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Pax7 is regulated by *cMyb* during early neural crest development through a novel enhancer

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SUMMARY

The neural crest (NC) is a migratory population of cells unique to vertebrates that generates many diverse derivatives. NC cells arise during gastrulation at the neural plate border (NPB), which is later elevated as the neural folds (NFs) form and fuse in the dorsal region of the closed neural tube, from where NC cells emigrate. In chick embryos, *Pax7* is an early marker, and necessary component of NC development. Unlike other early NPB markers, which are co-expressed in lateral ectoderm, medial neural plate or posterior-lateral mesoderm, *Pax7* early expression seems more restricted to the NPB. However, the molecular mechanisms controlling early Pax7 expression remain poorly understood. Here, we identify a novel enhancer of *Pax7* in avian embryos that replicates the expression of Pax7 associated with early NC development. Expression from this enhancer is found in early NPB, NFs and early emigrating NC, but unlike Pax7, which is also expressed in mesodermal derivatives, this enhancer is not active in somites. Further analysis demonstrates that cMyb is able to interact with this enhancer and modulates reporter and endogenous early *Pax7* expression; thus, cMyb is identified as a novel regulator of *Pax7* in early NC development.

KEY WORDS: Neural plate border, Neural crest, PAX7, cMyb, Enhancer, Chick

INTRODUCTION

Neural crest cells (NCCs) are multipotent migratory cells unique to vertebrates, that appear at the border of the neural plate (NP) and non-neural ectoderm (NNE), incorporate into the neural folds (NFs)/dorsal neural tube and migrate in stereotypic patterns throughout the embryo. NCCs differentiate at their final locations, generating different cell types, including cranial bone and cartilage, adipose tissue, neurons and glia of the peripheral nervous system, and pigment cells (Le Douarin and Kalcheim, 1999). Defects in NCCs lead to neurocristopathies, which include melanomas, neuroblastomas, cleft lip/palate and Hirschsprung's disease (Bolande, 1997; Farlie et al., 2004; Etchevers et al., 2006).

NC development is marked by the participation of signaling molecules and transcription factors (TFs) (Stuhlmiller and García-Castro, 2012b; Milet and Monsoro-Burq, 2012). Analyses of expression profiles, gain and loss of function, and *cis*-regulatory elements have been integrated into models of regulatory interactions known as neural crest gene regulatory network (NC-GRN) (Mayor et al., 1999; Meulemans and Bronner-Fraser, 2004; Steventon et al., 2005; Sauka-Spengler and Bronner-Fraser, 2008; Betancur et al., 2010b). The NC-GRN proposes that signaling molecules, including Wnts, bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs), establish the NPB by inducing the expression of a group of TFs known as border specifiers (*Msx1/2*, *Pax3/7*, *Dlx3/5*). Signaling molecules and border specifiers direct the expression of the next TFs known as neural crest specifiers (*Snail1/2*, *Sox8/9/10*, *FoxD3*, *AP-2*, *Twist*), which further induce expression of genes responsible for migration and differentiation.

The expression of the cohort of NC-TFs and markers is regulated in turn by their DNA-regulatory elements and the TFs with which they interact. Analysis of these components is recent and scarce. In *Xenopus*, the homeobox gene *Gbx2*, an early NCC marker, is directly activated by Wnt signaling (Li et al., 2009), and AP-2 is directly activated by *Pax3* (de Crozé et al., 2011). In zebrafish, Pax3 and Zic1 enhancers responsive to BMP, FGF and Wnt signaling have been identified (Garnett et al., 2012), and AP2 has been shown to regulates *Sox10* (Van Otterloo et al., 2012), whereas in chick embryos, cranial NC expression of *Sox10* depends on the synergistic activities of *Ets1*, *Sox9* and *cMyb* (Betancur et al., 2010a). Overall, in amniotes, little is known about the regulatory elements and TFs responsible for the initial formation of NCCs.

Pax7 is a transcription factor with an eminent role in muscle development and homeostasis (Buckingham and Relaix, 2007; Wang and Rudnicki, 2012), and important functions for NC development in avians, rodents and humans (Basch et al., 2006; Butali et al., 2013; Mansouri et al., 1996). In chick embryos, *Pax7* is required for early NC development (Basch et al., 2006), and has been recently shown to undergo post-translational modification by the SUMOylation enzyme Ubc9. The SUMOylation of Pax7 enables Pax7 functions in NC development, C2C12 myogenic differentiation and transcriptional activation (Luan et al., 2013). Importantly, Pax7 is one of the earliest markers of the NPB, and unlike other early NPB markers, which are co-expressed in lateral ectoderm (*AP-2*, *Hairy2*, *Msx1/2*, *Dlx5*), medial NP (*Zic1/2*, *Gbx2*, *Meis3*) or the primitive streak/prospective mesoderm (*Msx1*, *Pax3*, *cMyc*), *Pax7* early expression becomes quickly restricted to the NPB (Basch et al., 2006; Otto et al., 2006; Khudyakov and Bronner-Fraser, 2009). This restricted expression offers a unique opportunity to advance our understanding of early events of NC formation. The expression of *Pax7* has been analyzed in different contexts in various organisms, and it is known to be responsive to BMP, Wnt and FGF signals (Liem et al., 1997; Liem et al., 2000; Litingtung and Chiang, 2000; Briscoe et al., 2001; Basch et al., 2006; Otto et al., 2006; Maczkowiak et al., 2010; Stuhlmiller and García-Castro, 2012a). Analysis of mouse genomic sequences upstream of *Pax7* identified a 10 kb region able to recapitulate endogenous *Pax7* expression in only a few migratory cranial NC cells and the roof plate (Lang et al., 2003). However, the

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regulatory elements and TFs that initiate the expression of *Pax7* at the NPB have not been reported.

Here, we identify a novel *Pax7 cis*-regulatory element that directs expression in the avian NPB, NFs and early migrating NC cells, similar to the endogenous *Pax7* expression associated with early NC. This element does not provide expression in later NC or in mesoderm, where endogenous *Pax7* is seen. Through mutagenesis and overexpression studies, we unveil *cMyb* as a regulator of this enhancer and of endogenous early *Pax7* expression. Our study is the first to demonstrate the ability of *cMyb* to regulate a border specifier directly during NC development and thus proposes a fundamental role for *cMyb* during the early phases of the NC-GRN.

MATERIALS AND METHODS

Sequence conservation and TF-binding analysis

Sequence conservation was determined using VISTA Browser (Visel et al., 2007), over a 50 bp window with 70% conserved identity. Putative TF-binding sites were identified using the JASPAR core vertebrate database (Bryne et al., 2008).

Plasmid constructs

Enhancer constructs were PCR amplified (DNeasy Kit, Qiagen) from genomic chick or human DNA (a gift from J. Noonan, New Haven, CT, USA), and cloned into ptkEGFP (a gift from H. Kondoh, Osaka, Japan) or ptkmCherry (a gift from D. Meulemans, Boulder, CO, USA) using *Kpn*I and *Xho*I. pRFP was generated by removing the miRNA cassette from pRFPRNAi (ARK Genomics). Deletion constructs were generated by first amplifying each end with primers containing 15 bp of internal overlapping sequence followed by fusion by another round of PCR with each end fragment as a template and the original P7Ur8 primers. pCIR and cMyb pCIR overexpression constructs were donated by T. Sauka-Spengler (Oxford, UK). cMyb was blunt cloned into pCIG-*Sma*I. Dominant-negative cMyb (*DNcMyb*) was generated by PCR amplification of the DNA-binding domain, adding *Sma*I and *Xba*I sites to clone into pCIG. pcDNA, ETS1-pcDNA and Klf4, were gifts from C. Vercamer (Lille, France) and W. Lu (USC, Los Angeles, USA). Zeb1 and Klf4 pCDNA constructs were generated by Gateway cloning into pDEST40 (Invitrogen). Human full-length cDNA clones were used as PCR templates, provided by OpenBioSystems (clone IDs: Zeb1, 40036600; Klf4, 5111134). Sequences of all primers are listed in supplementary material Tables S2-S5.

Electroporation

Chick embryos (Hardy's Hatchery, Massachusetts, USA) were staged (Hamburger and Hamilton, 1951) and immobilized in filter paper for early culture (EC) (Chapman et al., 2001). DNA for electroporation was used at the following concentrations: ptkEGFP constructs, 750 ng/μl; pRFP, 50 ng/μl, all pCIR and pCDNA constructs, 1 μg/μl. Constructs were mixed with 0.01% Fast Green and injected between the vitelline membrane and the epiblast of HH3-4 embryos. Electroporation was carried out in Howard Ringers Solution (HR) in chambers containing a platinum plate below the embryo while holding a platinum electrode above, with five pulses of 6.5V (50 ms on, 100 ms off). Embryos were cultured at 38°C for 16-24 hours, fixed in freshly thawed 4% (w/v) paraformaldehyde for 30-45 minutes at room temperature, and rinsed in 0.1% Tween/PBS.

In situ hybridization and immunohistochemistry

Whole-mount immunofluorescence and *in situ* hybridization of *Pax7* were performed as previously described (Basch et al., 2006). *cMyb* probes were generated from a full-length clone in pBS2SK+ vector (XhoI, T3; 1930 bp, hydrolyzed with bicarbonate), and a 429 bp terminal fragment (*Bgl*II, T3). Gelatin cryosections (8-12 μm, Leica CM1900 Cryostat), washed in warm 0.5× PBS, immersed in PBS and permeabilized in 0.1% Tween/PBS. Sections were blocked with 4% FBS before adding anti-Pax7 AB (1:50 mIgG1, Developmental Studies Hybridoma Bank). Secondary antibodies used were of specific isotypes conjugated to Alexa 568 or Alexa 488 (1:2000) (Invitrogen). Nuclei were stained with 0.5 mg/ml DAPI, and slides were coverslipped in Vectashield (Vector Laboratories).

Images were acquired using a SPOT SE camera and software using a Nikon Eclipse 80i microscope and processed in Adobe Photoshop.

Site-directed mutagenesis

Site-directed mutagenesis was performed on P7eNPBE ptkEGFP using the standard protocol from QuikChange II Site-Directed Mutagenesis Kit (Stratagene), except PCR products were purified using QIAquick-PCR purification columns (Qiagen) prior to transformation.

EMSA

Protein extracts were produced using EasyXpress Protein Synthesis Kit (Qiagen). DNA probes (*cMyb* wild-type sense, 5′-AAGAGCCAACAG-CCCTCCCT; *cMyb* wt antisense, 5′-AGGGAGGGCTGTTGGCTCTT; mutated M2, 5′-AAGAGCCACACTAACTCCCT and 5′-AGGGAGTT-AGTGTGGCTCTT) were biotinylated (Biotin 3′ End Label Kit, Thermo Fisher Scientific), annealed at 98°C and slowly cooled to room temperature. EMSAs were performed using the LightShift Chemiluminescent-EMSA Kit (Thermo Fisher Scientific). Protein extracts (1/10 dilution) were mixed with 20 fmol labeled probe, 1× binding buffer (Thermo Fisher Scientific), 1 μg/ml salmon sperm DNA, 50% glycerol, 0.05% NP-40, 30 mM KCl, 5 mM $MgCl_2$ and 1 mM EDTA, and incubated at 4°C for 20 minutes. Competition assays with unlabeled competitor probes (100-400×) added sequentially were incubated as above.

RESULTS

Identification of putative enhancers of early Pax7 expression

In the chick embryo, *Pax7* expression is restricted to the NPB and early NC (Basch et al., 2006; Otto et al., 2006; Khudyakov and Bronner-Fraser, 2009). *Pax7* can be identified in late gastrula/early neurula stages emanating from the primitive streak, posterior to the node, in an anterior-ward diagonal pattern (Fig. 1F). At stages 6-7, *Pax7* is expressed in the NPB, in a broad domain excluded only from the anteriormost region of the NP (Fig. 1G). By stage 8, in addition to NFs and NPB, *Pax7* is also expressed in somites (Fig. 1H). Positive *Pax7* signal is detected in cranial dorsal neural tube and migrating NC at stage 10, and posterior signal (vagal, and trunk) is maintained in NFs, NPB and somites through stage 12 (Fig. 1I). Murine and human *Pax7* expression is also associated with early neural crest lineages (Jostes et al., 1990; Mansouri et al., 1996; Betters et al., 2010; Murdoch et al., 2012), and regulatory sequences controlling later *Pax7* expression have been identified (Murmann et al., 2000; Syagailo et al., 2002; Lang et al., 2003). However, no enhancer elements or TFs directly controlling its expression in the NPB/NC have been reported.

To identify putative enhancers replicating the early expression of *Pax7* in chick embryos, we focused on the genomic region upstream of *Pax7* transcriptional start site. We avoided the most proximal region, which displays considerable homology, because it failed to reveal early NPB activity in the mouse (Lang et al., 2003). Using VISTA browser (Visel et al., 2007), we identified sequences conserved between chick, human and mouse in a ~6 kb region of genomic DNA beginning ~10 kb upstream of chick *Pax7* (Fig. 1A). Enhancers are generally identified in conserved sequences; however, growing evidence advocates for conservation of regulatory function of genomic regions across species without sequence conservation or even common TF-binding sites (Ludwig et al., 2000; Romano and Wray, 2003; Ludwig et al., 2005; Fisher et al., 2006; Hare et al., 2008; Weirauch and Hughes, 2010; Swanson et al., 2011). Additionally, different species display slight variations in *Pax7* expression. Therefore, we aimed to analyze conserved and non-conserved sequences throughout the 6 kb region. To achieve this, we cloned 12 regions, covering 5870 bp (97.8%) of the targeted territory, and ranging in size from ~250 bp to ~700 bp

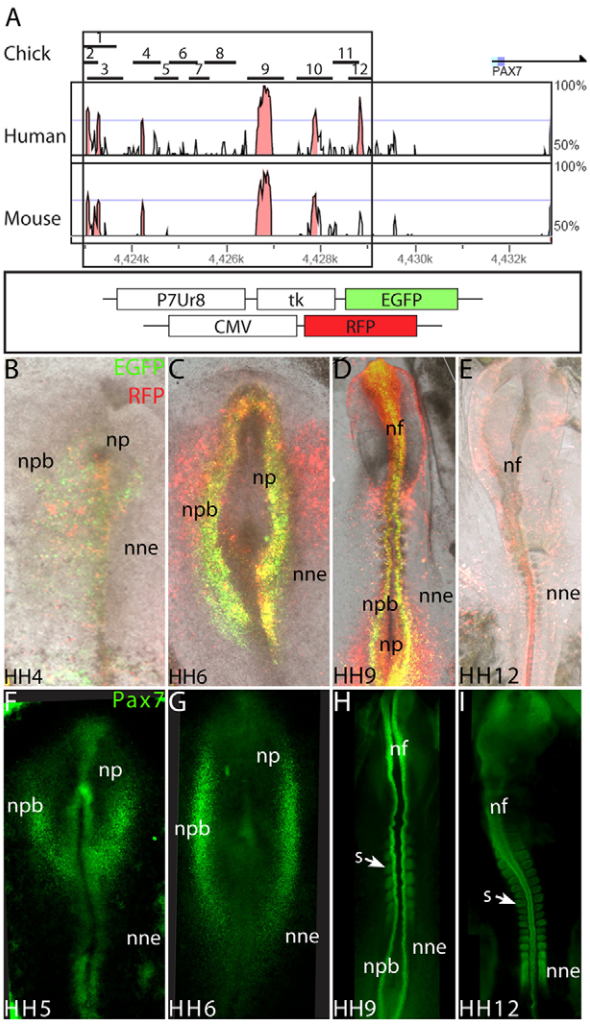

Fig. 1. Enhancer P7Ur8 activates reporter expression in a neural crest-like pattern. (**A**) VISTA plot of 12 kb from chicken chromosome 21, showing the percent identity (*y*-axis) between chicken-human (top) and chicken-mouse (bottom) sequences [conserved (>50% identity) non-coding peaks are highlighted in pink]. Position and relative size of 12 Pax7 upstream regions (P7Ur1 to P7Ur12) tested are numbered at the top of the plot. Each region was cloned in front of a weak promoter driving EGFP, and was co-electroporated with a ubiquitously expressed marker control (RFP). (B-E) P7Ur8-EGFP drives expression in a neural crest-like pattern. (**B**,**C**) P7Ur8-EGFP (green) is expressed as early as HH4-HH6 in the neural plate and neural plate border, but not in the NNE. (**D**) P7Ur8 is expressed in the neural plate, neural plate border and neural folds, but not in the NNE or somites through HH9. (**E**) By HH12, the oldest stage screened, P7Ur8 is no longer expressed. (**F-I**) Expression of Pax7 protein (green) is found in the neural plate border at HH5 (F) and later superimposes onto the neural folds (G-I). At HH9, somite expression of Pax7 is clearly visible (arrow, H). At HH12, Pax7 is expressed in the neural folds, somites (arrow) and presomitic mesoderm (I). np, neural plate; npb, neural plate border; nne, non-neural ectoderm; nf, neural folds; s, somites.

(Fig. 1A, labeled Pax7 Upstream regions, or P7Ur1 through P7Ur12). P7Urs were PCR amplified from chicken genomic DNA and inserted into the reporter construct ptkEGFP (Uchikawa et al., 2003). Enhancer reporter constructs were co-electroporated with an RFP marker plasmid (to determine electroporation extent) into the entire epiblast of Hamburger-Hamilton stage 3 (HH3) embryos and cultured between 16 and 24 hours, until HH6-12. EGFP expression was compared with RFP marker electroporation and endogenous *Pax7* from non-treated embryos (Fig. 1B-I).

Embryos electroporated with P7U regions 1-7 and 9-12 failed to reveal restricted expression at the stages analyzed (183 embryos analyzed, $n \geq 8$ per construct). Instead, embryos electroporated with P7Ur8 (~700 bp) exhibited NPB/NC reporter expression (Fig. 1B-D, $n=48$). Weak initial reporter activity driven by P7Ur8 can be identified at HH4 (Fig. 1B) distributed broadly in the central ectoderm; importantly, at this early stage reporter expression in prospective NPB territory matches endogenous *Pax7*. By HH6, reporter expression is seen in a broad NPB pattern excluded from the NNE and the more medial NP (Fig. 1C). At HH9, P7Ur8 replicates endogenous *Pax7* expression in the NFs, and lacks expression in medial neural and lateral NNE, and somites. However, additional expression is seen in the anterior primitive streak and in the caudal open NP, although it seems excluded from most medial territories (Fig. 1D). By HH12, the oldest stage screened, P7Ur8 no longer expressed the reporter signal (Fig. 1E).

To further establish the distribution of the EGFP expression driven by P7Ur8, we sectioned HH9 embryos co-electroporated with the RFP control plasmid (Fig. 2A-D). We found marker RFP and P7Ur8-EGFP signal in the NFs (Fig. 2B,C). However, unlike the marker RFP, P7Ur8-EGFP showed little expression in more medial neural tissue, was absent from the most ventral regions of the neural tube and no signal was detected in mesodermal tissues (Fig. 2C,D). We noticed a graded P7Ur8-EGFP signal, being stronger at dorsal regions and weaker towards ventral regions of the neural tube. In the caudal open NP, adjacent and posterior to the node, expression driven by P7Ur8 can be detected in the NFs, and additional signal is seen in the early NP/stem zone and remaining primitive streak (Fig. 2A). Equivalent sections from embryos immunostained for Pax7 (Fig. 2E-G) demonstrate a similar P7Ur8-EGFP signal to that of endogenous Pax7 in early migrating NC and dorsal neural tube (Fig. 2E) in NFs (Fig. 2F) and in the NPB in more caudal regions (Fig. 2G). However, unlike endogenous Pax7, Pax7Ur8-signal is clearly absent from somites (Fig. 2F). Furthermore, we directly immunostained Pax7 in embryos electroporated with P7Ur8-EGFP and cultured until HH10, when Pax7 is expressed in the dorsal neural tube, pre-migratory and migratory neural crest, and in the somites (Fig. 2H). The merged whole-mount image displaying endogenous Pax7 (red) with the P7Ur8-EGFP signal (green) clearly reveals exclusive expression of endogenous Pax7 in the somites, where P7Ur8 does not report. Furthermore, cranial sections reveal robust P7Ur8-EGFP signal in early migratory NC and dorsal neural tube cells co-expressing Pax7, and a few ventral cells with much weaker EGFP signal (Fig. 2I-I″).

These results suggest that the Pax7 upstream region P7Ur8 contains an enhancer element that is able to drive expression in a pattern similar to that of endogenous early Pax7 in the NPB, NF and early emigrating cranial NC, but does not elicit Pax7 expression in the mesodermal somites, nor does it sustain expression after HH12.

Identification of essential elements for P7Ur8 enhancer activity

To identify the minimal elements necessary and sufficient for the enhancer activity within P7Ur8, we performed a deletion analysis (Fig. 3). A series of ~100 bp overlapping internal deletions was generated and the expression profile of the resulting constructs monitored by electroporation (Fig. 3A). Deletions 1 to 4 (bp 90-352) and 8 (bp 478-593) had no effect on the expression of P7Ur8

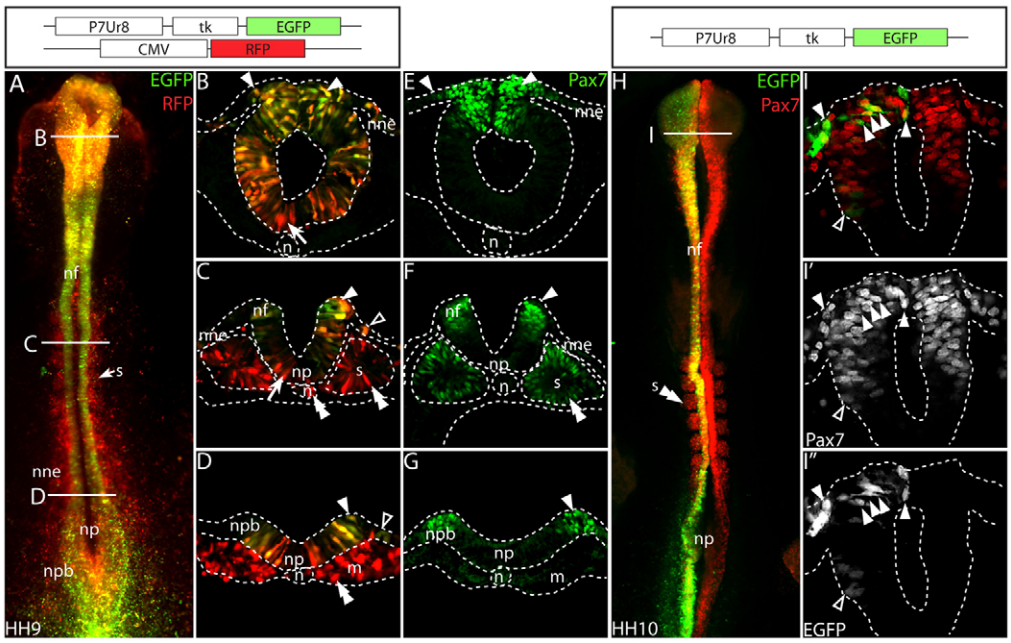

Fig. 2. Enhancer P7Ur8 is co-expressed with Pax7 in the neural crest, but not in the somites. (**A-D**) Co-electroporation of red-marker control and P7Ur8-EGFP reveal specific expression at HH9 in the dorsal most part of the neural tube (B, closed arrowheads) and neural folds (C and D, closed arrowheads), but not in the ventral neural tube (B, arrow) or neural plate (C, arrow). P7Ur8 is expressed in some NNE adjacent to the neural folds (C and D, open arrowheads), but is not expressed in the somites, the notochord (C, double arrowheads) or the pre-somitic mesoderm (D, double arrowhead). (**E-G**) Sections showing normal Pax7 expression (green) in a similarly staged embryo, in the tips of the forming neural folds (F,G, closed arrowheads), in the dorsal neural tube and in migrating NC (E), and in the somites (F, double arrowhead). (**H-I″**) At HH10, P7Ur8 (green) is co-expressed with Pax7 protein (red). Cells in the dorsal neural tube strongly express P7Ur8 (I-I″, closed arrowheads), whereas weak P7Ur8 expression is seen in more ventral cells (I-I″, open arrowheads). n, notochord; m, mesoderm; np, neural plate; npb, neural plate border; nne, non-neural ectoderm; nf, neural folds; s, somites.

(Fig. 3B); however, deletions 5-7 (bp 299-497) provided no reporter expression, suggesting high relevance for the intervening ~300 bp. However, removal of bp 251-352 from deletion 4 and bp 478-593 from deletion 8 had no effect on the expression, thus we proposed that the minimal elements necessary to drive expression of P7Ur8 are found in the 126 bp from 352 to 478. To specifically test this, we generated an additional deletion construct removing the intervening 130 bp between 350 and 480 (P7Ur8Δ9). As expected, this construct provided no reporter expression after electroporation (Fig. 3C).

Given that spacing of enhancer elements has been found to be essential for their function in some cases (Carey, 1998; Erives and Levine, 2004; Senger et al., 2004; Halfon, 2006), we replaced the deleted sequence in P7Ur8Δ9 with a random 130 bp sequence (P7Ur8Δ9S), re-establishing the original spacing of the flanking sequences. Similar to P7Ur8Δ9, P7Ur8Δ9S is unable to drive expression of EGFP (data not shown). To test whether the required 130 bp sequence is able to provide the reporter expression found in P7Ur8, we generated a construct inserting these 130 bp upstream of the tk promoter. When electroporated into chick embryos, this construct provides similar expression profile to the whole P7Ur8, demonstrating that this 130 bp sequence (termed Pax7 early neural plate border element, or P7eNPBE) is sufficient for enhancer activity during early developmental stages (Fig. 3D,E). To further limit the presence of relevant components within the P7eNPBE, we generated two smaller constructs. P7eNPBEΔ1 (first 80 bp from P7eNPBE) was unable to elicit reporter activity. Instead, P7eNPBEΔ2 (last 70 bp) efficiently reported enhancer activity, even in a single copy (Fig. 3E). This evidence suggests that within the 70 bp of P7eNPBEΔ2 lies information necessary and sufficient for the early NPB/NC reporter expression originally identified in P7Ur8.

Putative TF-binding sites necessary for P7eNPBEΔ2

In order to identify possible TF-binding sites involved in the regulation of P7eNPBEΔ2, we used the JASPAR database (Bryne et al., 2008), and found over 50 possible TFs that could bind more than a 100 recognition DNA-binding sites. To restrict this list, we generated eight 6-7 bp mutations, with each eliminating several potential binding sites. Mutated versions (M1 to M8) of the P7eNPBE were cloned into ptkEGFP, while the wild-type P7eNPBE enhancer was cloned upstream of the tk promoter driving mCherry. Co-electroporation of these constructs enables direct comparison and identification of possible effects triggered by the mutations. All of the mutations revealed some effect on enhancer activity (supplementary material Fig. S1). M2, M3 and M8 completely eliminated activity at all stages tested (supplementary material Fig. S1B-C′); M1 retained the spatiotemporal activity, but the robustness of expression, both in terms of intensity of signal and number of cells expressing the enhancer, was variable (supplementary material Fig. S1D-E′); M4-6 retained activity only before HH8 (supplementary material Fig. S1F-G′); and M7 retained activity only after HH8 (supplementary material Fig. S1H-I′). Fig. 4 provides two examples of mutated reporter expression: prior to HH8 there is almost unperturbed expression from M4 (yellow sequence Fig. 4A,B); conversely, M2 completely abrogated reporter expression (gray sequence Fig. 4A,C,C′). The fact that so many of the bases are crucial for the activity of P7eNPBE, and that the effects can be stage specific, indicates that the regulation of this enhancer element is complex and dynamic. It seems unlikely that one factor alone could fully regulate Pax7 expression, and instead these results suggest that multiple TFs recognizing the affected

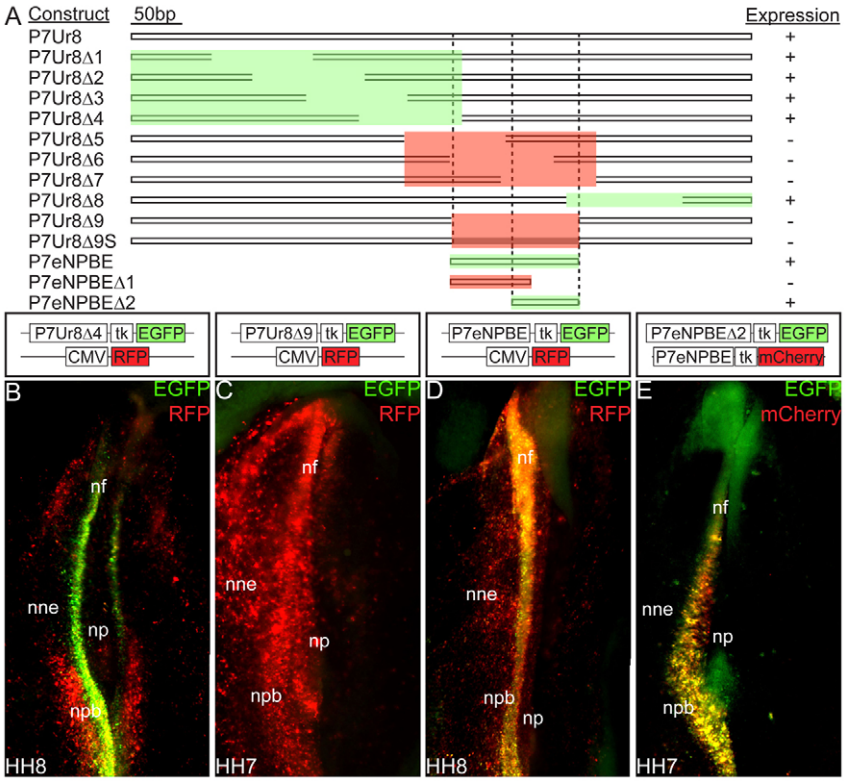

Fig. 3. Deletion analysis identifies the minimal element necessary and sufficient for the enhancer activity of P7Ur8. (**A**) Summary of the structure and activity of each deletion construct. Constructs highlighted in green retained enhancer activity, whereas constructs highlighted in red did not. (**B-E**) Examples of constructs with (B,D,E) and without (C) enhancer activity. np, neural plate; npb, neural plate border; nne, non-neural ectoderm; nf, neural folds.

sequences contribute in an important way to Pax7 regulation. Because all the mutations were found to be crucial in at least some fashion for the expression of P7eNPBE, and that many of the binding sites are overlapping, we decided to take a candidate approach to further analyze this element.

cMyb, but not ETS1, Klf4 or Zeb1, can regulate expression from the P7eNPBE

To restrict the list of putative TFs that could regulate Pax7 suggested through our mutagenesis effort (supplementary material Table S1), we focused on a few attractive candidates. *cMyb* mRNA and protein blots have been reported in chick as early as HH4 (Karafiat et al., 2005). At HH6, *cMyb* is expressed in the NPB, and at HH8-9 is expressed in dorsal NFs and migrating neural crest (Betancur et al., 2010a). *Ets1* is expressed in cranial neural crest cells at HH8, prior to emigration (Tahtakran and Selleck, 2003). *Zeb1* is expressed in the cranial neural crest and neural crest derivatives (Funahashi et al., 1993; Takagi et al., 1998). *Klf4* is expressed in prospective NP at HH4 and at HH8-9 is in the NFs and neural tube (Antin et al., 2010).

To test the effect of these candidate TFs on enhancer activity, we co-electroporated HH3 embryos with the P7eNPBE reporter and constructs ectopically expressing each gene. Expression of each protein was confirmed by western blot (data not shown). Ectopic expression of Klf4 (n=25; Fig. 4D), Zeb1 and ETS1 (n=20 and n=11; supplementary material Fig. S2D,E) had no effect on the activity of P7eNPBE, which was found in its normal distribution in the NFs. Instead, ectopic expression of cMyb disrupted the enhancer activity of P7eNPBE (Fig. 4E-G′). After incubation, the majority of embryos receiving cMyb and P7eNPBE displayed reduced expression the NFs (n=14/17) with a residual 'salt and pepper' activation pattern. Additionally, *cMyb* overexpression led to ectopic signal in the NP (n=37/49) and the NNE (n=47/51) (Fig. 4E-G), but did not trigger mesodermal expression (Fig. 4G,G′).

To further test whether cMyb could regulate *Pax7* expression through the P7eNPBE enhancer, we tested the capacity of cMyb to bind to this regulatory element *in vitro* through an electrophoretic mobility shift assay (EMSA, Fig. 4H). These experiments identified a shift of the labeled wild-type probe (lane 1) generated by an *in vitro* translated cMyb protein (38% bound probe, lane 2), outcompeted by excess unlabeled wild-type probe (2% bound probe, lane 3) but not by unlabeled mutated-M2 probe (31% bound probe, lane 4). Furthermore, labeled mutated-M2 probe (lane 5) displays a much weaker binding by cMyb (8%, lane 6) in comparison with that bound to the wild-type probe.

Overexpression and EMSA analyses support a possible role for *cMyb* in *Pax7* regulation through the P7eNPBE. At HH6, *cMyb* is expressed in the NPB (Betancur et al., 2010a) along with Pax7; however, its earlier spatial distribution has not been established, a premise for its participation in early Pax7 regulation. To achieve this, we performed *in situ* hybridization with two antisense probes for *cMyb* (full length and 300 bp) in gastrula embryos prior to *Pax7* expression. No specific signal was seen with corresponding sense control probes (not shown). Instead, in *cMyb* seems broadly expressed in the early ectoderm of HH3-4 embryos (Fig. 4I), confirming reported northern and western blots (Karafiat et al., 2005), and providing further support for the possible participation of *cMyb* in *Pax7* regulation.

cMyb and *ETS1* are known to cooperate in many different contexts (Dudek et al., 1992; McCracken et al., 1994; Melotti and Calabretta, 1994; Melotti et al., 1994; Postigo et al., 1997; Ratajczak et al., 1998; Brabletz et al., 1999; Betancur et al., 2010a). As there are *ETS1* sites present in P7eNPBEΔ2, we tested the possible cooperation between *ETS1* and *cMyb* to regulate P7eNPBE. To achieve this, we simultaneously electroporated both constructs and

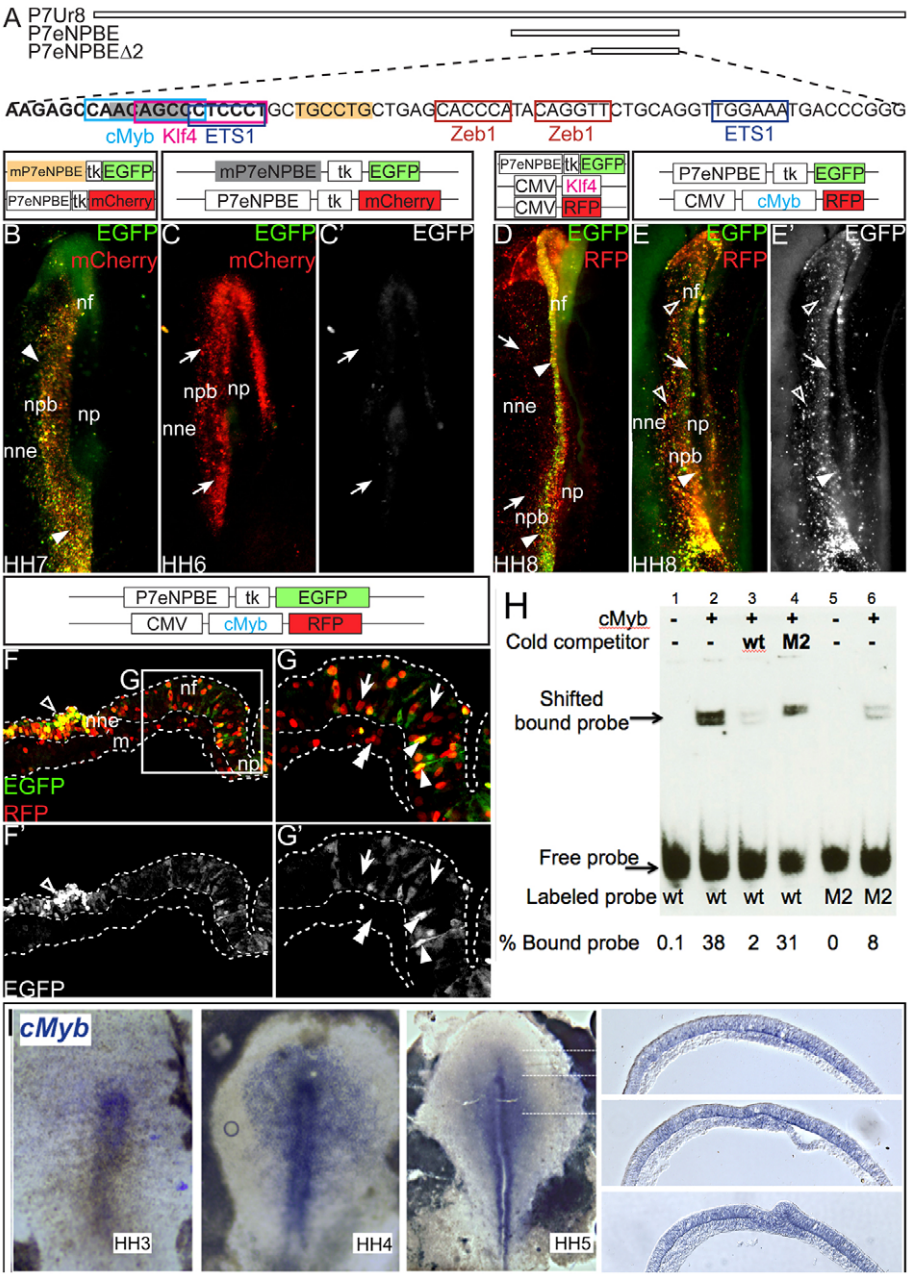

Fig. 4. The transcription factor cMyb is crucial for the enhancer activity of P7eNPBE. (**A**) Position size and sequence of the enhancer P7eNPBEΔ2 in comparison with P7Ur8 and P7eNPBE. (**B-C′**) Putative DNA-binding sites of four selected TFs shown in colored boxes (cMyb, light blue; Klf4 pink; ETS1, dark blue; Zeb1, red). Two examples of mutated areas are indicated, with yellow for early unaltered (B, arrowheads) and gray for abrogated (C,C′, arrows) reporter expression. (D-G′) These TFs were electroporated to assess effect on P7eNPBE enhancer activity. (**D**) Overexpression of Klf4 (red) led to normal P7eNPBE-EGFP expression in neural folds and neural plate border (arrowheads) and to exclusion from the NNE (arrows). (**E-G′**) cMyb (red) overexpression led to reduced P7eNPBE-EGFP signal in the neural folds (E-E′, arrow) with residual salt and pepper patterns, and expansion in the NNE (E,E′, open arrowheads). (F-G′) Sections of a similarly treated and staged embryo showing P7eNPBE expression in the NNE (open arrowheads, F,F′) and neural plate (arrowheads, G). P7eNPBE-EGFP is not ectopically activated in the mesoderm (double arrowheads G) and is much reduced in the neural folds, but displays a 'salt and pepper' pattern, with some cells lacking (arrows), and others presenting, expression (arrowheads) (compare with Fig. 2C,D). (**H**) EMSA performed using a 20 bp probe (bold sequence in A) containing the wild-type cMyb-binding site or the M2 mutated version. When cMyb is added, it binds to the wild-type probe, generating a clear shift (lane 2 ~38% bound probe), which is severely reduced when cold unlabeled wild-type probe is added (lane 3, ~2%); instead, adding the same excess amount of cold unlabeled mutated M2 probe still displays a robust shift (lane 4, ~31%); however, only 8% of the labeled mutated-M2 probe is bound by cMyb (lane 6). (**I**) *cMyb* is expressed in the early ectoderm of chick embryos prior to *Pax7*. Whole mount *in situ* hybridization on HH3-5 chick embryos. Dotted lines at HH5 indicate sections shown on the left. m, mesoderm; np, neural plate; npb, neural plate border; nne, non-neural ectoderm; nf, neural folds.

the reporter P7eNPBE, and examined for enhancer activity (supplementary material Fig. S2E,E′). As with cMyb alone, the enhancer expression is no longer restricted from the NNE (n=6/7) and appears in a reduced and 'salt and pepper' pattern in the NFs (n=5/6) and NP (n=3/5). This result suggests that ETS1 does not modify the activity of cMyb on P7eNPBE.

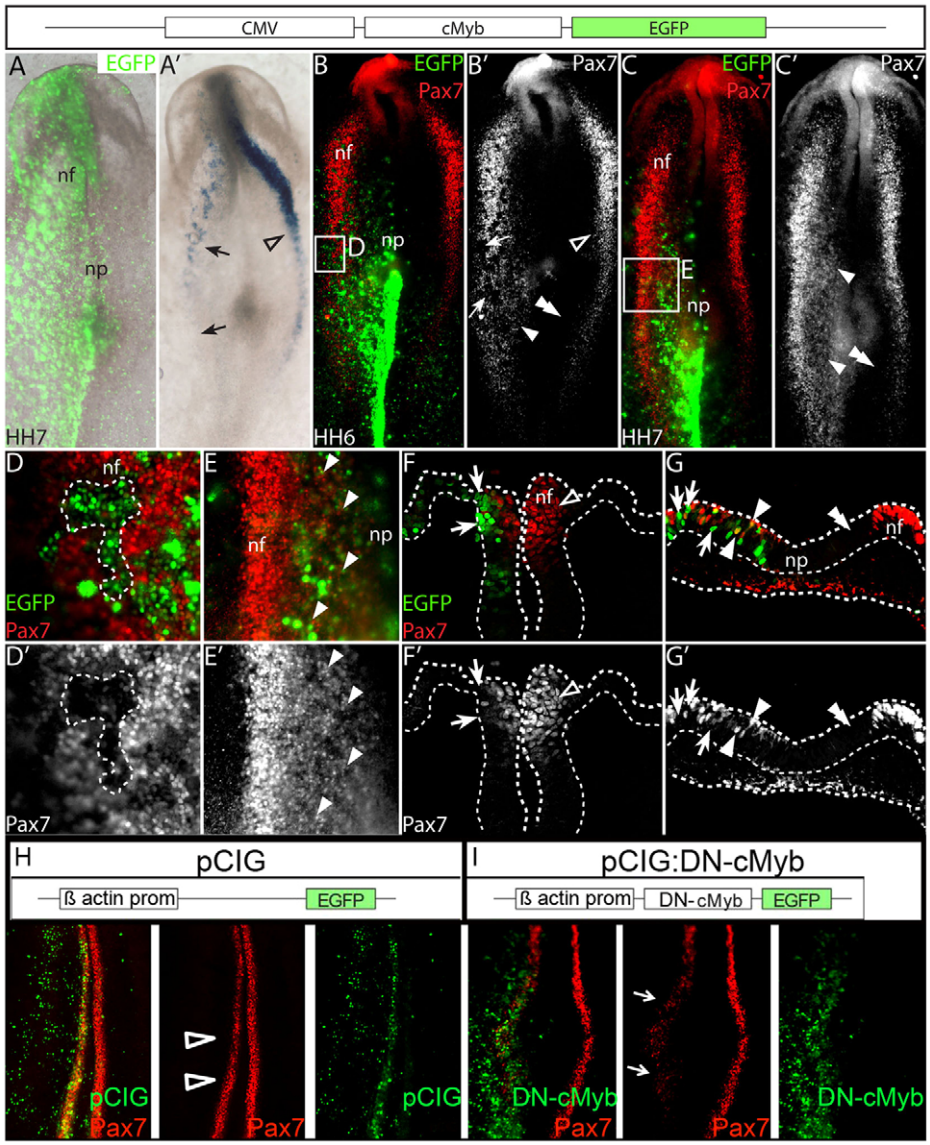

Fig. 5. Endogenous Pax7 mRNA and protein respond to cMyb modulation. The effect of cMyb overexpression was analyzed on endogenous Pax7 mRNA and protein. (**A**,**A′**) cMyb overexpression (green) leads to reduced levels of *Pax7* mRNA (blue) in electroporated neural folds (A′, arrows) compared with untreated neural folds (open arrowhead). (**B-G′**) Similar neural fold reduction of Pax7 protein (red) is seen upon cMyb overexpression (green) (B′,F,F′,G′, arrows; D,D′, outlined) compared with untreated neural folds (B′,F,F′, open arrowheads). Overexpression of cMyb in the neural plate instead leads to Pax7 ectopic expression (B′,C′,E,E′,G,G′, arrowheads) compared with untreated neural plates (B′,C′,G,G′, double arrowheads). Neither mRNA nor protein was expanded into the NNE. (**H**,**I**) Electroporation of pCIG empty vector control has no effect on Pax7 expression (H, open arrowheads); instead, the dominant-negative cMyb (DN-cMyb) construct leads to robust downregulation of endogenous Pax7 expression (I, arrows). np, neural plate; nf, neural folds.

Ectopic cMyb modulates endogenous Pax7 expression in the neural folds

To establish whether cMyb could also control endogenous expression of *Pax7*, we ectopically expressed cMyb and then examined both *Pax7* mRNA and protein expression (Fig. 5). Similar to the effect seen for the P7eNPBE, when cMyb is ectopically expressed, the levels of *Pax7* mRNA are reduced in the NPB/NFs (n=4/7) (Fig. 5A′), and ectopic *Pax7* expression was seen in the NP (n=3/8, not shown). However, no expansion into the NNE was observed (n=0/8). The effect of cMyb on Pax7 protein revealed an analogous pattern: ectopic cMyb lead to reduced Pax7 protein in NFs (Fig. 5B,B′,D,D′,F-G′, n=24/33) with expanded levels in the NP (n=24/31) (Fig. 5B-C′,D-E′,G,G′), whereas they rarely generated ectopic Pax7 expression in the NNE (n=3/23, not shown).

Given the reported interactions between cMyb and ETS1, and despite our lack of evidence for ETS1 modulation of the P7eNPBE, we decided to test their possible combinatorial effect on endogenous *Pax7* expression. ETS1-electroporated embryos showed normal *Pax7* mRNA distribution (n=4) (supplementary material Fig. S3B′), and co-electroporation of ETS1 and cMyb generated patterns seen with cMyb alone (n=6) (supplementary material Fig. S3C′). These results strongly suggest that ETS1 does not interact with cMyb to regulate early *Pax7* expression.

To further assess the possible role of *cMyb* in *Pax7* regulation, we turned to a previously reported dominant-negative approach (Kim et al., 2009; Kim et al., 2008; Lee et al., 2008; Taylor et al., 1996; Yi et al., 2002). cMyb contains a DNA-binding domain (DBD) on the N terminus, followed by a transactivation domain (TAD) and a negative regulatory domain (NRD) (Pattabiraman and Gonda, 2013; Ramsay and Gonda, 2008). The dominant-negative cMyb (DN*cMyb*) consists exclusively of the DBD; removing the TAD and the NRD is thought to prevent much of the protein-protein interactions of cMyb. We PCR amplified the DBD of cMyb and cloned it into pCIG, a vector with an IRES-EGFP. Then we performed unilateral electroporations (contralateral side serving as internal control) in HH3 chick embryos with either pCIG control or DN*cMyb*. Although control embryos displayed even numbers of $Pax7^+$ cells on either side (n=5, Fig. 5H), a robust reduction of $Pax7^+$ cells (>50% average) was found in in the electroporated side in 9 out of 10 embryos treated with DN*cMyb* (Fig. 5I). These results support a role for *cMyb* in modulating *Pax7*, and suggest it operates as an activator.

DISCUSSION

P7eNPBE directs expression in prospective neural crest

We have identified a novel enhancer of *Pax7* that is able to drive expression associated with early NC development. The reporter expression generated by P7eNPBE marks the NPB, becomes restricted to the NFs and early migratory NC, locations in which the reporter is co-expressed with endogenous *Pax7*. Unlike endogenous *Pax7*, P7eNPBE-driven expression fades after HH12 and never appears in somites, supporting a specific NPB-early NC character for this enhancer. Despite the striking similarity to the endogenous NC-related *Pax7* expression, a few deviations were noted. At HH4-5, the weak initial expression is unrestricted, and can be found in medial prospective NP and lateral NNE. Additional low-level P7eNPBE and P7Ur8-driven expression can be detected in more medial/ventral NP. These differences could easily be due to the lack of repressor elements in the P7eNPBE and P7Ur8 constructs. Further restriction of endogenous *Pax7* expression is achieved by miRNAs that regulate the translation and degradation of *Pax7* mRNA (Chen et al., 2010; Dey et al., 2011). The lack of these responsive elements in our constructs could account for some of the minor discrepancies. Overall, this small enhancer element reports accurately in the early NPB and early NC.

The P7eNPBE enhancer displays little sequence similarity to human but no homology to murine sequences, and thus highlights the importance of screening beyond highly conserved regions. Furthermore, there is growing evidence for the conservation of regulatory function of genomic regions across species in the absence of sequence conservation or even common TF-binding sites (Ludwig et al., 2000; Romano and Wray, 2003; Ludwig et al., 2005; Fisher et al., 2006; Hare et al., 2008; Weirauch and Hughes, 2010; Swanson et al., 2011). Therefore, functionally equivalent elements to P7eNPBE might exist in other species without obvious sequence similarity. Alternatively, the P7eNPBE enhancer could represent a regulatory element that is unique to chick. Slight variations in the expression of *Pax7* have been reported in different organisms (Bang et al., 1999; Monsoro-Burq et al., 2005; Sato et al., 2005; Basch et al., 2006; Hong and Saint-Jeannet, 2007; Minchin and Hughes, 2008; Khudyakov and Bronner-Fraser, 2009; Betters et al., 2010; Maczkowiak et al., 2010; de Crozé et al., 2011). Human embryonic expression of *Pax7* in dorsal neural tube and migrating NC has only been reported in relatively later stages and at trunk levels (Betters et al., 2010); however, the lack of earlier more anterior samples prevents equivalent comparison with the early NPB of the chick embryo. In the mouse, expression studies based on *in situ* hybridization had suggested that Pax7 expression started after neural tube closure at embryonic day 8 (Jostes et al., 1990; Mansouri et al., 1996). However, recent immunofluorescence analysis indicates that Pax7 appears earlier, in open NFs of the cranial region at embryonic day 7.5 (Basch et al., 2006; Murdoch et al., 2012). Preliminary work from our group supports functional conservation with limited sequence conservation between the equivalent P7eNPBE from human and chick (data not shown). Further studies will be necessary to establish whether this element is conserved in other species.

The regulatory elements of Pax7 in mammals

Previous studies of human and mouse *Pax7* regulatory elements have not yielded information regarding early NPB development (Murmann et al., 2000; Syagailo et al., 2002; Lang et al., 2003). However, studies of the regulation of *Pax3* in the mouse have been more successful, revealing several elements capable of driving reporter expression in endogenous early NPB/NC territories. Furthermore, specific TFs and their binding sites have been identified, and include direct and indirect Wnt-responsive elements (Natoli et al., 1997; Milewski et al., 2004; Pruitt et al., 2004; Degenhardt et al., 2010; Sanchez-Ferras et al., 2012). The exploration of the direct and indirect links between FGF, BMP and Wnt signals known to regulate *Pax7* expression and the P7eNPBE remain to be tested.

Large pool of putative Pax7 regulators derived from the P7eNPBE mutagenesis

Our enhancer element analysis uncovered a small sequence that is rich in TF-binding sites (>100); the mutagenesis performed in these sequences identified several of them as essential for the reporter function, thus many putative TFs could modulate the expression from the element here identified (supplementary material Table S1). From these, we focused on a small list of candidates, and identified a clear role for *cMyb* in regulating Pax7 expression. We suggest that cMyb is a key factor capable of regulating Pax7 transcription in the NPB and early NC through the P7eNPBE. Our analysis of other candidates, including KLF4, Zeb1 and ETS1, failed to identify their participation in *P7eNPBE* regulation. Therefore, a major task for the future will be to identify and characterize the interactions of TFs that bind this element and cooperate to modulate Pax7 expression in the early NPB and NC.

cMyb is a novel regulator of Pax7 expression in the chick

cMyb was first identified as the cellular counterpart to the *v-myb* oncogenes found in the avian acute leukemia viruses AMV and E26 (Klempnauer et al., 1982; Gonda and Bishop, 1983). Studies in hematopoietic cells have shown that *cMyb* controls proliferation, differentiation and survival, and is associated with stem cell regulation (Gewirtz and Calabretta, 1988; Mucenski et al., 1991; Ramsay et al., 1992; Walker et al., 1998; Weston, 1998; Oh and Reddy, 1999; Sun et al., 2008; Papathanasiou et al., 2010; Cheasley et al., 2011). In chick embryos, *cMyb* directly binds to a regulatory element that controls *Sox10* expression in the NFs (Betancur et al., 2010a). Previous studies showed that *cMyb* modulates the expression of *Msx1*, *Slug* and *c-Kit* during NC development (Karafiat et al., 2005; Karafiat et al., 2007). However, no direct interaction or regulatory elements were originally reported. More recently, a non-NC study using embryonic kidney, colon carcinoma, neuroblastomas and leukemia-blast cells identified a regulatory element recognized by *cMyb* controlling the expression of Snail2 and evocating an epithelial to mesenchymal transformation (Tanno et al., 2010). Collectively, these studies support an important role for *cMyb* within the NC-GRN.

Our results identify an essential role for a putative cMyb-binding site for P7eNPBE-driven expression, and we show that *cMyb* can bind to this sequence. We also demonstrate expression of *Myb* prior to *Pax7* and distinct effects of cMyb overexpression on P7eNPBE and endogenous Pax7. Finally, through a DN-*cMyb* construct, we reduced endogenous Pax7 expression. These results suggest that cMyb directly regulates Pax7 in a positive manner in the NPB, and is one of the earliest TFs that modulates neural crest development in chick embryos.

A balancing act of cMyb regulates the NPB expression of Pax7 in the early chick ectoderm

cMyb can activate or repress targets aided by other TFs and distinct co-factors including FLASH, p100, Mi-2α, Menin, CBP/p300, Mybp1a, N-CoR, mSin3a, c-Ski, Tif-1β, etc. (Emambokus et al.,

2003; Pattabiraman and Gonda, 2013; Ramsay and Gonda, 2008). Even when associated with p300, a co-factor traditionally seen as a co-activator, cMyb can function as activator or repressor (Zhao et al., 2011). Therefore, cMyb alterations trigger complex and context-dependent outputs.

Here, we have identified a putative cMyb DNA-binding site essential for the expression of the P7eNPBE, suggesting that cMyb may activate Pax7 early expression at the NPB. We showed sequence-specific binding of cMyb to this site, and that cMyb is expressed in the early ectoderm. If the role of cMyb is to activate Pax7 expression, one would expect that a loss-of-function approach should reduce endogenous Pax7 expression, precisely what we observed with our DN*cMyb*. These results strongly suggest that *cMyb* acts as a positive regulator of Pax7 expression in the early NPB.

Experiments in which P7eNPB reporter expression, Pax7 mRNA and Pax7 protein were clearly modulated by electroporation of full-length cMyb lend further support to the idea that cMyb is involved in Pax7 regulation through the P7eNPBE. However, the results are complex; instead of the anticipated increased expression, we observed an unexpected general reduction requiring a more elaborate explanation. Perhaps excess levels of cMyb antagonize its positive regulation of Pax7 via 'squelching' (Ptashne, 1988) of partner transcription factors and/or co-factors. Similar effects have been reported for Pax6, Stat5, VP16 and Sox2 among many other TFs (Boer et al., 2007; Czerny and Busslinger, 1995; Fischer and Gessler, 2007; Luo and Yu-Lee, 2000). Additionally, we observed residual 'salt and pepper' expression in the NPB. We suggest that most cells in the NPB already express cMyb, and electroporation leads to excessive levels, which could sequester partners away from the enhancer element (squelching). Occasional occupancy of the enhancer by cMyb and its partners could explain the residual salt and pepper pattern. Importantly, the expression of most NPB markers is not homogeneous, we have noted heterogeneous expression of most TFs associated with NC development in the NPB of chick, mouse, rabbit and human embryos. Therefore, salt and pepper expression could occur in the few cells where original cMyb-levels were low and reached appropriate levels after electroporation.

We suggest that, in the NP and the NNE, which normally have lower cMyb levels than the NPB (Betancur et al., 2010a), cMyb overexpression leads to 'functional' cMyb levels, favoring activation. Accordingly, we see ectopic reporter and Pax7 expression in the NP, suggesting that here cMyb is a limiting factor. Instead, in the NNE only the reporter was ectopically activated. Thus, negative regulators (transcription repressors or miRNAs) absent in the reporter may prevent ectopic expression of endogenous Pax7.

Based on this analysis, we propose a model for Pax7/P7eNPBE regulation in the early chick ectoderm (Fig. 6), where binding of cMyb and its partners is crucial for Pax7 and reporter expression (Fig. 6A). Mutagenesis of the P7eNPBE revealed the requirement of several sequences that could respond to several TFs. Among them we identify a crucial role for the DNA recognition site of

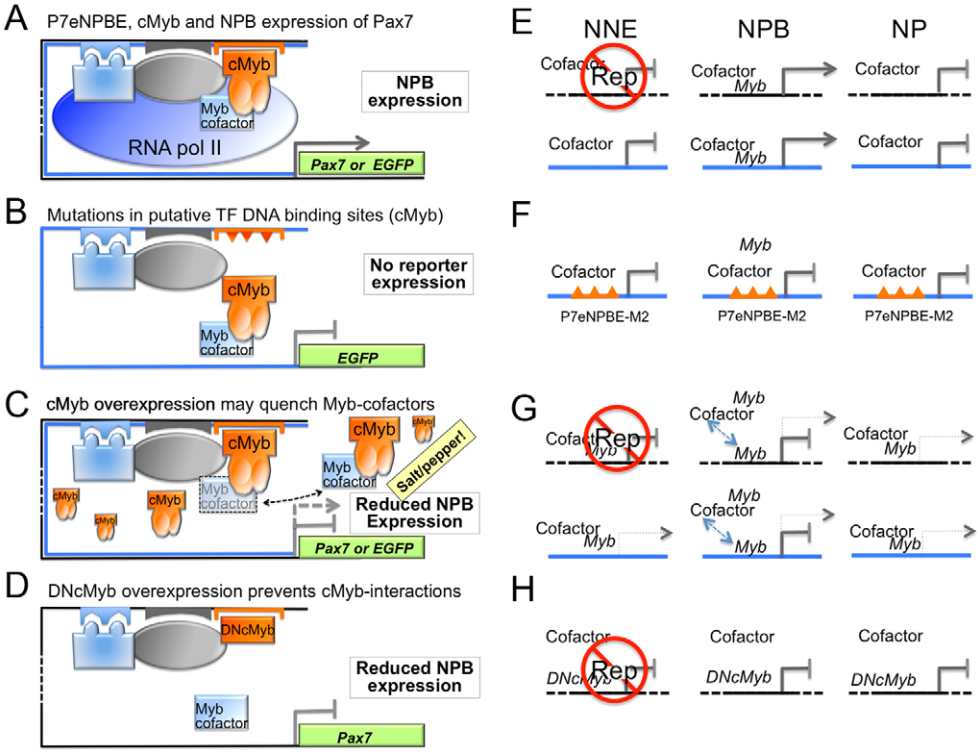

Fig. 6. cMyb Effect of cMyb on ectodermal regulation of *Pax7* expression through the P7eNPBE. (**A**) cMyb interacts with a sequence-specific DNA-binding site at the P7eNPBE to control Pax7 expression positively in the NPB of early embryos. (**B**) Mutation of the cMyb DNA-binding site prevents reporter expression. (**C**) Overexpression of cMyb quenches Myb co-factors, leading to global NPB downregulation with residual salt and pepper expression. (**D**) DNcMyb overexpression prevents cMyb co-factor(s) interactions and assembly at the enhancer, blocking Pax7 expression. (**E-H**) Context-dependent expression in the NNE, NPB and NP: components and interactions. A repressor mechanism absent from the reporter is proposed to prevent expression in the NNE (red circle, E,G,H). Myb co-factor(s) are broadly expressed, while the initial broad expression of cMyb becomes restricted to the NPB (Betancur et al., 2010a). Genomic Pax7 and P7eNPBE are indicated by black and blue lines, respectively.

cMyb (Fig. 6B). Overexpression of cMyb leads to downregulation of Pax7 and enhancer activity at the NPB due to squelching, with residual salt and pepper patterns due to occasional occupancy at the enhancer (Fig. 6C). DNcMyb overexpression instead triggered a reduction of Pax7 expression (Fig. 6D). We further suggest that context-dependent partners and/or mechanisms of repression (repressor TFs or miRNA-dependent regulation) could account for the behaviors seen in NNE, NPB and NP (Fig. 6F-I).

Murine Myb proteins and NC

cMyb-null mice display phenotypes related to hematopoietic development without any obvious effects on NC (Sitzmann et al., 1995; Mucenski et al., 1991). However, the close relative B-Myb is ubiquitously expressed and recognizes near identical sequences to those recognized by c-Myb. Although different functions and targets have been identified for cMyb and B-Myb (Rushton et al., 2003), B-Myb has been shown to experimentally modulate targets in undistinguishable fashion to cMyb, and is likely to share some transcriptional targets *in vivo* (Prouse and Campbell, 2012; Ramsay and Gonda, 2008). Importantly, cMyb and B-Myb have been reported to function redundantly in myeloid cells (Golay et al., 1997). B-Myb-null animals die before gastrulation (Tanaka et al., 1999), preventing any NPB/NC analysis. Therefore, the function of Myb proteins in murine NC development remains to be tested. Interestingly, a non-coding mutation affecting the binding of cMyb to a regulatory element of Zeb1 leads to craniofacial defects, including cleft palate (Kurima et al., 2011).

Concluding remarks

The early expression of *Pax7* in the avian early ectoderm is sharply restricted to the NPB; later on, *Pax7* is maintained in NC precursors and in early migratory NC, and is also expressed in mesoderm precursors. We recently have shown that FGF signaling can modulate Pax7 in the early chick ectoderm (Stuhlmiller and García-Castro, 2012a; Yardley and García-Castro, 2012). Among putative effectors of FGF/ERK signaling are the ETS TFs. We overexpressed ETS1 but were unable to identify any effects and thus FGF effectors modulating Pax7 expression remain to be identified. Although additional work is necessary to establish the precise components and arrangements of the active Pax7 enhancer in the NPB, here we have identified a regulatory element capable of reporting in the NPB in a fashion similar to that displayed by *Pax7*, and have demonstrated the participation of *cMyb* in the regulation of the isolated enhancer, and of endogenous Pax7.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

M.I.G.C. and S.V. designed the study. S.V. performed most experiments; J.M., M.T., N.A.S. and M.I.G.C. completed additional experiments. S.V. and M.I.G.C. analyzed results, interpreted data and wrote the manuscript.

Supplementary material

Supplementary material available online at
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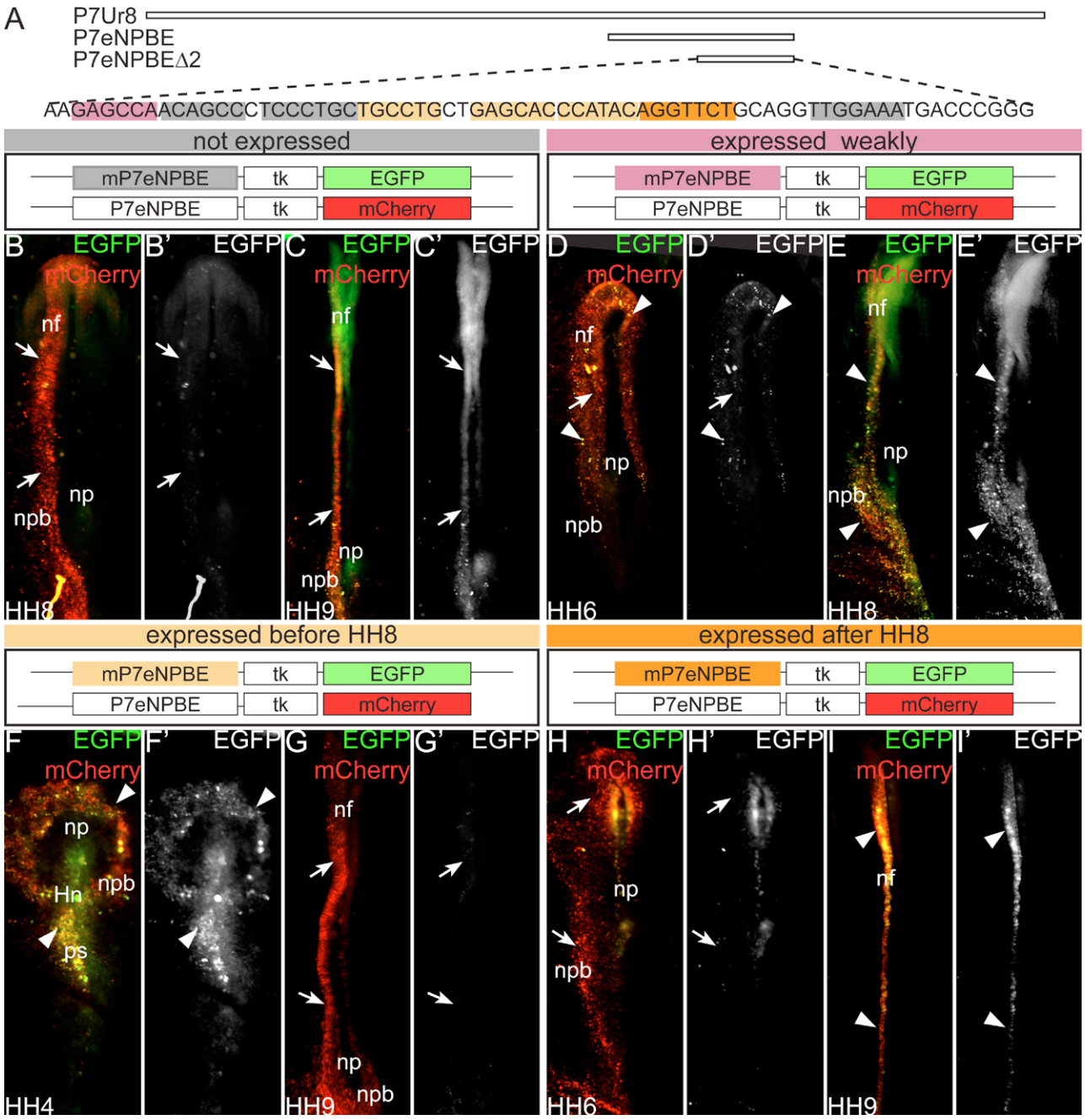

Fig. S1. Mutational analysis identified crucial bases for P7eNPBE enhancer activity. (**A**) A mutational analysis was performed to determine which binding sites are crucial for the enhancer activity of P7eNPBE. (B-I′) Embryos were co-electroporated with wild-type P7eNPBE driving mCherry and mutated P7eNPBE (termed mP7eNPBE) driving EGFP to directly compare wild type with mutated enhancer activity in the same embryo. (**B-C′**) The first type of mutation completely eliminated the activity of P7eNPBE at all stages screened. Although the wild-type enhancer (red) is expressed in the neural plate and neural plate border at HH8 (B) and HH9 (C), the mutated enhancers (green) are not expressed (B′,C′, arrows). (**D-E′**) The second type of mutation retains the spatiotemporal enhancer activity, but is generally expressed less brightly or in fewer cells. The wild-type enhancer (red) is robustly expressed in the neural plate and neural plate border at HH6 (D) and HH8 (E). The mutated enhancer (green) is also expressed in the neural plate and neural plate border (D′,E′, arrowheads), but often there are many cells not expressing the enhancer as robustly or even at all (D′, arrow). (F-I′) The remaining two types of mutations had stage-specific effects, with the mutated enhancers only expressed either before HH8 (F-G′) or after HH8 (H-I′). (**F-G′**) The wild-type (red) and mutated (green) enhancers are expressed at HH4 (F,F′) surrounding Hensen's node and in the primitive streak (arrowheads). But while the wild-type enhancer expression continues in the neural folds, neural plate and neural plate border at HH9 (G), the mutated enhancers are not expressed (G′, arrows). (**H-I′**) The final type of mutation is expressed only after HH8. The wild-type enhancer (red) is expressed in the neural plate border at HH6 (H) but the mutated enhancer (green) is not (H′, arrows). However, at HH9, both forms are expressed in the neural folds and neural plate border (I,I′, arrowheads). Hn, Hensen′s node; np, neural plate; npb, neural plate border; nne, non-neural ectoderm; nf, neural folds; ps, primitive streak.

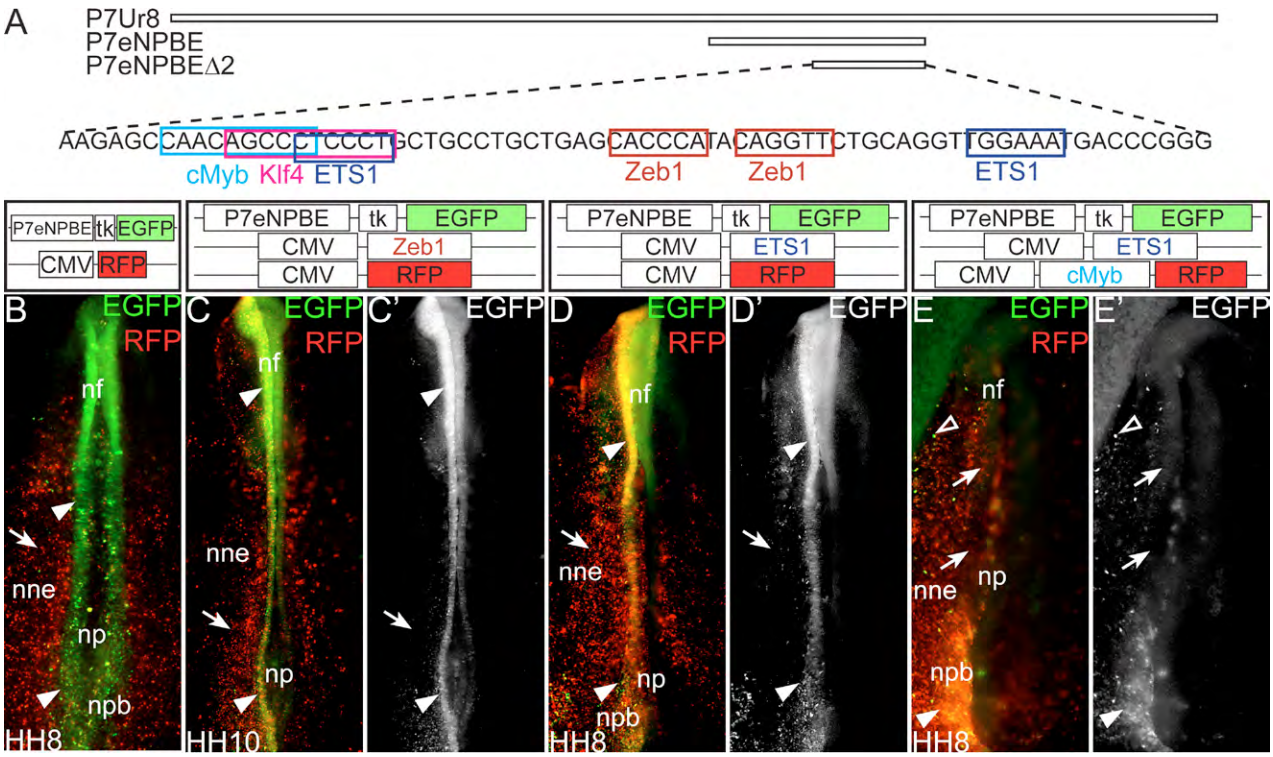

Fig. S2. Zeb1 and ETS1 are not sufficient to alter the enhancer activity of P7NCE. (**A**) Position size and sequence of the enhancer P7eNPBEΔ2 in comparison with the P7Ur8 and the P7eNPBE. Boxes in sequence from A indicate putative transcription factor-binding sites modulating enhancer activity, and include cMyb (light blue box), Klf4 (pink box), ETS1 (dark blue boxes) and Zeb1 (red boxes). (B-E′) These transcription factors were overexpressed by electroporation to assess possible effect on P7eNPBE enhancer activity. (**B**) At HH8, P7eNPBE (green) is normally robustly expressed in the neural plate and neural plate border (arrowheads) and is restricted from the non-neural ectoderm (arrows). (**C-D′**) Ectopic expression of either Zeb1 (C,C′, red) or ETS1 (D,D′, red) does not alter the normal expression of P7eNPBE (green), which is still present in the neural plate and neural plate border (arrowheads), and is restricted from the non-neural ectoderm (arrows). (**E**,**E′**) At HH8, when ETS1 and cMyb (red) are ectopically expressed together, P7eNPBE (green) expression is no longer restricted from the non-neural ectoderm (open arrowhead) and is in a ‘salt and pepper’ pattern. Although some cells in the neural plate and neural folds still express P7eNPBE (closed arrowhead), there are many cells that do not (arrows). np, neural plate; npb, neural plate border; nne, non-neural ectoderm; nf, neural folds.

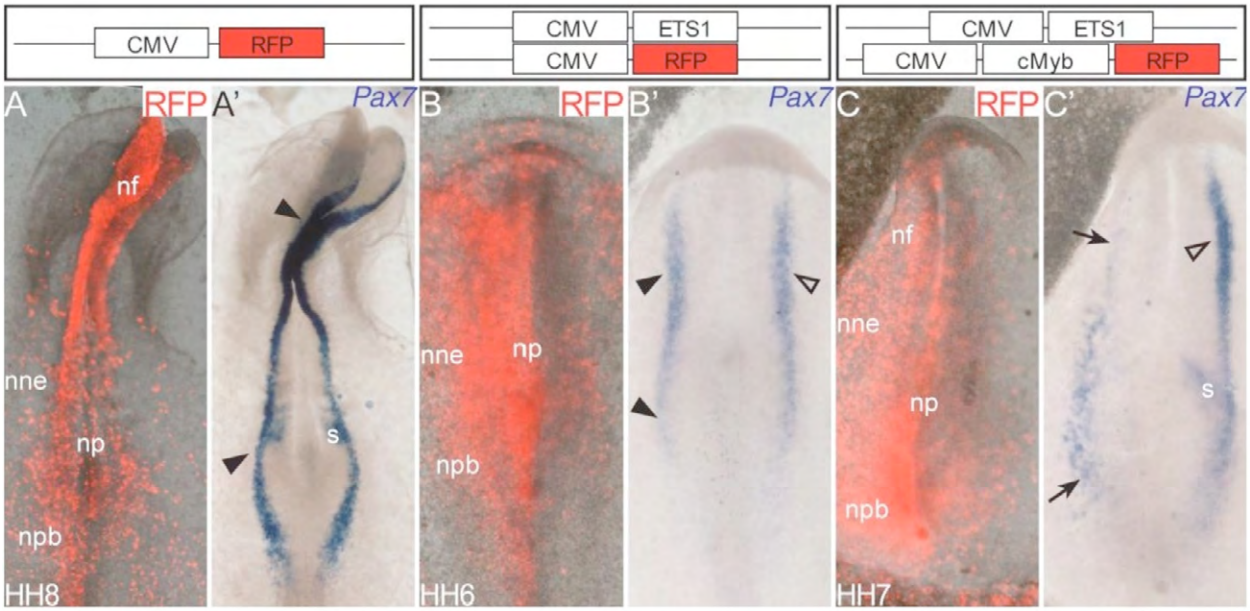

Fig. S3. Ectopic expression of ETS1 does not alter Pax7 mRNA expression. (A-C′) Empty vector control (A,A′), ETS1 (B,B′) or ETS1 and cMyb (C,C′) were ectopically expressed in embryos, which were subsequently stained for *Pax7* mRNA by in situ hybridization. (**A**,**A′**) At HH8, embryos misexpressing the control express *Pax7* (blue) in the neural folds and somites (closed arrowheads) as normal. (**B**,**B′**) At HH6, embryos misexpressing ETS1 display normal *Pax7* mRNA expression (blue) in the electroporated neural fold (closed arrowheads) when compared with the untreated neural fold (open arrowhead). (**C**,**C′**) At HH7, embryos treated with both ETS1 and cMyb show reduced levels of *Pax7* mRNA (blue) in the electroporated neural fold (arrows) and in the first forming somite (double arrowhead) compared with the untreated side (open arrowheads). The expression is not expanded into the neural plate or non-neural ectoderm. np, neural plate; npb, neural plate border; nne, non-neural ectoderm; nf, neural folds; s, somites.

Table S1. Summary of binding sites affected by mutagenesis

Mutation	1	2	3	4	5	6	7	8
Wild type sequence	GAGCCA	ACAGCC	TCCCTGC	TGCCTG	GAGCAC	CCATAC	AGGTTCT	TTGGAAA
Mutated sequence	TCTAAC	CACTAA	GAAAGTA	GTAAGT	TCTACA	AACGCA	CTTGGAG	GGTTCCC
Activity	+ (weak)	-	-	+/– (+ before HH8)	+/– (+ before HH8)	+/– (+ before HH8)	+/– (+ after HH8)	-
Sites deleted or moved	FOXA1 NFIC TFAP2A BRCA1 Mafb Myb	FOXA1 TFAP2A BRCA1 Mafb Myb BRCA1 Klf4	Klf4 ETS1 BRCA1 Mafb	SP1 BRCA1 Mfab Arnt::Ahr	INSM1 Arnt::Ahr Zeb1	INSM1 MZF1_5_13 Arnt::Ahr Zeb1 YY1 GATA2 FOXC1	Zeb1 SPIB	BRCA1 CEBPA ELF5 ETS1 NFIC HoxA5 NFATC2 REL REL
Sites added	GATA2 BRCA1	RUNX1 ZNF354C	ELK1 ELK1	FOXc1 AP1	ZNF354C FOXC1	RUNX1 En1	EBF1 Hltf	ESR1 En1

	GATA3	SOX10	E2F1	Arnt::Ahr	FOXL1	BRCA1	Zeb1	ESR2
	RUNX1	HoxA5	TFAP2	SPIB	Zeb1	SOX10	TFAP2A	NFKB1
	SOX10	GATA3	FEV	NKX3-1		Zfp423	NFIC	ETS1
		Pdx1	FOXC1			Zfp423	ZNF354C	
		ELF5	SP1			Arnt::Ahr	Znf143	
		FEV	NKX3-1			Hltf		
		SPI1	Prx2			ETS1		
			FOXL1			Nr2e3		
			GATA2			Nr2e3		

Sequence blocks of 6-7 bp were mutated in the enhancer P7eNPBE to determine the critical bases for enhancer activity. Putative binding sites were identified in wild-type and mutated forms of the enhancer using JASPAR (Bryne et al., 2008). All mutated forms of P7eNPBE modulated the activity, and all of them eliminated several overlapping binding sites while adding several new sites.

Table S2. Primers for *Pax7* upstream regions (putative enhancer regions)

Region	Primers (5′-3′)
P7Ur1	GAGCGGTACCCTCCTCTGAGCATTTCCACC GAGCCTCGAGTCACAACTTATTTCTCGGCG
P7Ur2	GAGCGGTACCCCTCTGAGCATTTCCACCC GAGCCTCGAGGAGCAGCATGGAAAATAGCC
P7Ur3	GAGCGGTACCATTTCCAGTTCCCATTCTGC GAGCCTCGAGACGTCCAAAGCAACTCTTCG
P7Ur4	GAGCGGTACCAGAGAAGGAAACCTCTCCCC GAGCCTCGAGGGACAAATCCTATCTGTAAGACGC
P7Ur5	GAGCGGTACCTGATGACCAAAACTGGGAGC GAGCCTCGAGCAATAATCACTGCTGCTTGGG
P7Ur6	GAGCGGTACCTAACCATGTCCCTCAGTGCC GAGCCTCGAGCACTTGGTTGTAGGAATGGG
P7Ur7	GAGCGGTACCCACCGAGTTTCACGTTAGGG GAGCCTCGAGGGAGGTTGCTACAATGAGGG
P7Ur8	GAGCGGTACCGCACAGAAAGGCAATAACCC GAGCCTCGAGAAAGCAACTATAAAACCCCGC
P7Ur9	GAGCGGTACCTTTTAGCAGTGTGTTTGCGG GAGCCTCGAGGACAGGGAAACACACCCAAC
P7Ur10	GAGCGGTACCGTTGTGTTCCATCACCTCCC GAGCCTCGAGGTTGGGTGAAAACACTTGCC
P7Ur11	GAGCGGTACCAAGGAAGAGGAAATGCAGGG GAGCCTCGAGTATTTCAGAGGGACTTCCGC
P7Ur12	GAGCGGTACCGAAGGAGCTCTCAAACACCG GAGCCTCGAGAATCACCCATACTTTCCCCC

Table S3. Primers used to generate deletions and smaller constructs from P7Ur8

Region	Primers (5'-3')
P7Ur8Δ1	AACATTTTTCCCTCTTCTTCCCCTGTGCT AGCACAGGGGAAGAAGAGGGAAAAATGTTT
P7Ur8Δ2	CCTCTCCCATTGGGGCCATCTGGGTTTTGG CCAAAACCCAGATGGCCCCAATGGGAGAGG
P7Ur8Δ3	TTTAACCTTTTCTTCGGCAGTTAGAAGGCA TGCCTTCTAACTGCCGAAGAAAAGGTTAAA
P7Ur8Δ4	CCAAAATTTCCATCTCAGGAGAGACGGAAC GTTCCGTCTCTCCTGAGATGGAAATTTTGG
P7Ur8Δ5	ATTGCAACGTGGCAGAGAGGATGAAAAGAG CTCTTTTCATCCTCTCTGCCACGTTGCAAT
P7Ur8Δ6	TGAATCTCACACAGGACAGGTTCTGCAGGT ACCTGCAGAACCTGTCCTGTGTGAGATTCA
P7Ur8Δ7	CACAAAAAGCAGAGGGAAACTCAACTCAAA TTTGAGTTGAGTTTCCCTCTGCTTTTTGTG
P7Ur8Δ8	GGAGAGCTCTGCTCGGACGAGTCCTAAGC GCTTAGGACTCGTCCGAGCAGAGCTCTTCC
P7Ur8Δ9	GAGCGGTACCGCACAGAAAGGCAATAACCC CTCAGAGCTCTGTGTGAGATTCACTGAT
P7Ur8Δ9G	CTCAGAGCTCTGTGTGAGATTCACTGAT CTGAGAGCTCTCGACTTCAAGGAGGACG CTGAGAGCTCTCCTCGATGTTGTGGCGGATCTTGAA
P7eNPBE	GACTGGTACCTTCATCCTCTGCTTTTTGTG GTAGCTCGAGTCACACAGGTCCAGGAGAGA
P7eNPBED1	GTAGCTCGAGTCACACAGGTCCAGGAGAGA ATTACCCGGGAGGTCCAGGAGAGACGGA
P7eNPBED2	ATTACDCCGGGAGCCAACAGCCCTCC GTAGCTCGAGTCACACAGGTCCAGGAGAGA

Primer pairs designed to generate and clone specific deletions (Δ) of Pax7 upstream region 8 (P7Ur8), as well as the smaller elements derived from it.

Table S4. Mutagenesis analysis of P7eNPBE

Region	Primers (5′-3′)
M1	GCTCAGCAGGCAGCAGGGAGGGCTGTGTTAGATTTTCATCCTCTGCTTTTTGTGATGC GCATCACAAAAAGCAGAGGATGAAAATCTAACACAGCCCTCCCTGCTGCCTGCTGAGC
M2	GGGTGCTCAGCAGGCAGCAGGGAGTTAGTGTGGCTCTTTTCATCCTCTGCTTTT AAAAGCAGAGGATGAAAAGAGCCACACTAACTCCCTGCTGCCTGCTGAGCACCC
M3	GCAGAACCTGTATGGGTGCTCAGCAGGCATACTTTCGGGCTGTTGGCTCTTTTCATCC GGATGAAAAGAGCCAACAGCCCGAAAGTATGCCTGCTGAGCACCCATACAGGTTCTGC
M4	GCAGAACCTGTATGGGTGCTCAGACTTACGCAGGGAGGGCTGTTGGCTCTTT AAAGAGCCAACAGCCCTCCCTGCGTAAGTCTGAGCACCCATACAGGTTCTGC
M5	CCAAACCTGCAGAACCTGTATGGTGTAGAAGCAGGCAGCAGGGAGGGCTGTT AACAGCCCTCCCTGCTGCCTGCTTCTACACCATACAGGTTCTGCAGGTTTGG
M6	TCGTTTCCAAACCTGCAGAACCTTGCGTTGTGCTCAGCAGGCAGCAGGGAGG CCTCCCTGCTGCCTGCTGAGCACAACGCAAGGTTCTGCAGGTTTGGAAACGA
M7	AGCCCGGGTCGTTTCCAAACCTGCCTCCAAGGTATGGGTGCTCAGCAGGCAGCAG CTGCTGCCTGCTGAGCACCCATACCTTGGAGGCAGGTTTGGAAACGACCCGGGCT
M8	TCTTACGCGTGCTAGCCCGGGTCGGGGAACCACCTGCAGAACCTGTATGGGTGCT AGCACCCATACAGGTTCTGCAGGTGGTTCCCCGACCCGGGCTAGCACGCGTAAGA

Mutations (red sequence) consisting of changes in groups of 6 to 7 bp at a time introduced in the P7eNPBE.

Table S5. Primers used to generate overexpression constructs

Clone	Primers (5′-3′)
Klf4	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGCCGCCATGGCTGTCAGCGACGCG GGGGACCACTTTGTACAAGAAAGCTGGGTAAAAATGCCTCTTCATGTG
Zeb1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGCCGCCATGGCGGATGGCCCCAGGTGTAA GGGGACCACTTTGTACAAGAAAGCTGGGTAGGCTTCATTTGTCTTTTC
DN-cMyb	GCGCTCTAGAAATGGGCCGGAGACCC CTTACCCGGGTTAGGAATTCCAGTGGTTCTT