarian granulosa cells. *DNA Cell Biol*., **9**, 479–485.
4. Faria,T.N., LaRosa,G.J., Wilen,E., Liao,J. and Gudas,L.J. (1998) Characterization of genes which exhibit reduced expression during the retinoic acid-induced differentiation of F9 teratocarcinoma cells: involvement of cyclin D3 in RA-mediated growth arrest. *Mol. Cell. Endocrinol*., **143**, 155–166.
5. Mukai,J., Hachiya,T., Shoji-Hoshino,S., Kimura,M.T., Nadano,D., Suvanto,P., Hanaoka,T., Li,Y., Irie,S., Greene,L.A. *et al*. (2000) NADE, a p75NTR-associated cell death executor, is involved in signal transduction mediated by the common neurotrophin receptor p75NTR. *J. Biol. Chem*., **275**, 17566–17570.
6. Koo,J.H., Saraswati,M. and Margolis,F.L. (2005) Immunolocalization of Bex protein in the mouse brain and olfactory system. *J. Comp. Neurol*., **487**, 1–14.
7. Mukai,J., Suvant,P. and Sato,T.A. (2003) Nerve growth factor-dependent regulation of NADE-induced apoptosis. *Vitam. Horm*., **66**, 385–402.
8. Alvarez,E., Zhou,W., Witta,S.E. and Freed,C.R. (2005) Characterization of the Bex gene family in humans, mice, and rats. *Gene*, **357**, 18–28.
9. Williams,J.W., Hawes,S.M., Patel,B. and Latham,K.E. (2002) Trophectoderm-specific expression of the X-linked Bex1/Rex3 gene in preimplantation stage mouse embryos. *Mol. Reprod. Dev*., **61**, 281–287.
10. Lee,N.H., Weinstock,K.G., Kirkness,E.F., Earle-Hughes,J.A., Fuldner,R.A., Marmaros,S., Glodek,A., Gocayne,J.D., Adams,M.D., Kerlavage,A.R. *et al*. (1995) Comparative expressed-sequence-tag analysis of differential gene expression profiles in PC-12 cells before and after nerve growth factor treatment. *Proc. Natl Acad. Sci. USA*, **92**, 8303–8307.
11. Behrens,M., Margolis,J.W. and Margolis,F.L. (2003) Identification of members of the Bex gene family as olfactory marker protein (OMP) binding partners. *J. Neurochem*., **86**, 1289–1296.
12. Koo,J.H., Gill,S., Pannell,L.K., Menco,B.P., Margolis,J.W. and Margolis,F.L. (2004) The interaction of Bex and OMP reveals a dimer of OMP with a short half-life. *J. Neurochem*., **90**, 102–116.
13. Yang,Q.S., Xia,F., Gu,S.H., Yuan,H.L., Chen,J.Z., Ying,K., Xie,Y. and Mao,Y.M. (2002) Cloning and expression pattern of a spermatogenesis-related gene, BEX1, mapped to chromosome Xq22. *Biochem. Genet*., **40**, 1–12.
14. McGuire,E.A., Hockett,R.D., Pollock,K.M., Bartholdi,M.F., O'Brien,S.J. and Korsmeyer,S.J. (1989) The t(11;14)(p15;q11) in a T-cell acute lymphoblastic leukemia cell line activates multiple transcripts, including Ttg-1, a gene encoding a potential zinc finger protein. *Mol. Cell. Biol*., **9**, 2124–2132.
15. Boehm,T., Foroni,L., Kaneko,Y., Perutz,M.F. and Rabbitts,T.H. (1991) The rhombotin family of cysteine-rich LIM-domain oncogenes: distinct members are involved in T-cell translocations to human chromosomes 11p15 and 11p13. *Proc. Natl Acad. Sci. USA*, **88**, 4367–4371.
16. Zeng,C., Justice,N.J., Abdelilah,S., Chan,Y.M., Jan,L.Y. and Jan,Y.N. (1998) The *Drosophila* LIM-only gene, dLMO, is mutated in Beadex alleles and might represent an evolutionarily conserved function in appendage development. *Proc. Natl Acad. Sci. USA*, **95**, 10637–10642.
17. Tsai,L.T., Bainton,R.J., Blau,J. and Heberlein,U. (2004) Lmo mutants reveal a novel role for circadian pacemaker neurons in cocaine-induced behaviors. *PLoS Biol*., **2**, e408.
18. Leuba,G., Vernay,A., Vu,D., Walzer,C., Belloir,B., Kraftsik,R., Bouras,C. and Savioz,A. (2004) Differential expression of LMO4 protein in Alzheimer's disease. *Neuropathol. Appl. Neurobiol*., **30**, 57–69.
19. Vu,D., Marin,P., Walzer,C., Cathieni,M.M., Bianchi,E.N., Saidji,F., Leuba,G., Bouras,C. and Savioz,A. (2003) Transcription regulator LMO4 interferes with neuritogenesis in human SH-SY5Y neuroblastoma cells. *Brain Res. Mol. Brain Res*., **115**, 93–103.
20. Hermanson,O., Sugihara,T.M. and Andersen,B. (1999) Expression of LMO-4 in the central nervous system of the embryonic and adult mouse. *Cell Mol. Biol. (Noisy-le-grand)*, **45**, 677–686.
21. Boehm,T., Spillantini,M.G., Sofroniew,M.V., Surani,M.A. and Rabbitts,T.H. (1991) Developmentally regulated and tissue specific expression of mRNAs encoding the two alternative forms of the LIM domain oncogene rhombotin: evidence for thymus expression. *Oncogene*, **6**, 695–703.
22. Foroni,L., Boehm,T., White,L., Forster,A., Sherrington,P., Liao,X.B., Brannan,C.I., Jenkins,N.A., Copeland,N.G. and Rabbitts,T.H. (1992) The rhombotin gene family encode related LIM-domain proteins whose differing expression suggests multiple roles in mouse development. *J. Mol. Biol*., **226**, 747–761.
23. Bulchand,S., Subramanian,L. and Tole,S. (2003) Dynamic spatiotemporal expression of LIM genes and cofactors in the embryonic and postnatal cerebral cortex. *Dev. Dyn*., **226**, 460–469.
24. Hinks,G.L., Shah,B., French,S.J., Campos,L.S., Staley,K., Hughes,J. and Sofroniew,M.V. (1997) Expression of LIM protein genes Lmo1, Lmo2,

and Lmo3 in adult mouse hippocampus and other forebrain regions: differential regulation by seizure activity. *J. Neurosci.*, **17**, 5549–5559.
25. Manetopoulos,C., Hansson,A., Karlsson,J., Jonsson,J.I. and Axelson,H. (2003) The LIM-only protein LMO4 modulates the transcriptional activity of HEN1. *Biochem. Biophys. Res. Commun.*, **307**, 891–899.
26. Wadman,I.A., Osada,H., Grutz,G.G., Agulnick,A.D., Westphal,H., Forster,A. and Rabbitts,T.H. (1997) The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *EMBO J.*, **16**, 3145–3157.
27. Retaux,S. and Bachy,I. (2002) A short history of LIM domains (1993–2002): from protein interaction to degradation. *Mol. Neurobiol.*, **26**, 269–281.
28. Zhou,H.J., Wong,C.M., Chen,J.H., Qiang,B.Q., Yuan,J.G. and Jin,D.Y. (2001) Inhibition of LZIP-mediated transcription through direct interaction with a novel host cell factor-like protein. *J. Biol. Chem.*, **276**, 28933–28938.
29. Grutz,G.G., Bucher,K., Lavenir,I., Larson,T., Larson,R. and Rabbitts,T.H. (1998) The oncogenic T cell LIM-protein Lmo2 forms part of a DNA-binding complex specifically in immature T cells. *EMBO J.*, **17**, 4594–4605.
30. Bao,J., Talmage,D.A., Role,L.W. and Gautier,J. (2000) Regulation of neurogenesis by interactions between HEN1 and neuronal LMO proteins. *Development*, **127**, 425–435.
31. Ono,Y., Fukuhara,N. and Yoshie,O. (1997) Transcriptional activity of TAL1 in T cell acute lymphoblastic leukemia (T-ALL) requires RBTN1 or -2 and induces TALLA1, a highly specific tumor marker of T-ALL. *J. Biol. Chem.*, **272**, 4576–4581.
32. Vitelli,L., Condorelli,G., Lulli,V., Hoang,T., Luchetti,L., Croce,C.M. and Peschle,C. (2000) A pentamer transcriptional complex including tal-1 and retinoblastoma protein downmodulates c-kit expression in normal erythroblasts. *Mol. Cell. Biol.*, **20**, 5330–5342.
33. Mao,S., Neale,G.A. and Goorha,R.M. (1997) T-cell oncogene rhombotin-2 interacts with retinoblastoma-binding protein 2. *Oncogene*, **14**, 1531–1539.
34. Lipkowitz,S., Gobel,V., Varterasian,M.L., Nakahara,K., Tchorz,K. and Kirsch,I.R. (1992) A comparative structural characterization of the human NSCL-1 and NSCL-2 genes. Two basic helix-loop-helix genes expressed in the developing nervous system. *J. Biol. Chem.*, **267**, 21065–21071.
35. Brown,L., Espinosa,R.III, Le Beau,M.M., Siciliano,M.J. and Baer,R. (1992) HEN1 and HEN2: a subgroup of basic helix–loop–helix genes that are coexpressed in a human neuroblastoma. *Proc. Natl Acad. Sci. USA*, **89**, 8492–8496.
36. Matthews,J.M. and Visvader,J.E. (2003) LIM-domain-binding protein 1: a multifunctional cofactor that interacts with diverse proteins. *EMBO Rep.*, **4**, 1132–1137.
37. Wadman,I., Li,J., Bash,R.O., Forster,A., Osada,H., Rabbitts,T.H. and Baer,R. (1994) Specific *in vivo* association between the bHLH and LIM proteins implicated in human T cell leukemia. *EMBO J.*, **13**, 4831–4839.
38. Begley,C.G., Lipkowitz,S., Gobel,V., Mahon,K.A., Bertness,V., Green,A.R., Gough,N.M. and Kirsch,I.R. (1992) Molecular characterization of NSCL, a gene encoding a helix–loop–helix protein expressed in the developing nervous system. *Proc. Natl Acad. Sci. USA*, **89**, 38–42.
39. Gobel,V., Lipkowitz,S., Kozak,C.A. and Kirsch,I.R. (1992) NSCL-2: a basic domain helix–loop–helix gene expressed in early neurogenesis. *Cell Growth Differ.*, **3**, 143–148.