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The posteriorizing gene *Gbx2* is a direct target of Wnt signalling and the earliest factor in neural crest induction

Bo Li*, Sei Kuriyama*, Mauricio Moreno and Roberto Mayor†

Wnt signalling is required for neural crest (NC) induction; however, the direct targets of the Wnt pathway during NC induction remain unknown. We show here that the homeobox gene *Gbx2* is essential in this process and is directly activated by Wnt/β-catenin signalling. By ChIP and transgenesis analysis we show that the *Gbx2* regulatory elements that drive expression in the NC respond directly to Wnt/β-catenin signalling. *Gbx2* has previously been implicated in posteriorization of the neural plate. Here we unveil a new role for this gene in neural fold patterning. Loss-of-function experiments using antisense morpholinos against Gbx2 inhibit NC and expand the preplacodal domain, whereas Gbx2 overexpression leads to transformation of the preplacodal domain into NC cells. We show that the NC specifier activity of *Gbx2* is dependent on the interaction with *Zic1* and the inhibition of preplacodal genes such as *Six1*. In addition, we demonstrate that Gbx2 is upstream of the neural fold specifiers *Pax3* and *Msx1*. Our results place *Gbx2* as the earliest factor in the NC genetic cascade being directly regulated by the inductive molecules, and support the notion that posteriorization of the neural folds is an essential step in NC specification. We propose a new genetic cascade that operates in the distinction between anterior placodal and NC territories.

KEY WORDS: Neural crest induction, Posteriorization, Gbx2, Wnt, Pax3, Msx1, Zic1, Genetic cascade, *Xenopus*

INTRODUCTION

Neural crest (NC) is a transient embryonic population, unique to vertebrates, which arises at the border of the neural plate and gives rise to a huge variety of cell types, such as neurons and glia in the peripheral nervous system, connective tissues of the craniofacial structures and pigment cells of the skin (Le Douarin and Kalcheim, 1999). This cell population is induced at the gastrula stage by signals produced by the mesoderm, neural plate and epidermis (Raven and Kloos, 1945; Selleck and Bronner-Fraser, 1996; Mancilla and Mayor, 1996; Marchant et al., 1998; Bonstein et al., 1998; Monsoro-Burq et al., 2003). Studies in chick, zebrafish and *Xenopus* embryos have identified many NC-inducing signals, including BMPs, Wnts, FGF, Notch and RA (reviewed by Basch et al., 2004; Steventon et al., 2005). From all these molecules the most compelling evidence exists for Wnt and BMP signals. Work in *Xenopus* and zebrafish shows that inhibition of BMP signalling combined with Wnt activation is required for NC induction (Saint-Jeannet et al., 1997; La Bonne and Bronner-Fraser, 1998), and in chick Wnt signalling has clearly been implicated in NC specification (Garcia-Castro et al., 2002). However, the mechanism by which BMP and Wnt signalling interact to induce NC is not clear. It has been proposed that Wnts act as posteriorizing signals during this process, but this remains controversial (Villanueva et al., 2002; Wu et al., 2005).

The anteroposterior axis of the neural plate is specified in a two-step process (Nieuwkoop, 1952) (reviewed by Gamse and Sive, 2000). First, all neural tissue is induced as anterior neural plate; this effect is driven by BMP antagonists. In the second step, called transformation or posteriorization, the anterior neural plate is posteriorized by Wnt, RA and FGF coming from the caudal end of the embryo. Interestingly, the same combination of signals induces NC (Villanueva et al., 2002). Moreover, only the posterior neural fold develops into neural crest, whereas the anterior neural fold gives rise to placodal tissue. In analogy with neural tissue, it has been proposed that initially all the neural fold is induced with an anterior (preplacodal) character dependent on attenuation of BMP signals (Brugmann et al., 2004; Litsiou et al., 2005; Ahrens and Schlosser, 2005). Subsequent posteriorization by Wnt, FGF and RA signals permits the development of NC tissue from the medioposterior neural-fold region (Papalopulu and Kintner, 1996; Villanueva et al., 2002). The anterior neural-fold area does not express NC markers, but can be transformed into NC by the activation of Wnt, FGF or RA (Villanueva et al., 2002). The role of posteriorization during neural crest induction has been recently challenged. It has been shown that activation of Wnt/β-catenin signalling leads to NC induction without affecting the anteroposterior axis of the neural plate, suggesting that NC induction is independent of posteriorization (Wu et al., 2005).

In order to explore further a possible role of posteriorization in NC induction, we have analysed the function of the homeobox gene *Gbx2*. *Gbx2* has been implicated in mediating caudalization of the neural plate by Wnt, FGF and RA, and in establishing the midbrain-hindbrain boundary (MHB) (Simeone, 2000).

Here we show that *Gbx2* is expressed in a broad ectodermal domain, including the area from which NC arises. Loss-of-function experiments show for the first time that *Gbx2* is required for NC induction, whereas gain-of-function experiments indicate that *Gbx2* induces NC genes. Our results indicate that *Gbx2* is essential for the anteroposterior division of the neural folds: expression of Gbx2 promotes posterior neural fold fate (NC) while inhibiting anterior neural fold fate (preplacodal). Surprisingly we found that, although controlled by the same signals, the anteroposterior specification of the neural folds is independent from the anteroposterior specification of the neural plate; reconciling previous discrepant results (Villanueva et al., 2002; Wu et al., 2005; Carmona-Fontaine et al., 2007). We show that the NC specification by *Gbx2* depends on its interaction with the neural fold gene *Zic1*. *Zic1* alone drives the expression of preplacodal genes, whereas a combination of *Zic1* and

Department of Cell and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK.

*These authors contributed equally to this work
†Author for correspondence (r.mayor@ucl.ac.uk)

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Gbx2 inhibits this fate and induces NC genes. Furthermore, we show that *Gbx2* is an immediate direct target of the Wnt signalling, and that β-catenin/TCF3 binds to the *Gbx2* promoter. Importantly *Gbx2* exerts its action upstream of the earliest expressed NC genes *Pax3* and *Msx1*. Our results place *Gbx2* on the top of the NC genetic cascade, being the earliest factor that is directly regulated by the NC inductive signals.

MATERIALS AND METHODS

Xenopus embryos, micromanipulation, whole-mount in situ hybridization and cartilage staining

Xenopus embryos were obtained as described previously (Gómez-Skarmeta et al., 1998) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Animal caps were performed as described by Mancilla and Mayor (Mancilla and Mayor, 1996). For in situ hybridization, antisense digoxigenin or fluorescein-labelled RNA probes were used. Specimens were prepared, hybridized and stained using the method of Harland (Harland, 1991), and NBT/BCIP or BCIP alone were used as substrates for the alkaline phosphatase. The genes analysed were *Snail2* [formerly *Slug* (Mayor et al., 1995)]; *FoxD3* (Sasai et al., 2001); *Gbx2* (von Bubnoff et al., 1996); *Otx2* (Blitz and Cho, 1995); *Sox2* (Kishi et al., 2000); *Six1* (Ghanbari et al., 2001); *Cpl1* (Richter et al., 1998); *En2* (Hemmati-Brivanlou et al., 1991); *Krox20* (Bradley et al., 1993); *Pax3* (Bang et al., 1997); *Msx1* (Suzuki et al., 1997) and cytokeratin *Xk81A* (Jonas et al., 1985); tbx2 (Takabatake et al., 2002). For cartilage staining, embryos were fixed at stage 45-47 and processed as previously described (Tribulo et al., 2004).

RNA synthesis in vitro and microinjection of mRNAs

All plasmids were linearized and RNA transcribed as described by Harland and Weintraub (Harland and Weintraub, 1985), using SP6 or T7 RNA polymerases, and the GTP cap analogue (New England Biolabs). After DNAse treatment, RNA was purified (BD Biosciences) and resuspended in DEPC-water for injection and lineage tracing. RNA was co-injected with FLDx (Molecular Probes) using 8-12 nl needles into 8- or 32-cell embryos as described by Aybar et al. (Aybar et al., 2003). The use of FDX as a lineage marker allows the identification of the injected side of the embryo but not the injected cells, as usually only the strongest fluorescent is visible and many times the precipitate from the in situ hybridization quenches the fluorescence. The constructs used were: DD1 (Sokol, 1996); Wnt8 (Sokol, 1996); β-catenin-GR (Domingos et al., 2001); Gbx2 (Glavic et al., 2002); Gbx2EnRGR (Glavic et al., 2002); tBR [truncated BMP receptor (Graff et al., 1994)]; Pax3 (Sato et al., 2005), HD-Msx1 (Tribulo et al., 2003); Msx1 (Tribulo et al., 2003); Zic1 (Nakata et al., 1998). Treatment with dexamethasone was performed as described previously (Tribulo et al., 2003).

Gbx2 morpholinos

A translation-blocking morpholino (ATG MO) and a splicing MO (Spl MO) against *Gbx2* were designed, and both of them produced the same neural crest phenotype. The sequence of the translation-blocking MO (ATG MO) is: 5′-GCTGAAAGGCTGCACTCATATAAGC-3′. A seven-mismatch Gbx2 mutant (7mutGbx2) was generated to perform the rescue of the ATG MO. To amplify the 7mutGbx2, the following primers were used: forward primer: 5′-GAATTCACC *ATGtcgGCtGCtTTcCAa* CCCCCTCTCATGAT-3′ and reverse primer: 5′-TTGACTCGAGTCAAGGTCTTGCTT-GCTCCAGC-3′. The italic letters show the morpholino target sequences. The lower case shows mismatching nucleotides inserted by PCR without changing amino acid sequences. The amplified Gbx2 open reading frame (ORF) cDNA was subcloned into pCS2+ expression vector. To design the splicing MO we cloned the first intron of the *Xenopus laevis Gbx2* gene. We predicted the structure of *X. laevis Gbx2* allele from that of *X. tropicalis Gbx2*. The primers for exon1 (5′-GAGAAGAAGGAAACAA-GACCTACAT-3′) and exon2 (5′-AGTTTGGCAGGAGATATTGTCATCT-3′) were designed to amplify 200 bp in the ORF of cDNA, and 1.7 kb in genomic DNA. The isolated genomic fragment was subcloned in pBluescript II vector, and the sequences of exon-intron boundary were read from several different clones. The sequence of the splicing MO is: 5′-GTGATGGTTGCTACACTTACCTAGA-3′ (Gene Tools). To check the efficiency of splicing MO, we performed RT-PCR with exon1-exon2 primers as described above. The shifted RNA was not amplified by RT-PCR using Go-Taq (Promega) (data not shown).

Chromatin immunoprecipitation and promoter analysis

Chromatin immunoprecipitation (ChIP) was based on the method described previously (Stewart et al., 2006). Briefly, 100 embryos from each stage (stages 11 and 14) were homogenized in 1 ml of nuclei extraction buffer, and fixed in 1% formaldehyde/nuclei extraction buffer A [0.5% TritonX-100, 10 mM Tris-HCl (pH 7.5), 3 mM calcium chloride, 0.25 M sucrose, 1 mM DTT, 0.2 mM PMSF, 1 tablet of complete mini (Roche applied science)] at room temperature for 15 minutes. The fixation was quenched by 0.125 M glycine for 5 minutes. The lysate was filtered with 100 μm cell strainer, centrifuged at 2000 ***g***, resuspended in 1 ml of nuclei extraction buffer C [10 mM Tris-HCl (pH 7.5), 3 mM calcium chloride, 2.2 M sucrose, 1 mM DTT, 0.2 mM PMSF], layered on 9 ml of nuclei extraction buffer B [0.5% TritonX-100, 10 mM Tris-HCl (pH 7.5), 3 mM calcium chloride, 2.2 M sucrose, 1 mM DTT, 0.2 mM PMSF], and the nuclei were extracted by ultracentrifugation (40,000 ***g***, 4°C, 3 hours). The nuclei were re-suspended in 3 ml SDS lysis buffer. Chromatin-protein complexes were sonicated in 15 ml Falcon tubes, and 100 μl aliquots were flash frozen in liquid nitrogen. The DNA concentration in one of the aliquots was determined after proteinase K treatment. ChIP was done with Protein G-dynabeads (Invitrogen). Protein G-dynabeads were blocked with 200 μg/ml salmon testis DNA (SIGMA) and 0.5 mg/ml BSA in ChIP buffer at 4°C overnight. All the samples were diluted by adding ChIP buffer and pre-cleared with Protein G-dynabeads. After pre-clear 20 μl of chromatin was taken as an input control. Antiserum against β-catenin (final 1:1000, anti-rabbit polyclonal, Calbiochem ab6302) was used to precipitate DNA-Tcf/Lef/β-catenin complex, and pan-cadherin type I antiserum was used as an IgG negative control (final 1:1000, anti-rabbit polyclonal, Sigma C3678). Antibodies were incubated with Protein G-dynabeads at room temperature for 30 minutes, and washed once. Then, 1 ml of 3-5 ng/μl (estimated) of pre-cleared chromatin was added to 40 μl slurry of antibody-Protein G beads complex. The eluted samples were treated with RNase A (RPA grade, Promega) at 37°C for 15 minutes and proteinase K (Sigma) and incubated at 65°C overnight. The DNA was purified using the MinElute Reaction Cleanup Kit (QIAGEN), and the target sequences were amplified by Go-Taq Flexi (Promega). PCR cycles; 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds, for 32-34 cycles. The primers used for ChIP are as follows: Gbx2-ChIP-U: 5′-GAATCCCAACCACAGAAGGA-3′, Gbx2-ChIP-D: 5′-GTAGGCACCAGAGCCACAGT-3′ (–346 to –75, 272 bp/32 cyc); Snail2A-3kb U: 5′-TGTTTCCATCCCAACACCTG-3′, Snail2A-3kb D: 5′-CTTTCACAGGCTGAGGCATT-3′ [–3005 to –2802, 204 bp/33 cyc, GenBank AF368040.1 (Vallin et al., 2001)]; Xen-2 GS-3 U: 5′-GGCT-CGAGGCTTCTCCCCAAGCCC-3′, Xen-2 GS-3 D: 5′-CCAAGCTT-GTCGCCTCCAGACCCG-3′ (33 cyc). This GS primer is used for amplification of competitor fragments in gel-shift assay as described in McGrew et al. (McGrew et al., 1999); alpha tubulin intron-U: 5′-TGAA-ACAGGAGCAGGAAAGC-3′, alpha tubulin intron-D: 5′-GCTCTGG-GTGGAATAACTGC-3′ (34 cyc) (Dunican et al., 2008).

For promoter analysis the 5′ upstream region of *Gbx2* was cloned and fused to GFP as a reporter gene. Different deletion constructs were injected into *Xenopus* embryos, and transient transgenics were generated using the Tol2 system (Kawakami, 2007).

RNA isolation from embryos and RT-PCR analysis

Total RNA was isolated from whole embryos or embryonic tissues after microdissection and cDNA were synthesized as previously described (Aybar et al., 2003). The primers used in this study were: Gbx2U 5′-AAACTGCCCACAAAGAGGAGGAC-3′, Gbx2D 5′-TGGTGTTGGC-TCCGTATGGCAAA-3′; Six1 (Pandura and Moody, 2000); ODC (Heasman et al., 2000); Pax3 (Hong and Saint-Jeannet., 2007); Nrp1 (Richter et al., 1990); Snail2 (Aybar et al., 2003); FoxD3 (Sasai et al., 2001); Sox9 (Monsoro-Burq et al., 2003); EpK (Jonas et al., 1989). PCR amplification with these primers was performed over 23-27 cycles and the PCR products were analysed on 1.5% agarose gels. As a control, PCR was performed with RNA that had not been reverse-transcribed to check for DNA contamination.

RESULTS

Gbx2 is expressed in the prospective neural crest

We hypothesized that the NC is induced in posterior ectoderm by Wnt signals (Fig. 1A) and that the posteriorizing activity of Wnt is mediated by *Gbx2* (Fig. 1C). An essential aspect of this proposal is the demonstration that *Gbx2* is expressed in the NC at the time of its induction (Fig. 1B). The expression of *Gbx2* was compared with the expression of the NC marker *Snail2*. At the late gastrula stage (stage 12), when NC induction takes place (Mancilla and Mayor, 1996), *Snail2* and *Gbx2* were clearly co-expressed by NC cells (Fig. 1D,E). This was further confirmed by double in situ hybridization for *Snail2* and *Gbx2* (Fig. 1F,G). Sections show that *Gbx2* expression was restricted to the ectoderm; it was not expressed in the mesoderm and it overlapped with *Snail2* expression (Fig. 1H-K). *Gbx2* was expressed in a wide domain of ectoderm, which included the prospective NC domain (Fig. 1L-O) and was absent only from the anteriormost region of the embryo (Fig. 1M-O). It should be noted that later in development, at the neurula stages, the expression of *Gbx2* was inhibited in the midbrain posterior domain (Glavic et al., 2002); however, at gastrula stages this area still expressed *Gbx2* and included the most anterior prospective NC cells (Fig. 1F,N) (Glavic et al., 2002). We noticed at the neurula stage a gap between the neural and epidermal expression of *Gbx2* (Fig. 1Q, arrow). Double in situ hybridization with *Snail2* showed that this gap in *Gbx2* expression was adjacent to the NC (Fig. 1R-T), corresponding presumably to the preplacodal region (PPR). In summary, *Gbx2* is expressed in the prospective NC territory at the time of NC induction (Fig. 1B).

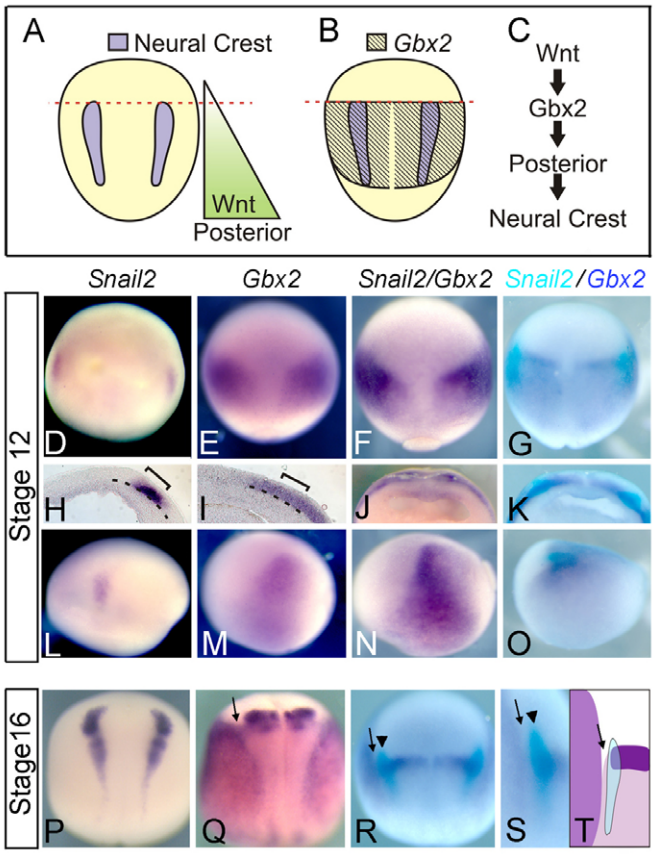

Fig. 1. *Gbx2* is expressed in posterior ectoderm that includes the prospective neural crest. (**A-C**) Hypothesis of neural crest (NC) induction by the posteriorizing activity of *Gbx2*. (**D-S**) In situ hybridization at the indicated stages for the indicated genes. (D-G) Dorsal view, anterior to the top. (H-K) Transverse sections. (L-O) Lateral view, anterior to the left. (P-R) Dorsal view, anterior to the top. Arrowhead, NC; arrow, gap in *Gbx2* expression. (S) Detail of the neural fold region in a lateral view, anterior to the top, midline to the right. (**T**) Summary of *Gbx2* and *Snail2* expression at stage 16. Anterior to the top, midline to the right. Different tones of purple denote different levels of *Gbx2* expression. Blue, NC.

Gbx2 is essential for NC induction

In order to analyze *Gbx2* function in NC induction, we undertook an MO loss-of-function approach. MOs were injected into one animal blastomere of eight-cell stage embryos. In all injected embryos shown in this work the injected side is shown to the right side. FLDx was used to recognize the injected side. Although injection of control MO did not affect the expression of *Snail2* (Fig. 2A,I), injection of splicing (Spl) – or translation (ATG) blocking – MOs produced a strong inhibition of this NC marker (Fig. 2B,C,I).

Injection of Gbx2 mRNA led to a modest but consistent expansion of *Snail2* expression (Fig. 2D); a similar expansion was observed when mRNA coding for Gbx2EnR [Gbx2 homeodomain fused to Engrailed repressor domain of *Drosophila* (Glavic et al., 2002)] or Gbx2 mutated in the MO-binding site were injected (Fig. 2F,H). Injection of Gbx2EnR or the mutated *Gbx2* mRNA were able to rescue the inhibition of *Snail2* expression produced by translation-blocking MO (Fig. 2E,G,I), showing its specificity.

To measure the efficiency of the MO treatment we analyzed the splicing of *Gbx2* when embryos were injected with control MO or Gbx2 splicing MO. A strong inhibition of *Gbx2* splicing was observed with the Spl MO (Fig. 2J). As the splicing and translational MO produced the same effect on NC induction we used the translational MO in most of the subsequent experiments (from now on referred to as Gbx2 MO).

As *Gbx2* is expressed in the neural plate and epidermis in addition to the NC, we performed targeted injections of Gbx2 MOs at the 32-cell stage into blastomeres fated to become neural plate, NC or epidermis (Fig. 2M-P). Only Gbx2 depletion in the neural crest was able to inhibit *Snail2* (Fig. 2L,O), and no effect was observed when injected into the neural plate (Fig. 2M,O) or epidermis adjacent to the NC (Fig. 2N,O).

Gbx2 is a direct target of Wnt signalling during neural crest induction

We previously hypothesized that one of the first steps in NC induction was posteriorization of anterior ectoderm by NC-inducing signals (Aybar and Mayor, 2002). As *Gbx2* has been involved in posteriorization, we propose that it could be one of the earliest factors activated in the NC induction cascade. Accordingly, we asked whether *Gbx2* is a direct target of NC-inducing/posteriorizing signals.

Activation of Wnt signalling by injection of Wnt8 or β-catenin-GR mRNA in the prospective NC region led to expansion of endogenous *Snail2* expression (Fig. 3A), whereas inhibition of Wnt signalling by expression of a dominant negative of Dsh (DD1) (Tada and Smith, 2000) led to NC inhibition (Fig. 3B) (DeCalisto et al., 2005). A similar expansion or inhibition of *Gbx2* was observed after activation or inhibition of the Wnt pathway (Fig. 3C,D), suggesting that NC induction by Wnt may be Gbx2 dependent. We tested whether *Gbx2* was a direct target of Wnt signalling during neural crest induction by inhibiting protein

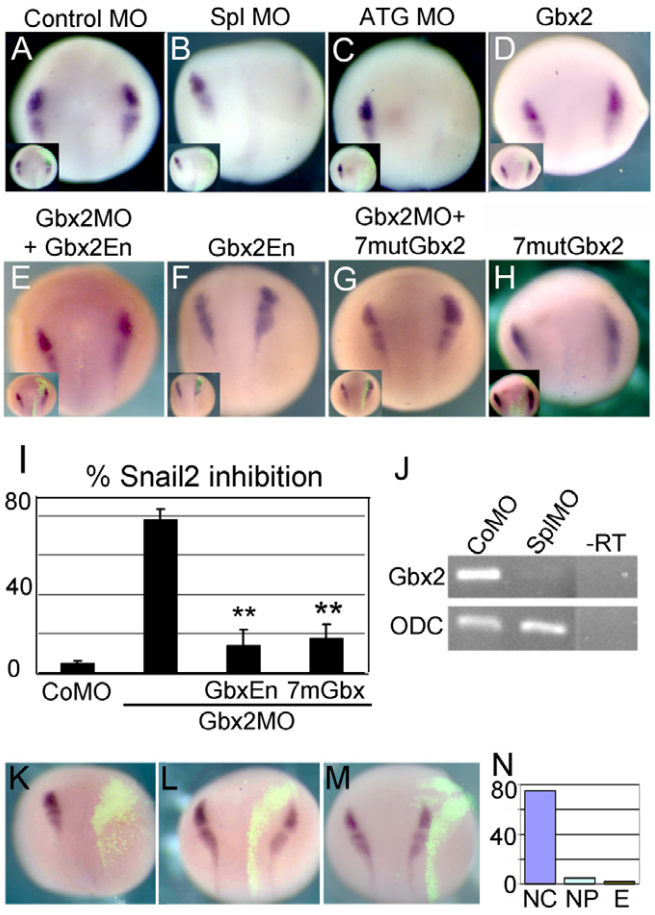

Fig. 2. *Gbx2* is required for NC induction. Embryos were injected in animal blastomeres at the eight-cell stage with the indicated MO, and the expression of *Snail2* was analysed between stages 12 and 13. In all the images, in situ hybridizations are shown in dorsal view with anterior to the top and the inset corresponds to the overlay of in situ hybridization and fluorescence to show the injected side to the right. (**A**) Control MO (20 ng). (**B**) Gbx2 splicing MO (20 ng). (**C**) Gbx2 translational MO (16 ng). (**D**) *Gbx2* mRNA (1 ng). (**E**) Gbx2 translational MO (16 ng) and Gbx2EnR-GR (1 ng). Dexamethasone was added at stage 10. (**F**) Gbx2EnR-GR (1 ng). Dexamethasone was added at stage 10. (**G**) Gbx2 translational MO (16 ng) and seven-mismatch (7mismatch) Gbx2 mRNA (1 ng). (**H**) 7mismatch Gbx2 mRNA (1 ng). (**I**) Summary of rescue experiment showing percentage of embryos with *Snail2* inhibition. ** $P<0.001$. (**J**) Efficiency of splicing MO. RT-PCR of embryos injected with 20 ng of control MO or 20 ng of Gbx2 splicing MO. Gbx2 and ODC were analysed. ODC, loading control. (**K-N**) Targeted injection of Gbx2 translational MO. A1, A3 or A4 blastomeres were injected with Gbx2 MO to target neural plate, NC or epidermis, respectively. (K) NC injection. (L) Neural plate injection. (M) Epidermis injection. (N) Summary of targeted injection, showing percentage of Snail2 inhibition after injecting in prospective NC, neural plate or epidermis. A minimum of 30 embryos was analysed in each experiment. E, epidermis; NP, neural plate.

synthesis in explants previously injected with a combination of *tBR* and *β-catenin-GR* mRNAs to induce NC. Injected animal caps were treated with the protein synthesis inhibitor cyclohexamide (CHX) at stage 11.5, and 30 minutes later β-catenin activity was triggered by addition of dexamethasone (DEX). After 2 hours of culture, mRNA was extracted and RT-PCR was performed for *Gbx2*, *Sox9* and *Snail2*.

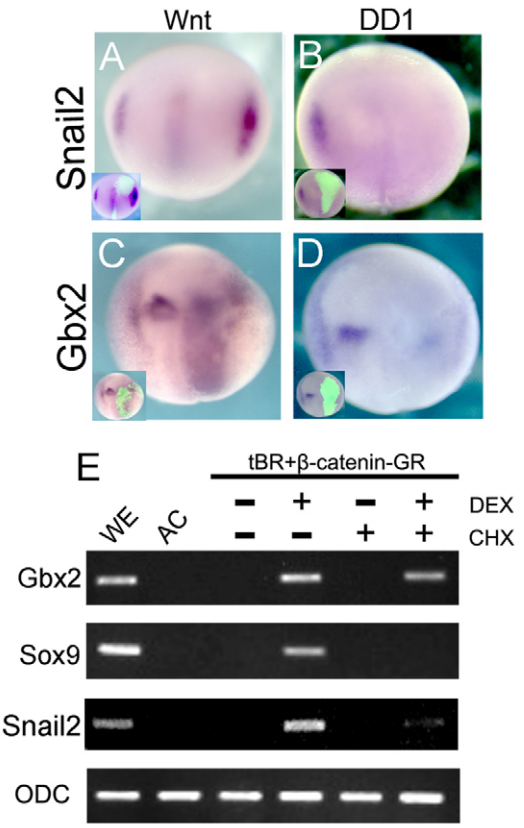

Fig. 3. *Gbx2* is a direct target of Wnt signaling. (**A**,**C**) Embryo injected in animal blastomeres of an eight-cell-stage embryo with 1 ng of *Wnt8* mRNA. (**B**,**D**) Embryo injected with 1 ng of *DD1* mRNA into animal blastomere of an eight-cell embryo. *Snail2* (A,B) or Gbx2 (C,D) expression was analysed at stage 12.5. (**E**) RT-PCR of animal caps analysing *Gbx2, Snail2* and *Sox9* expression. AC, animal cap; CHX, cycloheximide added 0.5 hours before DEX; ctBR, 1 ng of dominant-negative of BMP4 receptor; DEX, dexamethasone added at stage 11.5; ODC, loading control; WE, whole embryo.

NC induction by inhibition of BMP and activation of Wnt signalling was confirmed by *Sox9* and *Snail2* expression after DEX treatment (Fig. 3E, fourth lane). In the same situation, *Gbx2* expression was induced (Fig. 3E). However, treatment with CHX completely blocked *Sox9* expression (Fig. 3E, sixth lane), indicating that protein synthesis is being inhibited and that it is required for *Sox9* expression. A strong reduction in *Snail2* expression was observed, suggesting that most of the *Snail2* expression analysed at this early stage is not directly regulated by Wnt signalling, as it can be deduced from several reports that place *Snail2* downstream of *Pax3*, *Msx1* and *Zic1* (Kuo et al., 1998; Tribulo et al., 2003; Monsoro-Burq et al., 2005; Sato et al., 2005; Hong and Saint-Jeannet, 2007; Zhao et al., 2008) (and see Discussion). Interestingly, no effect on *Gbx2* expression was observed in the presence of CHX (Fig. 3E, sixth lane). These results show that once Wnt signalling is activated protein synthesis is not required for *Gbx2* upregulation, suggesting that *Gbx2* transcripts are directly regulated by β-catenin.

To further investigate whether *Gbx2* is directly regulated by Wnt signalling, we analysed the presence of TCF/LEF binding sites in the 5′ region of the *Gbx2* gene. We identified a region of 500 bp upstream of *Gbx2* that contains three (1-3) LEF/TCF consensuses (Fig. 3A), with site 1 as a perfect TCF/LEF consensus (Eastman and Grosschedl, 1999).

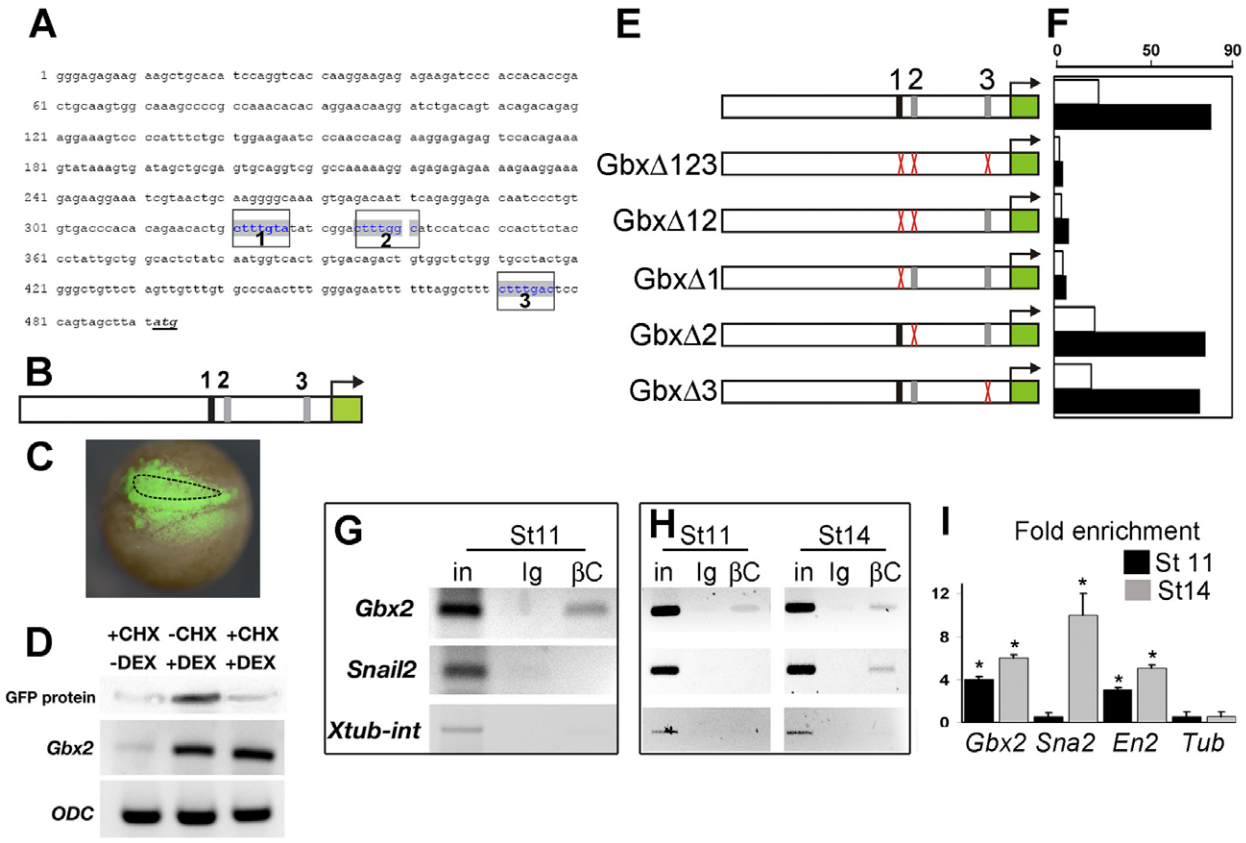

Fig. 4. Analysis of *Gbx2* regulatory region. (**A**) 5′ region upstream of *Gbx2*. Boxes 1 to 3 indicate TCF/LEF consensus binding sites. Start codon is underlined. (**B**) Fusion construct of *Gbx2* putative enhancer (containing the three TCF/LEF consensus sites) and GFP as a reporter gene. (**C**) Transgenic embryos were generated and GFP fluorescence was visualized. A stage 12 embryo is shown. Dashed line, prospective NC. (**D**) Animal caps taken from transgenic embryos injected with tBMPR and β-catenin-GRto were treated with cyclohexamide and 0.5 hours later with dexamethasone. GFP protein was assayed by western blot and *Gbx2* expression by RT-PCR. ODC, loading control. Note that CHX did not inhibit *Gbx2* transcription but inhibited GFP synthesis. (**E**) Deletion constructs used to develop transgenic embryos. Red X indicates deletion in the TCF/LEF binding site. (**F**) Percentage of transgenic embryos showing GFP expression. Embryos were injected with β-catenin-GR, dexamethasone was added at stage 11 and GFP fluorescence was analysed at stage 12. White bar, GFP fluorescence in absence of dexamethasone; black bar, GFP fluorescence in the presence of DEX. Note that the TCF/LEF binding site 1 is essential for *Gbx2* enhancer activity. (**G-I**) ChIP assay. (G) ChIP assay on chromatin of stage 11 embryos. (H) ChIP assay on chromatin of stage 11 and 14 embryos. (I) Quantification of fold enrichment of ChIP at stages 11 (black bars) and 14 (gray bars) for *Gbx2* and *Snail2*. *En2* enhancer, positive control; *Tubulin* intron, negative control. βC, β-catenin antibody; CHX, cyclohexamide; DEX, dexamethasone; *Gbx2*, *Gbx2* enhancer; Ig, IgG pan-cadherin antibody; in, input; *Snail2*, *Snail2* promoter; *Xtub-Int*, *Tubulin* intronl.

To analyse whether this 500 bp region of *Gbx2* works as a regulatory region, we fused it to GFP (Fig. 3B) and transient transgenic frogs were generated. Fluorescence analysis shows a clear expression of GFP in the NC region (Fig. 3C, dashed line), in addition to other domains that are consistent with *Gbx2* expression. This observation suggests that the 500 bp contains NC regulatory elements of *Gbx2*, and we will call it a *Gbx2* enhancer. To test whether the *Gbx2* enhancer responds directly to Wnt signalling, we induced NC in animal caps taken from transgenic embryos injected with a combination of tBMPR and inducible β-catenin, as described for Fig. 3E. The presence of GFP protein was analysed by western blot and *Gbx2* by RT-PCR (Fig. 4D). Activation of β-catenin by dexamethasone treatment led to a strong increase in GFP protein and *Gbx2* mRNA, but only GFP protein was sensitive to the protein synthesis inhibitor CHX (Fig. 4D, lane 3). This experiment suggests that the *Gbx2* enhancer is able to respond to Wnt signalling and that this response is direct, as no protein synthesis is required.

In order to identify which of the three putative TCF/LEF binding sites of the *Gbx2* enhancer were functional, we performed specific deletions for each of them (Fig. 4E) and tested their activity in transgenic embryos (Fig. 4F). Control embryos (Fig. 4F, white bars) or embryos injected with β-catenin (Fig. 4F, black bars) were co-injected with each deletion construct, and GFP fluorescence was analysed. We identified site 1 as essential for the β-catenin response in the NC cells (Fig. 4E,F).

Then, to analyse whether β-catenin/TCF interacts physically with site 1 of the *Gbx2* enhancer, we conducted ChIP assays using chromatin extracted from embryos at two different stages and an antibody against β-catenin. We found that at the early gastrula stage the antibody specifically precipitated site 1 of *Gbx2*, but did not precipitate the *Snail2* promoter or a *tubulin* intron used as control (Fig. 4G). These findings indicate that β-catenin/TCF does indeed associate physically with the putative *Gbx2* regulatory region at the early gastrula stage. We performed a second ChIP at neurula stage (stage 14) and found that in addition to *Gbx2* precipitation, the *Snail2* promoter was also precipitated (Fig. 4H). Quantification of these results (Fig. 4I), including *En2* enhancer as positive and *tubulin* intron as negative control, respectively, showed that β-catenin/TCF binds to the *Gbx2* gene during early NC induction, and later binds to the *Snail2* promoter,

as has been shown by Vallin et al., 2001 (Vallin et al., 2001). Taken together, these results demonstrate that *Gbx2* is a direct target of Wnt signalling.

Next we tested whether NC induction by Wnt signalling is *Gbx2* dependent. Injection of β-catenin-GR (DEX stage 11) led to an expansion of the NC markers *Snail2*, *Pax3* and *Msx1* (Fig. 5A-C). This effect was reverted in the presence of Gbx2 MO (Fig. 5D-F). Moreover, inhibition of the NC markers *Snail2*, *Pax3* and *Msx1* (Fig. 5G-I) by Wnt inhibition (Dsh dominant-negative DD1) was rescued by co-injection of Gbx2 mRNA (Fig. 5J-L). Taken together, these results show that NC induction by Wnt signalling is *Gbx2* dependent and makes Gbx2 the earliest target of this pathway during this process.

Gbx2 is upstream in the NC genetic cascade

In the genetic cascade controlling NC development *Pax3* and *Msx1* have been proposed as the first factors activated by secreted inducing signals (Mayor and Aybar, 2001; Meulemans and Bronner-Fraser, 2004; Steventon et al., 2005). As *Gbx2* is a direct target of the NC inducer Wnt, we asked whether *Pax3* and Msx1 expression are regulated *by Gbx2*. First, we observed that *Gbx2* was expressed earlier than *Pax3*/*Msx1* in the prospective NC area (Fig. 6A-F). Second, loss of *Gbx2* led to inhibition of *Msx1* and *Pax3* expression (Fig. 6G-J), but the effect was stronger when the embryos were analysed at the gastrula stage (stage 11; Fig. 6G,H), than at the neurula stage (stage 14, Fig. 6I,J). We also observed that overexpression of Gbx2 mRNA produced an expansion of NC markers (not shown). In order to determine when *Gbx2* was required for NC development, an inducible Gbx2 construct was used, in which Gbx2 was fused to the repressor domain of engrailed and to the glucocorticoid receptor elements, which can be exogenously activated (Glavic et al., 2002). Activation of Gbx2Enr-GR affected the expression of the NC marker *Snail2* only if it was activated before the end of gastrulation (stage 12), with no effect after stage 15 (Fig. 6K). Taken together, these results indicate that *Gbx2* is required for the early phase of NC specification and not for the late maintenance after stage 15 (Steventon et al., 2009).

Next we performed a series of epistasis experiments aimed to confirm that *Gbx2* was upstream of *Pax3* and *Msx1*. Inhibition of *Snail2* and *FoxD3* by Gbx2 MO (Fig. 7A,D) could be almost completely rescued by co-injection of *Pax3* or *Msx1* mRNA (Fig. 7B,C,E,F). However, inhibition of NC induction by loss of function of Pax3 (Fig. 7G) or Msx1 (Fig. 7J) could not be rescued by co-injection of *Gbx2* mRNA (Fig. 7H,I,K,L). Taken together, these results indicate that *Gbx2* is upstream of *Pax3* and *Msx1* in the NC genetic cascade.

Gbx2 works as a posteriorizing factor of the neural fold

Previous evidence demonstrates that *Gbx2* acts as posteriorizing factor in the neural plate (Wurst and Bally-Cuif, 2001). Therefore, we investigated whether *Gbx2* also interferes with anteroposterior patterning of the neural folds. As shown above, NC markers were inhibited by Gbx2 MOs (Fig. 8A,C) and slightly expanded by Gbx2 overexpression (Fig. 8B,D). Interestingly, these effects were accompanied by changes in the expression of the preplacodal marker *Six1*. *Six1* is normally expressed in the PPR of the anterior neural fold. Upon Gbx2 MO injection, *Six1* expression was expanded posteriorly (Fig. 8E), and shifted anteriorly if Gbx2 was overexpressed (Fig. 8F). *Gbx2* repression of *Six1* was further confirmed in explant experiments. An animal cap explant expressed *Six1* when BMP signalling was inhibited [injection of a dominant-negative form of the type II BMP receptor (tBR)] (Fig. 8G) (Brugmann et al., 2004). However, this effect was reverted when animal caps were co-injected with *Gbx2* mRNA (Fig. 8G, fourth lane). These experiments suggest that *Gbx2* is a repressor of the PPR.

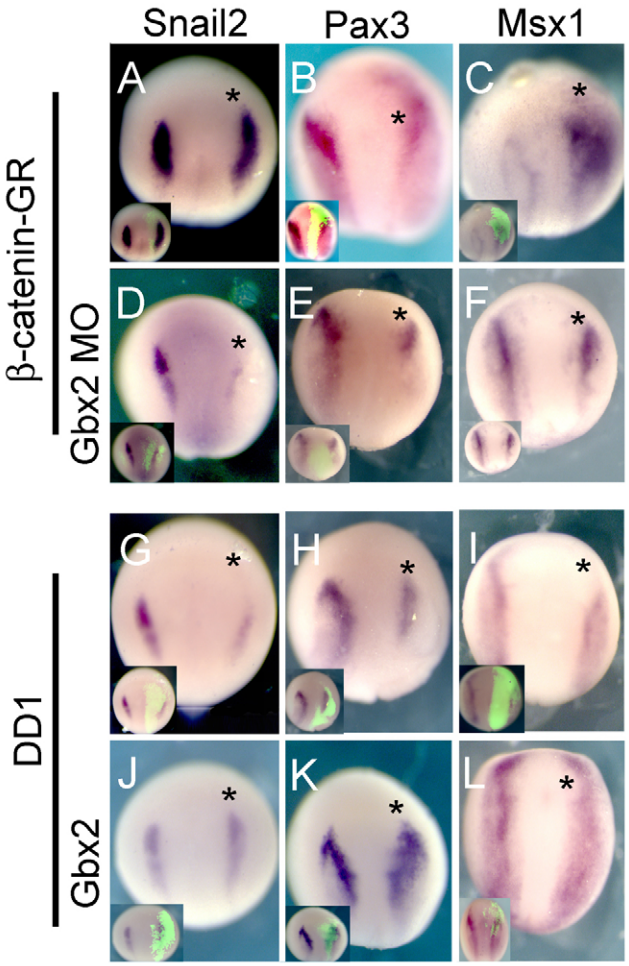

Fig. 5. NC induction by Wnt signalling is *Gbx2* dependent. Embryos were injected in animal blastomeres at the eight-cell stage as indicated. The expression of *Snail2*, *Pax3* and *Msx1* was analysed at stage 12. (**A-C**) β-catenin-GR (1 ng) and induced at stage 10 with DEX. Between 75 and 86% of NC expansion; *n*=153 embryos. (**D-F**) β-catenin-GR (1 ng), induced at stage 10 with DEX and 16 ng of Gbx2 MO. NC expansion was reduced to less than 2%; *n*=174. (**G-I**) 1 ng of Dsh dominant-negative DD1. Between 78 and 82% of inhibition of NC genes; *n*=124. (**J-L**) Dsh dominant-negative DD1 (1 ng) and 1 ng of *Gbx2* mRNA. NC inhibition was reduced to less than 2%; *n*=120.

These results indicate that *Gbx2* is a posteriorizing factor for the neural fold. We then asked whether the anteroposterior axis of the neural plate is also affected in these conditions. Surprisingly, we found that the anteroposterior patterning of the neural plate was normal. Regionally restricted neural plate markers such as *Cpl1*, *En2* and *Otx2* were expressed normally after similar Gbx2 MO or *Gbx2* mRNA injections (Fig. 8H-M; see Fig. S1A,C in the supplementary material). Only much higher levels of Gbx2 MO than those used here affect neural plate patterning (see Fig. S1B in the supplementary material). In addition, Gbx2 knockdown did not change the expression of the pan-neural plate marker *Sox2*, or the epidermal marker Keratin (Fig. 8N,O). In conclusion, reduction of Gbx2 produces an enlargement of

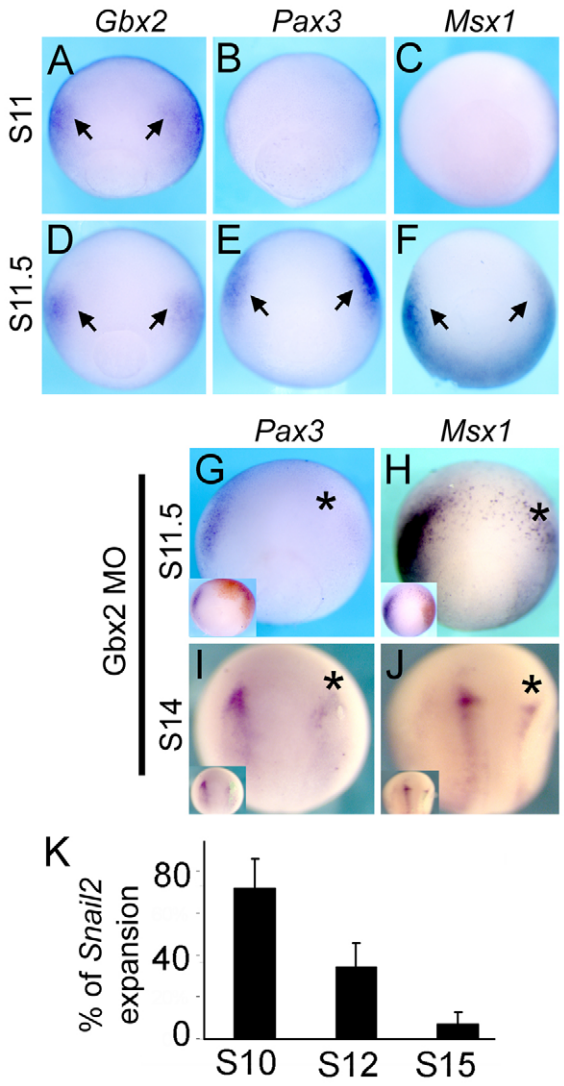

Fig. 6. Early requirement of *Gbx2* for NC induction. (**A-F**) In situ hybridization for *Gbx2*, *Pax3* and *Msx1* at the indicated stages. Note that only *Gbx2* is observed at stage 11. (**G-J**) Gbx2 MO was injected at the eight-cell stage and the expression of *Pax3* and *Msx1* was analysed at the indicated stages. Asterisks indicate the injected side (visualized in the inset). Note almost complete inhibition of *Pax3* and *Msx1* at stage 11.5 (43% of total and 31% of partial inhibition; *n*=83), and partial inhibition at stage 14 (70% of partial inhibition; *n*=110). (**K**) Percentage of embryos with defects in *Snail2* expression after activation of GbxEnR-GR with dexamethasone at the indicated stages (s).

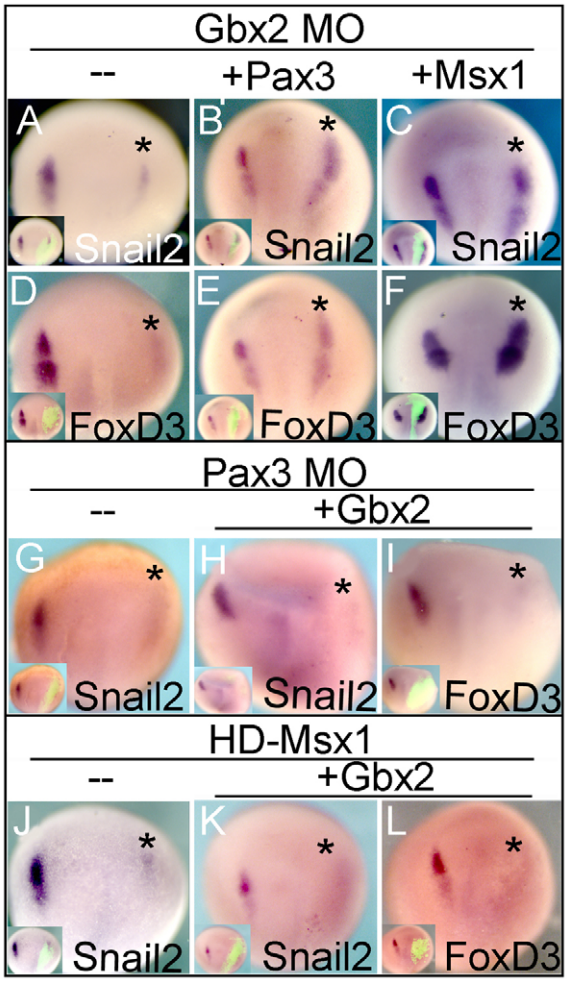

Fig. 7. *Gbx2* is upstream of *Pax3* and *Msx1* in the NC genetic cascade. Embryos were injected as indicated and the expression of the indicated genes was analysed at stage 12. (**A-F**) Gbx2 MO alone (16 ng) (A,D) or co-injected with 1 ng of *Pax3* mRNA (B,E) or 1 ng of *Msx1* mRNA (C,F). Eighty-one to 83% of NC inhibition by Gbx2 MO (*n*=178) was rescued to less than 1% of inhibition by co-injection with *Pax3* (*n*=78) or *Msx1* (*n*=69) mRNA. (**G-I**) Pax3 MO alone (20 ng) (G) or co-injected with 1 ng of *Gbx* mRNA (H,I). Seventy-eight percent (*n*=90) of NC inhibition by Pax3 MO was not rescued by *Gbx2* MRNA (75-79% inhibition; *n*=189). (**J-L**) Msx1 dominant-negative HD-Msx1 alone (1 ng) (J) or co-injected with 1 ng of *Gbx2* mRNA (K,L). Sixty-eight percent (*n*=89) of NC inhibition by HD-Msx1 alone was not rescued by *Gbx2* mRNA (73-77% of inhibition; *n*=124).

the PPR at the expense of NC cells (Fig. 8P), whereas Gbx2 overexpression has the opposite effect (Fig. 8Q). Moreover, the posteriorizing effect of *Gbx2* in the neural folds is independent of the anteroposterior patterning of the neural plate.

These observations suggest that *Gbx2* is involved in the anteroposterior differences of the neural fold, as the absence of *Gbx2* is required for specification of anterior placodes and its presence is needed for development of NC and probably posterior placodes that are at the same anteroposterior level as the NC, such as the otic placode. NC and placode derivatives were analysed after interfering with *Gbx2* activity. Gbx2 MO produced a strong inhibition of NC derivatives, such as cartilage and melanocytes (see Fig. S2A,B,E in the supplementary material), while that Gbx2 mRNA injection leads to inhibition of anterior placode such as lenses without major effect in more posterior placodes such as otic (see Fig. S2C,D in the supplementary material).

Gbx2 interacts with *Zic1* to induce NC

We have shown that *Gbx2* is essential to induce NC in the posterior neural folds. However, our data suggest that *Gbx2* is not sufficient for NC induction, as its overexpression only can expand the NC in the neural fold region. For this reason, we hypothesized that *Gbx2* may interact with another factor in the neural folds to induce NC. As there is evidence that attenuation of BMP signalling is necessary for neural fold specification (Marchant et al., 1998; Nguyen et al., 1998; Streit and Stern, 1999), we

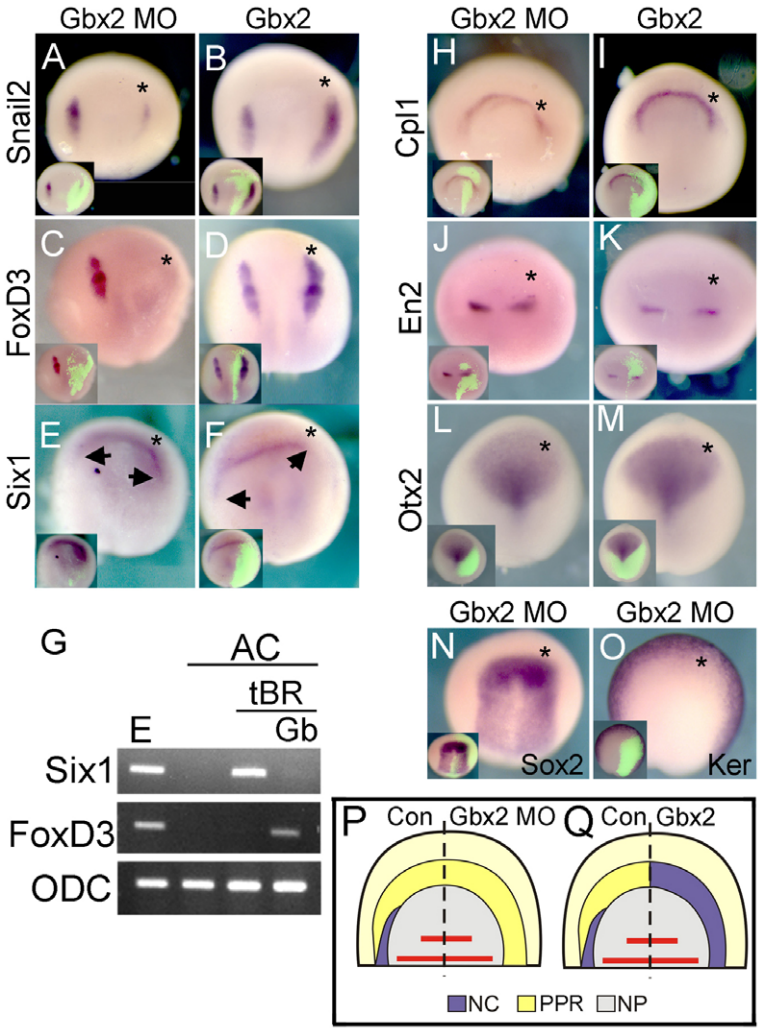

Fig. 8. *Gbx2* is required for the posteriorization of neural folds. Embryo injections and expression of indicated genes were analysed at stage 12. (**A**,**B**) *Snail2*. (**C**,**D**) *FoxD3*. (**E**,**F**) *Six1*. Arrows, posterior end of *Six1* expression. (**G**) RT-PCR of animal caps analysing *Six1* and *FoxD3* expression. (**H**,**I**) *Cpl1*. (**J**,**K**) *En2*. (**L**,**M**) *Otx2*. (**N**) *Sox2*. (**O**) *Keratin*. (**P**,**Q**) Summary phenotypes produced by Gbx2 MO (P) and *Gbx2* mRNA (Q). Anterior part of the embryo is represented, with left side as control and right-hand side as that injected. Percentages of phenotypes are shown in Fig. S1 in the supplementary material. A minimal of 35 embryos was analysed in each experiment. AC, animal cap; E, whole embryo; Gb, 1 ng of *Gbx2* mRNA; ODC, loading control; tBR, 2 ng of dominant-negative of BMP4 receptor.

reasoned that a factor induced by BMP attenuation could be required together with *Gbx2* for NC induction. Indeed, although NC markers were not induced in animal caps by BMP inhibition alone (Fig. 9A, third lane), inhibition of BMP in the presence of Gbx2 led to the induction of NC markers (Fig. 9A, fifth lane), similar to the effect obtained by BMP inhibition and Wnt activation (Fig. 9A, fourth lane). The latter was completely abolished when *Gbx2* was inhibited by Gbx2 MO (Fig. 9A, sixth lane). Taken together, our experiments indicate that attenuation of BMP induces a factor that in turn interacts with *Gbx2*, which is induced by Wnt signalling, to activate NC marker expression.

In order to identify the unknown factor, we tested several candidate genes expressed in the early neural folds for their ability to induce NC markers when co-expressed with Gbx2 in animal caps. From these, only *Zic1* was able to do so (Fig. 9B). In addition, we observed that the induction of Six1 by Zic1 (Fig. 9B, fourth lane) was completely inhibited by co-injection of Gbx2 (Fig. 9B, fifth to seventh lanes). These results suggest that *Zic1* is at least one of the factors that interact with *Gbx2* to induce NC in the posterior neural fold and to inhibit preplacodal fates.

DISCUSSION

We found that *Gbx2* plays an essential role in NC induction and propose a new genetic cascade that operates in the distinction between NC and anterior PPR. *Gbx2* resides at the top of the NC genetic cascade, being directly activated by the NC inducer Wnt. Several lines of evidence support this idea: (1) *Gbx2* is expressed in the prospective NC at the time of its induction; (2) inhibition of *Gbx2* expression leads to a complete loss of the NC region, concomitant with an expansion of the PPR; (3) *Gbx2* is a direct downstream target of Wnt/β-catenin signalling during NC induction; (4) *Gbx2* is upstream of the earliest transcription factors, *Pax3* and *Msx1*, in the NC genetic cascade; and finally, (5) interaction between *Gbx2* and *Zic1* is sufficient to induce NC and inhibit the PPR in animal caps.

We propose the following model of NC induction (Fig. 9C-F). Initially, a specific level of BMP activity induces *Zic1* along the entire neural plate border (Fig. 9C,F). The direct regulation of *Zic1* by BMP has been previously reported (Mizuseki et al., 1998; Rohr et al., 1999; Tropepe et al., 2006; Hong and Saint-Jeannet, 2007). Then, Wnt signalling induces directly the expression of *Gbx2* in the posterior region of the embryo (Fig. 9D,F). *Zic1* by itself induces *Six1* and specifies the anterior placodal domain next to the anterior neural folds (Fig. 9E,F), whereas *Zic1* in combination with *Gbx2* induces NC (and posterior placode, such as otic) in the posterior neural folds, where both genes are co-expressed (Fig. 9E,F). In addition, *Gbx2* inhibits *Six1* expression and the PPR (Fig. 9F). In summary, the presence of *Gbx2* at the neural plate border defines the region that becomes NC, and in its absence the region develops into anterior placode territory.

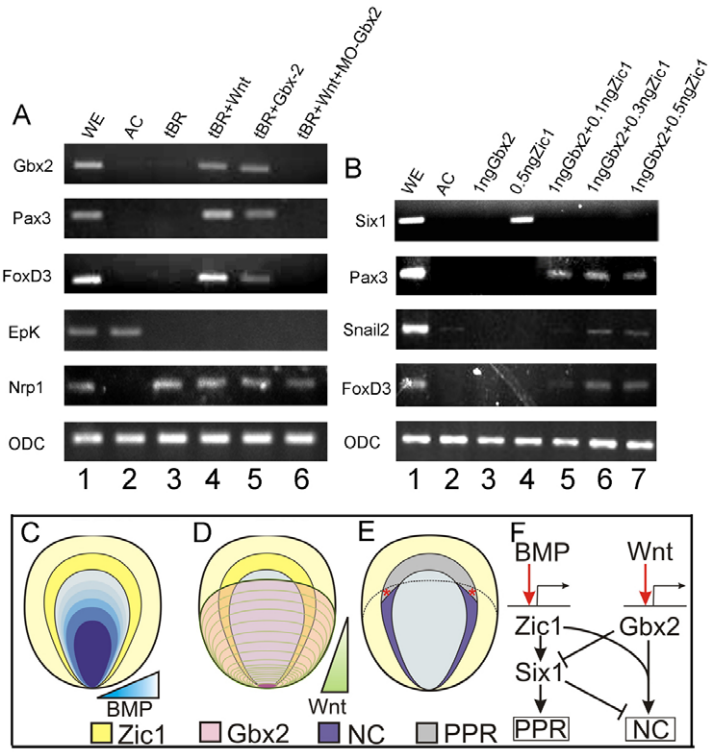

Fig. 9. Interaction between *Gbx2* and *Zic1* is sufficient to induce NC. (**A**,**B**) RT-PCR of animal caps analysing the expression of the indicated genes at the equivalent of stage 12. (A) *Gbx2* interacts with a factor induced by attenuation of BMP activity. (B) Interaction of *Gbx2* and *Zic1* induces NC. Animal caps expressing the indicated amounts of *Gbx2* and *Zic1* mRNA. (**C-F**) Model of NC induction by *Gbx2*. See text for details. Red asterisks in E indicate the placodes that are at the same anteroposterior level as the neural crest, such as the otic placode, and are dependent on *Gbx2* activity. (F) Network of genetic interactions that specify PPR and NC. Red arrows, direct regulation of *Zic1* by BMP (Tropepe et al., 2006) and of *Gbx2* by Wnt (this work). AC, animal cap; Gbx2, 1 ng of *Gbx2* mRNA; Gbx2 MO, 8 ng of Gbx2 MO; ODC, loading control; WE, whole embryo; Wnt8, 1 ng of *Wnt8* mRNA.

Gbx2 is upstream of the NC genetic cascade

Since the discovery of the transcription factor *Slug* (Nieto et al., 1994; Mayor et al., 1995), more than a dozen transcription factors required for NC development have been identified. Several attempts to organize these factors into a genetic cascade have been performed (Mayor et al., 1999; Mayor and Aybar, 2001; Meulemans and Bronner-Fraser, 2004; Steventon et al., 2005; Sauka-Spengler and Bronner-Fraser, 2008). From all these proposals the idea emerges that secreted molecules (BMPs, Wnts and FGF) activate the expression of a first set of transcription factors, among these *Pax3* and *Msx1*. Evidence that any of these factors, or any other known transcription factor, is directly regulated by the inducing signals has so far been lacking. Here we show for the first time that *Gbx2* is the earliest element of the cascade, is directly regulated by Wnt signals and participates in NC induction.

Several lines of evidence indicate that *Gbx2* is one of the most upstream factors in the NC genetic cascade. First, activation of *Gbx2* by Wnt signalling does not require protein synthesis. Second, *Pax3* and *Msx1*, usually described as the most upstream factors in the genetic cascade (Sauka-Spengler and Bronner-Fraser, 2008; Meulemans and Bronner-Fraser, 2004; Steventon et al., 2005), are downstream of *Gbx2*. Third, β-catenin/TCF/LEF factors seem to bind directly to the *Gbx2* enhancer. It is interesting to note that in our ChIP analysis we found that β-catenin/TCF/LEF does not bind to the *Snail2* promoter during early NC induction, but it binds at later stages. This observation is consistent with the *Snail2* promoter study that shows activity only after stage 14 (Vallin et al., 2001), and with several publications that clearly show *Snail2* as a factor downstream of *Pax3*, *Msx1* and *Zic1* during early NC induction (Kuo et al., 1998; Tribulo et al., 2003; Monsoro-Burq et al., 2005; Sato et al., 2005; Hong and Saint-Jeannet, 2007; Zhao et al., 2008). These data suggest that inductive signals such as Wnt are likely to work at different steps during NC development, such as in the early NC induction by controlling genes such as *Gbx2* and later during NC maintenance by controlling genes such as *Snail2*. This idea is consistent with the recent identification of an early induction and a later maintenance step for NC specification and with the demonstration that both steps are Wnt dependent (Steventon et al., 2009).

Gbx2 has been implicated in the formation of the MHB and in the posteriorization of the neural plate (Joyner et al., 2000). In this work we show that during gastrulation, when NC induction starts (Mancilla and Mayor, 1996), *Gbx2* is expressed in a broad domain of the ectoderm including the prospective NC. Later, at neurula stages, when some additional interactions are required to refine the position of the induced NC, *Gbx2* is absent from the most anterior NC domain. This loss of *Gbx2* is likely to be due to the repressor activity of *Otx2* in the MHB (Glavic et al., 2002). It is known that at the late gastrula/early neurula stages *Gbx2* and *Otx2* expression overlaps in a domain wider than the MHB (Garda et al., 2001; Glavic et al., 2002). At this stage, *Gbx2* is expressed in the entire NC population, and we show here that it plays an essential role in early NC induction. Gbx2 knockout mice exhibit defect in NC derivatives, such as heart and head (Byrd and Meyers, 2005). However, this phenotype has been explained as a defect in NC pattering or migration, instead of NC induction. Our results support a role for *Gbx2* in the very early specification of NC cells that explain the reported deficiencies in NC derivatives in these mutants.

Gbx2 works as a neural fold posteriorizing factor

Gbx2 has also been implicated in posteriorization of the neural plate, and its role in hindbrain specification has been widely studied (Millet et al., 1999; Hidalgo-Sanchez et al., 1999). Our results support a similar role for *Gbx2* as a neural-fold posteriorizing factor. We show here that anterior neural fold, defined by the expression of the preplacodal marker *Six1*, is transformed into NC by the action of *Gbx2*, corresponding to the posteriorization process (Nieuwkoop,

1952; Cox and Hemmati-Brivanlou, 1995; Villanueva et al., 2002). Furthermore, we show that *Gbx2* is sufficient to repress the expression of *Six1* and promotes the expression of the NC markers in vitro. Our results provide molecular support to the hypothesis that NC induction requires posteriorization of the neural fold (Aybar and Mayor, 2002; Villanueva et al., 2002). Surprisingly we found that neural fold and neural plate posteriorization can be dissociated: anteroposterior patterning of the neural fold is possible without affecting this process in the neural plate. Moreover, the neural fold seems to be more sensitive to the posteriorizing agents *Gbx2* and Wnt than neural plate (this work) (Carmona-Fontaine et al., 2007). The different sensitivity offers an explanation to apparently contradictory results. We have previously shown that addition of posteriorizing factors, such as Wnt, FGF and RA, transform the anterior neural fold into NC, supporting a role for posteriorization in NC induction (Villanueva et al., 2002). This has been challenged by the observation that activation of Wnt signalling induced NC markers without any effect in the anteroposterior axis of the neural plate, concluding that NC induction was independent of posteriorization (Wu et al., 2005). The results presented here show that NC induction is independent from neural plate posteriorization, and that posteriorization of the neural fold can be dissociated from posteriorization of the neural plate. We propose that NC induction at the anterior neural fold described by Wu et al. (Wu et al., 2005) is the result of neural fold posteriorization.

Gbx2 makes the distinction between NC and anterior PPR along the anteroposterior and mediolateral axis

The PPR forms in the outer border of the anterior neural fold and contributes to sense organs and cranial sensory ganglia (Streit, 2004; Schlosser, 2006). A recent model for its induction has been proposed, in which inhibition of Wnt signalling is an essential component (Brugmann et al., 2004; Litsiou et al., 2005). This model is consistent with our results showing that *Gbx2* is an inhibitor of PPR and is a Wnt target. Inhibition of *Zic1* leads to a depletion of the NC and PPR population (Hong and Saint-Jeannet, 2007). However, activation of Zic1 inhibits NC induction and promotes PPR development (Hong and Saint-Jeannet, 2007). The observation that activation and inhibition of *Zic1* leads to NC inhibition can be explained with our finding that *Zic1* plays a dual role that is context dependent. In the posterior region of the embryo, *Zic1* interacts with *Gbx2* to promote NC specification, whereas anteriorly *Zic1* induces *Six1* required for specification of the placode territory (Brugmann et al., 2004). In addition, different levels of *Zic1* may also be important to specify different territories, as it has been shown for the distinction between hatching gland, NC and placodes (Hong and Saint-Jeannet, 2007) and by the direct upregulation of NC markers (Sato et al., 2005).

We have previously shown that Dkk1 is required to inhibit NC specification at the anterior neural fold by inhibiting cell proliferation (Carmona-Fontaine et al., 2007). The data shown here suggest that in addition to cell proliferation Wnt signalling controls the specification of NC versus anterior placode. Interestingly, we have previously shown that placode markers, and not neural plate markers, are affected by Wnt/Dkk1 (Carmona-Fontaine et al., 2007). Taken together, these observations suggest that Dkk1 could work as an inhibitor of *Gbx2* at the anterior neural fold region.

We show that injection of *Gbx2* leads to a modest lateral expansion of NC. We propose that this expansion could be due to the overlap of *Gbx2* and *Zic1*. It is known that *Zic1* is expressed in a wider domain than the NC at the neural plate border that includes the PPR region (Hong and Saint-Jeannet, 2007), where *Gbx2* expression is downregulated (Fig. 1T). Thus, injection of *Gbx2* generates a new region of *Gbx2*/*Zic1* co-expression in the PPR domain. This new interaction leads to the small mediolateral expansion of the NC observed. It is interesting to notice that the same genetic network specifies both the anteroposterior and mediolateral border between NC and PPR.

The model presented here supports the idea that initial patterning of the ectoderm is determined by positional information dependent on two orthogonal gradients. A mediolateral BMP gradient specifies neural plate, neural plate border and epidermis, whereas an anteroposterior Wnt gradient divides the neural plate border into PPR and NC. Interestingly, a similar orthogonal double gradient of decapentaplegic and Wingless specify the anteroposterior and mediolateral axis of the *Drosophila* imaginal disc (Strigini and Cohen, 1999), suggesting that a BMP/Wnt orthogonal gradient is an ancient mechanism to generate positional information.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/19/3267/DC1

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