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Sequence alteration in the enhancer contributes to the heterochronic *Sox9* expression in marsupial cranial neural crest

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ABSTRACT

Neonates of marsupial mammals are altricial at birth, because their gestation period is relatively short compared to placental mammals. Yet, as they need to travel to the teat from the birth canal, and suckle on the mother's milk, forelimbs and jaws develop significantly early. Previous studies in opossum (*Monodelphis domestica)*, an experimental marsupial model, have revealed that cranial neural crest cells are generated significantly early compared to those in placental mammals, such as mouse, leading to an early development of jaw primordia. We have previously found that *Sox9*, an important neural crest-specifier gene, is expressed in the future cranial neural crest of the opossum embryonic ectoderm significantly earlier than that in mouse or quail embryos. As *Sox9* is essential for neural crest formation in various vertebrates, it seems likely that the heterochronic expression of *Sox9* is critical for the early cranial neural crest formation in the marsupial embryos. In this study, we show a marsupial-specific sequence in the *Sox9* neural crest enhancer *E3*. We also reveal that the mouse *E3* enhancer is activated in the cranial neural crest cells of quail embryos, that the *E3* enhancer with marsupial-specific sequence is activated earlier in the *Pax7*-expressing neural border prior to the onset of endogenous *Sox9* expression, and that a mis-expression of *cMyb*, which is also a transcriptional activator of *Pax7*, in the neural border can ectopically activate the "marsupialized" enhancer. Thus, we suggest that the modification of the *E3* enhancer sequence in the marsupial ancestor would have promoted the early expression of *Sox9* in the neural border, facilitating the early formation of the cranial neural crest cells and the subsequent heterochronic development of the jaw primordia.

1. Introduction

Heterochrony is a concept of temporal shift of developmental program, and has been suggested as a strong driving force of morphological changes/modifications during evolution (Hall, 2003; Held, 2014). One of the well-known examples is axolotl (*Ambystoma mexicanum*), which reaches its sexual maturity without metamorphosis (e. g. Tompkins, 1978). Such retention of the juvenile traits into adult is termed neoteny, within the heterochrony concept, paedomorphosis. While the axolotol neoteny affects the entire body, certain heterochronies only occur in specific body parts. Neonates of marsupial (metatherian) mammals, such as kangaroo and koala, are altricial at birth, because their gestation period is relatively short compared to placental (eutherian) mammals. Yet, as they need to travel to the teat from the birth canal, and suckle on the mother's milk, forelimbs and jaws develop significantly early (Vaglia and Smith, 2003; Keyte and Smith, 2010; Debora and Sears, 2010. See also Smith, 2001 and references therein).

Neural crest (NC) cells generate a wide variety of cell types and tissues in vertebrates, and cranial neural crest (CNC) cells are, in particular, the essential mesenchymal component of the jaw primordia (Le Douarin and Kalcheim, 1999; Hall, 2000). Mechanisms of NC formation have been extensively studied in vertebrate experimental models, and in recent years, a "two-step model" has been proposed for NC specification (Betancur et al., 2010; Stuhlmiller and García-Castro, 2012; Pla and Monsoro-Burq, 2018). Thus, under the influence of FGF, Wnt, and BMP signals, the neural border (NB) is specified between non-neural and neural ectoderm, and expresses NB-specifier genes, such as *Pax7*. Then, subsequent activation of Wnt and BMP signals induces expression of NC-specifier genes, such as *Snail1/2*, *Foxd3*, and *Sox9/10* in the NB. These NC-specifier genes then promote the following NC cell developmental events, including epithelial-mesenchymal transition (EMT), migration, and differentiation.

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Previous studies in gray short-tailed opossum (*Monodelphis domestica*), the most commonly used experimental marsupial model, showed that CNC cells are generated significantly early compared to those in placental mammals, such as mouse (Vaglia and Smith, 2003; see also Smith, 2001, 2006 for reviews). Thus, among the concepts of heterochrony, marsupial CNC development can be categorized as pre-displacement within peramorphosis. We have previously examined the expression of genes known to be important for the CNC development, and found that the *Sox9* gene, which encodes a HMG-type transcription factor, is expressed in the future CNC of the opossum embryonic ectoderm significantly earlier than in mouse or quail embryos (Wakamatsu et al., 2014). Since the importance of *Sox9* function in NC specification, EMT, and differentiation of cranial cartilages has been extensively studied in other experimental vertebrate models, such as mouse, chick, frog, and fish (Mori-Akiyama et al., 2003; Akiyama et al., 2004; Sakai et al., 2006. See also Sakai and Wakamatsu, 2005; Milet and Monsoro-Burq, 2012 for reviews), it seems likely that the heterochronic expression of *Sox9* is critical for the early CNC cell formation in the marsupial embryos (Wakamatsu et al., 2014).

It has not been fully understood how *Sox9* expression is regulated in the CNC cells, but previous efforts have identified the "*E3* neural crest enhancer" in mouse, by comparing genomic sequences of mouse and puffer fish, and subsequently by making a transgenic mouse line carrying the *E3* enhancer and *Sox9* promoter in the upstream of *LacZ* reporter gene (Bagheri-Fam et al., 2001, 2006). In this study, we have compared the genomic sequences of *E3* among various mammals including marsupial species, and found a short stretch of sequence only conserved among marsupial *E3* sequences. We have subsequently compared the enhancer activities of mouse *E3* and *E3* with marsupial-specific nucleic acid substitutions in avian embryos, and found that the "marsupialized" *E3* enhancer is activated in NB, the future CNC/NC cells, much earlier than the wild type *E3* reporter or the endogenous *Sox9* gene. Our results indicate that the "marsupialized" *E3* enhancer might, in part, use the NB-specification machinery for its activation. We therefore suggest that the modification of the *E3* enhancer sequence in the marsupial ancestor would have promoted the early expression of *Sox9*, facilitating early formation of the CNCs, and the subsequent heterochronic development of the jaw primordia.

2. Materials and Methods

2.1. Experimental animal

Animal experiments were conducted in accordance with the guidelines of Tohoku University (Regulations for Animal Experiments and Related Activities at Tohoku University), with approval of the Tohoku University Medical School Animal Experiment Committee (2015MdA-129-1, 2017MdA-209, 2018MdA-063).

Fertilized Japanese quail (*Coturnix japonica*) eggs were obtained from local farm (Quail Factory-Tydess). Eggs were incubated at 38 °C in a humidified incubator, and embryos were staged as previously described (Hamburger and Hamilton, 1951; Ainsworth et al., 2010).

2.2. Antibodies and immuno-staining

Anti-Sox9 rabbit monoclonal antibody (Abcam, EPR14335), anti-cMyb rabbit polyclonal antibody (Novus Biologicals, NBP1-80306), and anti-Pax2 polyclonal rabbit antibody (Zymed, see also Wakamatsu, 2011; Shida et al., 2015), were commercially obtained. Anti-Pax7 mouse monoclonal antibody was obtained from Developmental Study Hybridoma Bank (Wakamatsu, 2011). HNK1 (mouse IgM, Tucker et al., 1988) was used as described previously (Wakamatsu et al., 2004). Cy3-conjugated secondary antibodies were purchased from Jackson Immuno Research.

Immunological staining on sections was performed as described previously (Wakamatsu et al., 1993; Wakamatsu and Weston, 1997). Sections treated with the antibodies were also exposed to DAPI (Sigma) to visualize nuclei, and subsequently mounted with VectaShield mounting medium (Vector Laboratories). Fluorescent images were captured by AxioCam CCD camera on AxioplanII microscope (Zeiss).

2.3. Expression vectors

An expression vector of *monomeric red fluorescent protein* (*pCAGGS-mRFP1*) was previously described (Wakamatsu, 2011; Shida et al., 2015). A *LacZ* reporter plasmid carrying otic/neural crest enhancers (*E5*, *E4*, *E3*) and promoter of mouse *Sox9* gene (*pCMVβ-E5-E3Sox9^{p}-LacZ*, Bagheri-Fam et al., 2006) was kindly provided by Dr. Gerd Scherer. *LacZ* was replaced with *EGFP* derived from *pEGFP-N1* (*pE5-E3Sox9^{p}-EGFP*). A part of the *E3* enhancer sequence (CACATGCCACA) was replaced with a corresponding opossum sequence (TATGTATACTCAGTT) by PCR-amplifying *pE5-E3Sox9^{p}-EGFP* with primers (mSox9Enhancer1: TATACATATCAATCATCCTTTGACA, mSox9Enhancer2 CTCAGTT-CAGCCCTTGAAACACCA) and subsequent self-ligation (*pE5-E3-^{mut}Sox9^{p}-EGFP*, See also Fig. 1 for the position of the replaced sequence). *E5-E3Sox9^{P}* and *E5-E3^{mut}Sox9^{P}* sequences were further transferred to *pGL3-basic Luciferase* reporter vector (Promega) for luciferase assays (see below). An *EGFP*-reporter vector carrying *Pax7*-NB enhancer (*pP7Ur8-t-k-EGFP*) and an expression vector of *cMyb* (*pCIG-cMyb-IRS-EGFP*), were kindly provided by Dr. Martin García-Castro (Vadasz et al., 2013). The protein-coding region of *cMyb* cDNA was transferred to *pmiwSV* (*pmiw-cMyb*), and the expression of the cMyb protein was confirmed by anti-cMyb immuno-staining in transfected NIH3T3 cells. A *red fluorescent protein* reporter with chicken *Sox2 N2* enhancer was kindly provided by Dr. Masanori Uchikawa (Uchikawa et al., 2003). Expression vectors of *MybL1* and *MybL2* were generously provided by Dr. Fumihiko Okumura (Okumura et al., 2016).

2.4. Ex ovo electroporation

Electroporation of stage 3.5–5 quail embryos was essentially performed as described (Wakamatsu, 2011). In brief, albumen was carefully removed, and vitelline membrane-enveloped yolk and embryo were transferred to an HBSS-filled Petri dish. HBSS-diluted ink was injected underneath the embryo for a better visualization of the embryo. A rod–like positive electrode was inserted underneath the embryo. DNA solution (less than 1 μl of 5 μg/μl in PBS containing 0.025% Fast Green) was locally injected in between vitelline membrane and epiblast/embryonic ectoderm, and a tungsten needle as a negative electrode was placed over the vitelline membrane. Electroporation was performed with CUY21 electroporator (BEX, condition: 3 V, 25 ms duration, 225 ms interval, and 3 pulses). The electroporated embryos on the vitelline membrane-enveloped yolk were transferred to an egg white-filled glass jar, and incubated at 38 °C.

2.5. Luciferase assay

NIH3T3 cells were transfected with a *Luciferase* reporter and effecter plasmid DNAs with LipofectAMINE Plus reagent (Invitrogen). An expression vector of *Renilla luciferase* (*pGL4.74 [hRluc/tk]*, Promega) was always co-transfected to normalize the transfection efficiency. Cell lysates were collected and assayed for luciferase activity with PicaGene Dual Sea Pansy Luminescence Kit (Toyo Ink Manufacturing) by Lumat LB9507 Luminometer (Berthold Technologies) after 24 h of culture. For luciferase assay with embryonic tissues, electroporation to stage 3.5–4 embryos were performed as described above. Seven hours after electroporation of the *Renilla* and firefly *luciferase* reporter plasmids, and *pCAGGS-mRFP*, with or without the *cMyb* expression vector, strips of electroporated tissues with NB was excised with a tungsten needle, and the tissue fragments were subsequently incubated in a pancreatin solution. Dissociated ectoderm and mesoderm tissues (5–6 fragments/assay) were separately processed for luciferase assay as indicated above.

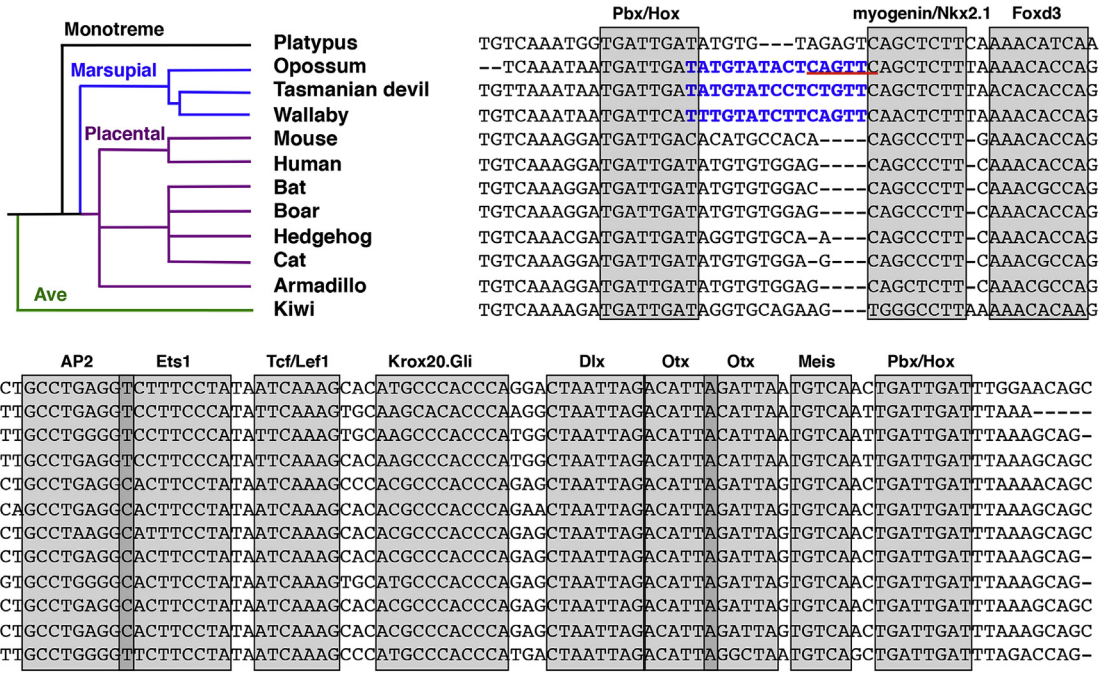

Fig. 1. Phylogenetic tree and a sequence comparison of *Sox9 E3* enhancers. Sequences of following species are used as representatives of each group. Platypus as a monotreme, opossum (Didelphimorphia), Tasmanian devil (Dasyuromorphia), and wallaby (Diprotodontia) as marsupials, mouse (Rodentia), human (Primate), bat (Chiroptera), boar (Artiodactyla), hedgehog (Eulipotyphla), cat (Carnivora), and armadillo (Cingulata) as placentals, and kiwi as an ave. Predicted transcription factor binding sites indicated in a previous study (Bagheri-Fam et al., 2006), are boxed. DNA sequences conserved among marsupial species are indicated in blue, and a potential binding site for Myb family protein is underlined in red.

3. Results

3.1. Interspecies comparison of the Sox9 "neural crest enhancer" sequences

Previous studies identified the *E3* NC enhancer for *Sox9* in the mouse genome (Bagheri-Fam et al., 2001, 2006). While the *E3* enhancer drove a *LacZ* reporter in the CNC of transgenic mouse embryos, the *E4* and *E5* enhancers amplified the activity of *E3* (Bagheri-Fam et al., 2006). We thus compared the corresponding *E3* enhancer sequences among mammalian species, including a monotreme (platypus: *Ornithorhynchus anatinus*), a few marsupials (gray short-tailed opossum: *Monodelphis domestica*, Tasmanian devil: *Sarcophilus harrisii*, and tammar wallaby: *Macropus eugenii)*, as well as several placentals spanning various orders (house mouse: *Mus musculus*, human: *Homo sapiens*, Seba's short-tailed bat: *Carollia perspicillata*, wild boar: *Sus scrofa*, four-toed hedgehog: *Atelerix albiventris*, cat: *Felis catus*, nine-banded armadillo: *Dasypus novemcinctus*). While the *E3* NC enhancer sequences were found and highly conserved in these mammals, we have also noticed that a short stretch of sequences was only conserved among marsupials (Fig. 1 in blue). This sequence might simply reflect a kinship of marsupial species, and might not have any functional significance. Alternatively, this unique sequence may modify the *E3* activity, contributing to the marsupial-specific expression of *Sox9* in the CNC cell lineage. We also found the *E3* sequence in the kiwi (*Apteryx australis*) genome, and included it as an avian example for a comparison (Fig. 1). The kiwi *E3* does not show a homology to the marsupial-specific sequence.

3.2. Temporal shift of the NC enhancer activity in vivo

Next, we aimed to test if the activity of *Sox9* NC enhancers would differ between placentals and marsupials. For this purpose, quail embryo was chosen as an assay system, because birds are evolutionary neutral to both placental and marsupial mammals, and would likely have similar regulatory mechanism(s) through the *E3* enhancer (see above) for *Sox9* expression in the NC lineage. We first constructed *EGFP* reporters carrying the mouse *E5-E3* enhancers and the *Sox9* promoter (*pSox9^{p}-EGFP*, *pE3Sox9^{p}-EGFP*, *pE5-E3Sox9^{p}-EGFP*, Fig. 2A). To visualize electroporated cells, a *red fluorescent protein* expression vector with non-specific enhancer and promoter (*pCAGGS-mRFP1*) was co-electroporated. When quail embryonic ectoderm around the future cranial neural folds at stage 5 was electroporated (Fig. 2B), mRFP1 expression was broadly observed in the ectodermal tissues, such as epidermal ectoderm, otic placode, brain, neural tube, trunk NC cells, and CNC cells 17 and 24 h after electroporation (Hamburger and Hamilton (HH) stage 9–10 and stage 11, respectively, Fig. 2C', D′, E′, F′, G′). While EGFP expression was barely detected in *pSox9^{p}-EGFP*–electroporated embryos (Fig. 2C-C"), a specific expression of EGFP was observed in the otic placode (see also Shida et al., 2015) and some CNC cells in *pE3Sox9^{p}-EGFP*– electroporated embryos (Fig. 2D-D″). Such expression was more pronounced in the *pE5-E3-Sox9^{p}-EGFP*–electroporated embryos (Fig. 2E-F″), suggesting that *E4* and *E5* sequences enhanced *E3* activity in the avian context, as previously observed in the transgenic mouse embryos (Bagheri-Fam et al., 2006). In particular, EGFP expression in the migrating CNC cells was clearly detected 24 h after electroporation (Fig. 2F-G"').

Next, we have generated a reporter with a sequence replacement in the *E3* enhancer, so that the sequence conserved among marsupials was introduced (*pE5-E3^{mut}Sox9^{p}-EGFP*, Fig. 2A, see also Materials and Methods for details). When *pE5-E3^{mut}Sox9^{p}-EGFP* was electroporated into the ectoderm of stage 5 embryos, however, unlike *pE5-E3Sox9^{p}-EGFP*-electroporated embryos (Fig. 2F-F″), EGFP expression was only observed in the trunk NC cells, but not in the CNC cells 24 h after electroporation (HH stage 11, Fig. 2H-H″). These results indicate that the wild type mouse *E3* enhancer and "marsupialized" enhancer have non-identical enhancer activities at least in the avian context, but it was unclear if the "marsupialized" enhancer could contribute to *Sox9* expression in the CNC cells.

Our previous study had revealed that the onset of *Sox9* expression in

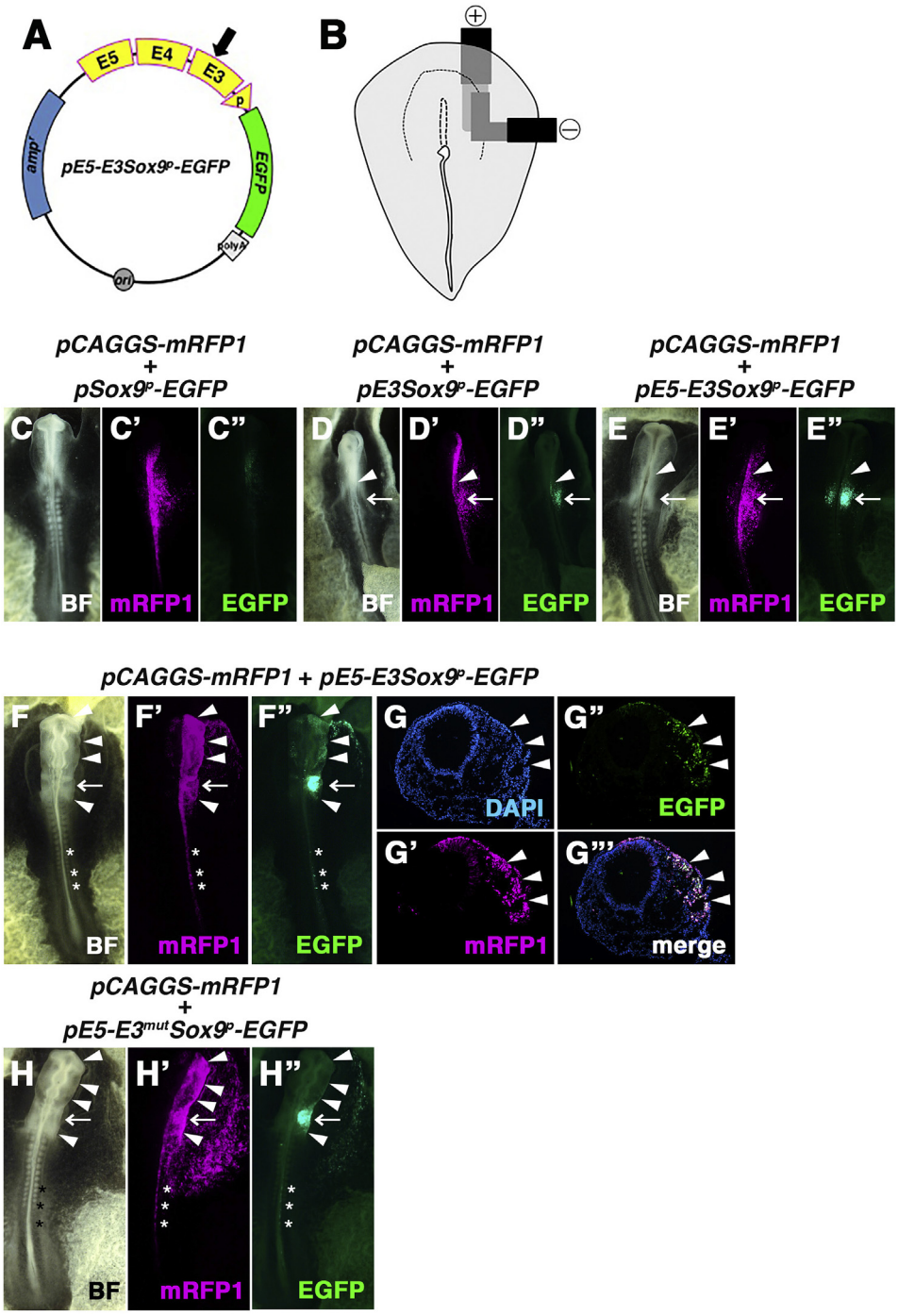

Fig. 2. Evaluation of mouse NC enhancer-*EGFP* reporters in quail embryos. A. *pE5-E3Sox9ᵖ-EGFP* reporter plasmid. An arrow indicates the position of the sequence substitution in the "marsupialized" *pE5-E3mutSox9ᵖ-EGFP* reporter. B. Position of electrodes for electroporation to stage 5 quail embryo. DNA will be overlaid onto the ectoderm, a positive electrode will be inserted underneath the embryo, and a negative electrode will be placed over the future neural fold. C-E". *EGFP* reporter expressions in whole-mount dorsal views 17 h after electroporation at stage 5. BF in C, D, E: bright field. Expression of mRFP1 indicates electroporated areas (C′, D′, E′). While EGFP fluorescence is barely detectable in an enhancer-less *pSox9ᵖ-EGFP*-electroporated embryo (n = 3/3, C″), a weak EGFP expression was detected in CNC (arrowhead) and otic placode (arrow) in a *pE3Sox9ᵖ-EGFP*-electroporated embryo (n = 3/3, D″). EGFP fluorescence in the CNC (arrowhead) and the otic placode (arrow) is more pronounced in a *pE5-E3Sox9ᵖ-EGFP*- electroporated embryo (n = 4/4, E″). F–F". *EGFP* reporter expression 24 h after *pE5-E3Sox9ᵖ-EGFP* electroporation at stage 5. EGFP is expressed in the CNC cells (arrowheads, n = 5/6), trunk NC cells (asterisks, n = 4/6), and otic cup (arrow, n = 6/6). G-G‴. EGFP expression in the midbrain CNC cells on a transverse section 24 h after electroporation of *pE5-E3Sox9ᵖ-EGFP*. Migrating CNC cells (arrowheads) are EGFP-positive. DAPI indicates nuclei. H–H". The "marsupialized" *pE5-E3mutSox9ᵖ-EGFP* reporter is not activated in the CNC cells of quail embryos electroporated at stage 5. EGFP is expressed in the trunk NC cells (asterisks) and otic cup (arrow), but not in the CNC cells (arrowheads, n = 5/5).

the cranial region of the opossum embryos was similar to that of *Pax7* (Wakamatsu et al., 2014), while in avian embryos *Pax7* expression well preceded *Sox9* expression, as a NB-specifier gene (Wakamatsu et al., 2014. See also Basch et al., 2006). A NB enhancer of chicken *Pax7* was previously identified by testing the activity of *EGFP* reporter with this enhancer in chicken embryos (Vadasz et al., 2013). In that study, the reporter genes were electroporated into the epiblast cells nearby primitive streak, because the NB is specified as soon as epiblast cell differentiate to ingressing mesoderm/endoderm and ectodermal tissues. We thus electroporated reporter genes to the similar epiblast region at stage 3.5–4 of quail embryos (Fig. 3A). Embryos younger than this stage were not examined, because embryos did not develop normally after electroporation. We first tested the *EGFP* reporter construct carrying the *Pax7*-NB enhancer (*pP7Ur8-tk-EGFP*, see Vadasz et al., 2013) with *pCAGGS-mRFP1*. While mRFP1 expression was observed both in the epiblast/ectoderm cells and the ingressing mesoderm/endoderm cells, EGFP expression was highly restricted to the NB, 7 h after electroporation (HH stage 6–7, Fig. 3B-B"). We also co-electroporated *pP7Ur8-tk-EGFP* along with RFP reporter carrying chicken *Sox2* neural plate enhancer *N2* (Uchikawa et al., 2003), and observed the *Pax7* enhancer activity along the lateral edge of the neural plate (Supplemental Fig. 1), further confirming that our procedure was fit to detect the enhancer activity in the

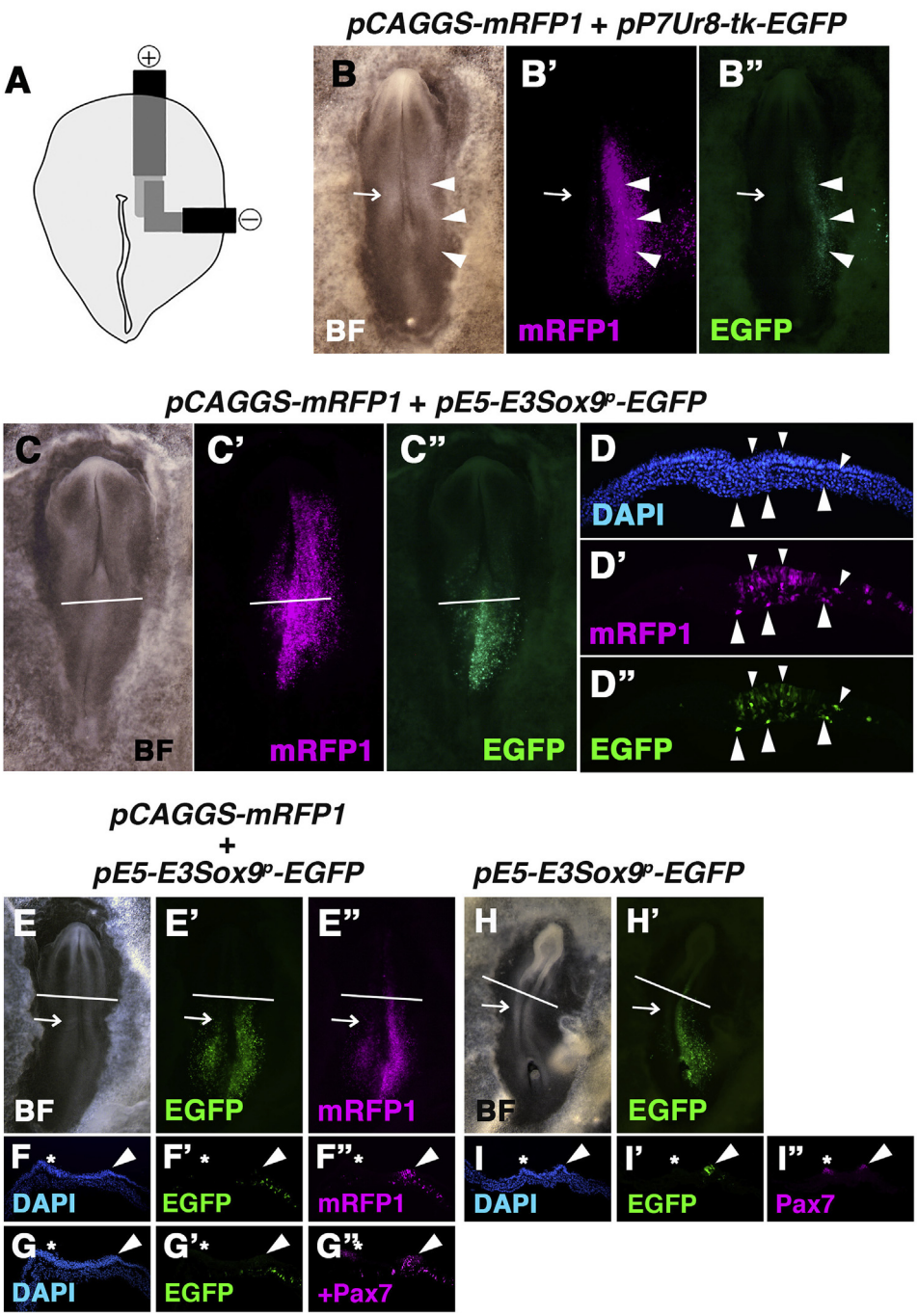

Fig. 3. The *pE5-E3Sox9^{p}-EGFP* reporter is not activated in the NB of quail embryos electroporated at stage 3.5–4. A. Position of electrodes for the electroporation. The negative electrode will be placed over the epiblast nearby the primitive streak. B–B". Expression of a *Pax7* NB-enhancer reporter (*pP7Ur8-tk-EGFP*) 7 h after electroporation. While mRFP1 expression is broadly detected (B′), EGFP fluorescence is highly restricted in the NB (n = 6/7, arrowheads in B″). BF: bright field. Arrows indicate the position of the first somite. C–C". Expression of *pE5-E3Sox9^{p}-EGFP* reporter 7 h after electroporation. EGFP expression is detected in/around the primitive streak (n = 6/6). Lines indicate approximate section plane in D-D". D-D". A transverse section of *pE5-E3Sox9^{p}-EGFP*-electroporated embryo, similar to C–C″ at the level of primitive streak. Epiblast/ectoderm cells close to the streak (small arrowheads) and ingressing mesoderm/endoderm cells (large arrowheads) are EGFP-labeled. DAPI indicates nuclei. E-E". Expression of *pE5-E3Sox9^{p}-EGFP* reporter 7 h after electroporation in an embryo slightly older than C. Lines indicate approximate section plane in F-G". Arrows indicate the position of the first somite. F–F". A transverse section showing the lack of EGFP-positive cells in the electroporated (arrowheads) and not electroporated (*) neural folds. G-G". A transverse section showing Pax7 expression in the electroporated (arrowheads) and contra-lateral (*) neural folds. H–H". Expression of *pE5-E3Sox9^{p}-EGFP* reporter 12 h after electroporation. mRFP1 was not co-electroporated. Lines indicate approximate section plane in I–I". Arrows indicate the position of the first somite. I–I". A transverse section showing Pax7 expression in the EGFP-positive (arrowheads) and contra-lateral (*) neural folds.

NB. Next, we electroporated *pE5-E3Sox9^{p}-EGFP* into the epiblast. EGFP expression was detected in the epiblast cells nearby the streak and ingressing cells of the electroporated embryos along with co-electroporated mRFP1, 7 h after electroporation (HH stage 6–7, Fig. 3C-D″). These expressions likely reflect the *Sox9* expression in mouse and chicken embryos (Akiyama et al., 2005; Sakai et al., 2006), as well as in the *E5-E3Sox9^{p}-LacZ* transgenic mouse (Bagheri-Fam et al., 2006). In such embryos, EGFP expression was not detected in the Pax7-positive NB of 7 h embryos (HH stage 6–7, Fig. 3E-G″). In the neural folds of embryos 12 h after electroporation, however, EGFP expression was observed in the Pax7-positive premigratory CNCs (HH stage 8, Fig. 3H-I"), suggesting that this timing was the approximate onset of *pE5-E3Sox9^{p}-EGFP* reporter activation in the CNC lineage.

In case of *pE5-E3^{mut}Sox9^{p}-EGFP*-electroporated embryos 7 h after electroporation, in addition to some expression nearby the primitive streak, EGFP was observed in the ectoderm (HH stage 6–7, Fig. 4A-A″), similar to the NB-specific activation of the *Pax7* NB reporter (Fig. 3B"). In fact, EGFP-positive cells co-expressed Pax7, further supporting our idea that the marsupialized *E3* enhancer was activated in the NB cells (HH stage 6, Fig. 4B-B″) prior to the onset of the endogenous *Sox9* expression at stage 6.5 at the midbrain level, and around stage 8 at the hindbrain level (See Sakai et al., 2006). Co-expression of Pax7 and EGFP was still maintained in the elevating neural folds anterior to the first somite 12 h after electroporation (HH stage 7.5, Fig. 4C-C″). These EGFP-positive cells in the neural folds of 7 and 12 h after electroporation were found next to posterior pre-placodal region expressing Pax2 (Supplemental

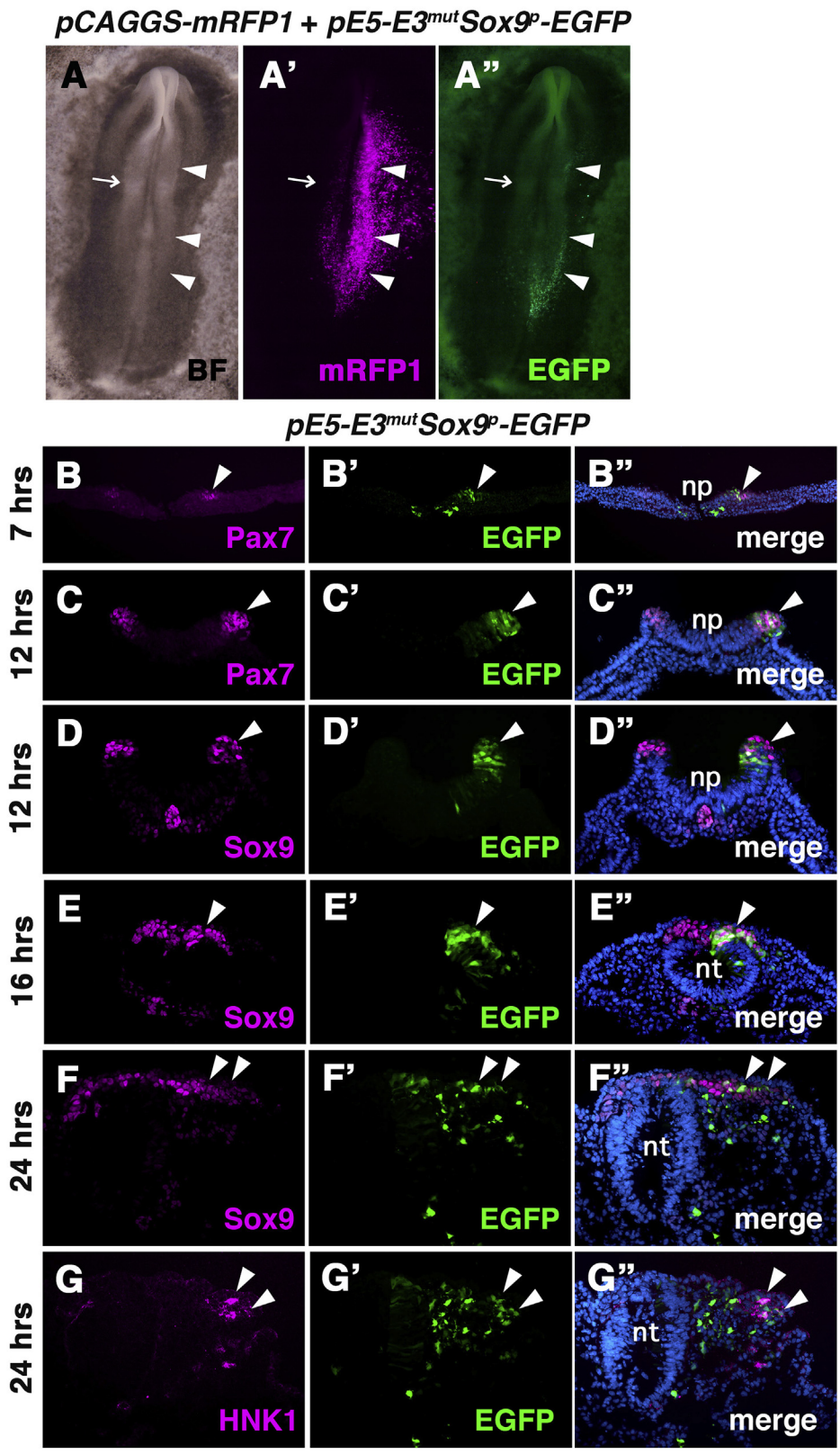

Fig. 4. The "marsupialized" reporter is activated in the NB of quail embryos electroporated at stage 3.5–4. A-A". Expression of *pE5-E3^{mut}Sox9^{p}-EGFP* reporter 7 h after electroporation. While a large area is electroporated (mRFP1 expression in A′), EGFP expression is highly restricted to the NB (n = 6/6, arrows in A″). Arrows indicate the position of the first somite. B-G". The EGFP-positive cells in the *pE5-E3^{mut}Sox9^{p}-EGFP*-electroporated quail embryos co-express NB and NC markers. Embryos electroporated at stage 3.5–4 were fixed 7, 12, 16, and 24 h after electroporation, and transversely sectioned. Sections at the level of hindbrain are stained with anti-Pax7 (NB marker, B, C), anti-Sox9 (pre-migratory, emigrating, and early migrating NC marker, D, E, F), and HNK1 (migratory NC cell marker, G) antibodies. Merged pictures also show nuclei with DAPI staining. Arrowheads indicate EGFP-positive NB/CNC cells. np: neural plate, nt: neural tube.

Fig. 2. See Wakamatsu, 2011; Shida et al., 2015), indicating the activation of the enhancer at the hindbrain level. To further characterize the fate of EGFP-positive cells, in which the marsupialized enhancer had been activated, we have examined expressions of endogenous Sox9 and HNK1, as NC-specifier and migratory NC cell markers anterior to the first somite 12, 16, and 24 h after electroporation (HH stage 8, 9, and 10, respectively, Fig. 4D-G″). The Sox9-positive CNC cells prior to EMT and emigration from the neural tube expressed EGFP (Fig. 4D-E″). EGFP-positive migrating CNC cells also expressed Sox9 (Fig. 4F-F″), and HNK1 (Fig. 4G-G"). These results suggested that the marsupial-specific sequence in the *E3* enhancer would contribute to the expression of *Sox9*, as early as the NB specification in the future CNC.

3.3. Marsupial E3 enhancer is regulated by NB inducing mechanisms

The marsupial-specific sequence in the *E3* enhancer possessed a potential binding site of Myb family proteins (see Fig. 1, underlined in red). Previous studies have revealed that the transcription factor cMyb is involved in NB specification and subsequent CNC formation (Vadasz et al., 2013; Betancur et al., 2014). It was also suggested that the *Pax7* expression in the NB is directly activated by cMyb (Vadasz et al., 2013). Thus, an expression vector of *cMyb* was co-electroporated into stage 3.5–4 quail embryos along with *pE5-E3^{mut}Sox9^{p}-EGFP* reporter. Compared to the embryos electroporated with *pE5-E3^{mut}Sox9^{p}-EGFP* alone (HH stage 6, Fig. 5A-A″), EGFP expression was more broadly observed in the *cMyb*-electroporated embryos (Fig. 5B-B”), suggesting that cMyb could activate the marsupialized enhancer either directly or indirectly.

Next, we quantitatively examined the enhancer activation by Myb family transcription factors (cMyb, MybL1, MybL2) (Fig. 5C). To do so, we constructed *Luciferase* reporter genes carrying *E5-E3* or *E5-E3^{mut}* enhancers, and performed dual Luciferase assays in NIH3T3 cells (See Materials and Methods). We did not detect, however, a specific activation of the reporter in *Myb* family transfection (Fig. 5C). In contrast, when we co-electroporated the *Luciferase* reporter genes into the epiblast of stage 3.5–4 embryos, *cMyb* could activate the *E5-E3^{mut}* reporter in the ectoderm (Fig. 5D). Thus, although the mechanism underlying the early activation of the marsupialized *E3* enhancer remains to be determined, this activation seemed to be context dependent (See also Discussion). A previous study showed that *cMyb* mRNA was broadly expressed in early chicken embryos, such as stage 4–5 (Vadasz et al., 2013. Betancur et al., 2014). Thus, we have examined cMyb protein expression at similar stages of quail embryos (Fig. 5E-F′). Although cMyb proteins were broadly detected (Fig. 5E’), their intracellular localization was often biased in the cytoplasm or in the nucleus (Fig. 5F, F’), suggesting post-translational regulations of cMyb function in vivo.

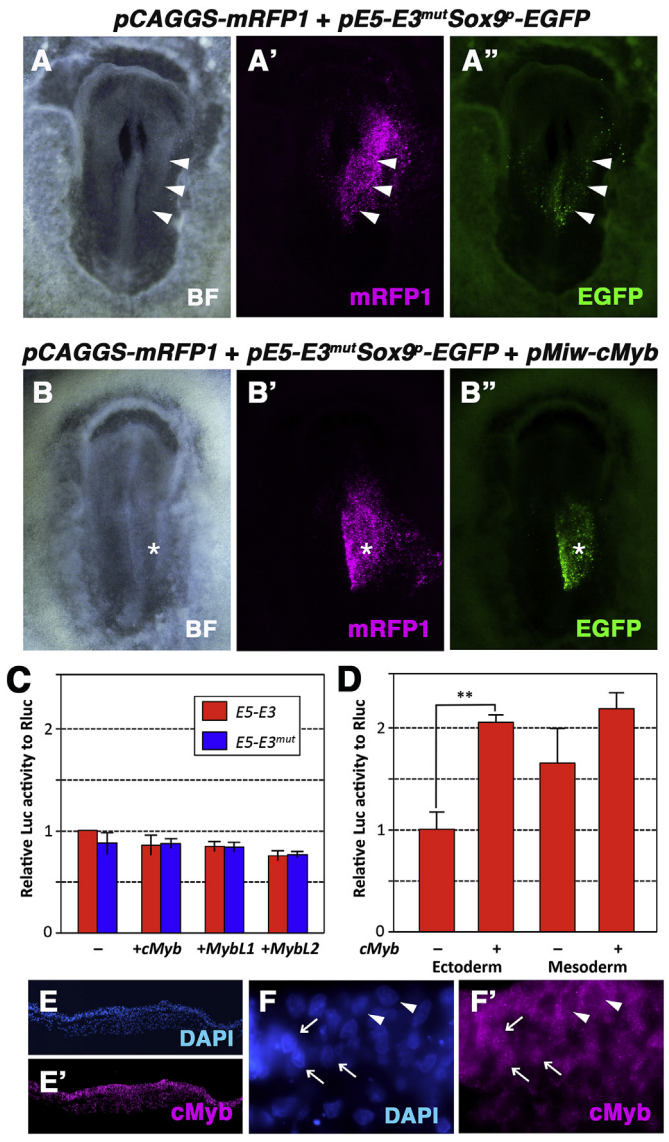

Fig. 5. Activation of the “marsupialized” *E3* enhancer by *cMyb*. A-A”. Seven hours after electroporation of the *pE5-E3^{mut}Sox9^{p}-EGFP* reporter to stage 4 quail embryos, the reporter is activated in the NB (arrowheads, n = 6/6). B–B”. When a *cMyb* expression vector is co-electroporated, in contrast, the reporter is ectopically activated (asterisk in B″, n = 7/7). C. Luciferase assay in NIH3T3 mouse fibroblast cell line. Results are shown as a fold-induction compared with the value obtained by a co-transfection of *pE5-E3Sox9^{p}-Luciferase* and empty *pmiwSV* vector. Neither *pE5-E3Sox9^{p}-Luciferase* nor *pE5-E3^{mut}Sox9^{p}-Luciferase* reporters are activated by co-transfection of *cMyb*, *MybL1*, or *MybL2* expression vectors. Error bars indicate standard deviations obtained from data of 3–4 independent experiments. D. Luciferase assay in electroporated embryonic ectoderm and mesoderm. Error bars indicate standard deviations obtained from data of 5 independent experiments. **<0.001 for student’s T-test. E and E’. Expression of cMyb protein in a transverse section of stage 4 embryo. F and F’. High magnification views of the section in E and E’. Arrows and arrowheads indicate nuclear and cytoplasmic localizations of anti-cMyb immuno-reactivity, respectively.

4. Discussion

The *Sox9* NC enhancer *E3* was originally identified in mouse (Bagheri-Fam et al., 2001, 2006), and in this paper, we have identified a marsupial-specific sequence alteration in the *E3*, by comparing corresponding sequences among monotreme, marsupial, and placental mammalian species. We also showed that, while the mouse *E3* enhancer was activated in the NC/CNC cells of quail embryos, the *E3* enhancer with the marsupial-specific sequence was activated in the *Pax7*--expressing NB significantly earlier than the mouse *E3* enhancer, ahead of the onset of endogenous *Sox9* expression (Fig. 6 for a summary, see also Sakai et al., 2006). These results are consistent with the fact that, in opossum embryos, *Sox9* is expressed in the NB/premigratory CNC cells as early as *Pax7* expression, unlike in the mouse or quail embryos (Wakamatsu et al., 2014). Thus, we suggest that the modification of the *E3* enhancer sequence in the marsupial ancestor would have promoted the early expression of the *Sox9* in the NB, and would have contributed to the early formation of the CNC cells and subsequent pre-displacement of the jaw development (see also Wakamatsu et al., 2014).

Because transgenic techniques are not available in any marsupials to date, we assayed the enhancer activities in the heterologous avian system. Yet, as the marsupial-specific sequence alteration in the *E3* enhancer resulted in the temporal shift of the reporter activation, this sequence

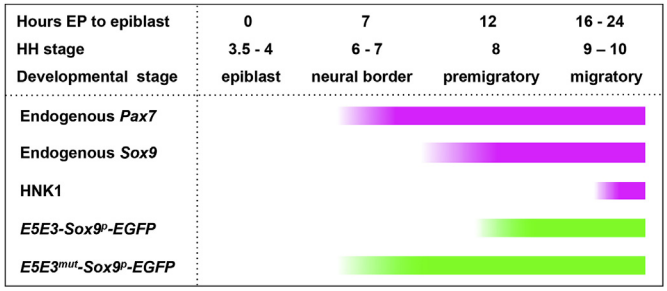

Fig. 6. Summary. Temporal correlation of developmental stages, hours after electroporation to stage 3.5–4 embryos, and expression of NB/NC markers and EGFP-reporters.

alteration could be sufficient to recapitulate the endogenous *Sox9* expression in the NB/CNC cells of marsupial embryos. However, in the transgenic mouse embryos carrying the *E3* enhancer-*LacZ* reporter gene, the reporter expression was only detected in a subset of the CNC cells (Bagheri-Fam et al., 2006). This limited expression of the transgene might be explained by a positional effect in the transgenic line in that study. Alternatively, there could be additional enhancers required to drive *Sox9* expression in the CNC cells. If there is any, theoretically, by comparing the genomic sequence of the *Sox9* topographically associated domain (TAD) (Franke et al., 2016) among marsupials and placental mammals, we would be able to identify such sequence conserved in the marsupials but not in the placentals. Yet, the genomic sequences of the *Sox9* TAD in three marsupials (opossum, wallaby, and Tasmania devil) appear to be too conserved to narrow-down candidate sequences (YW, unpublished observation). Therefore, to identify any additional heterochronic enhancers, the TAD of marsupial *Sox9* should be comprehensively examined for the enhancer activity in a marsupial context.

Previous studies have revealed that *cMyb* gene, which encodes one of the Myb family transcription factor, is involved in the NB specification and subsequent NC formation (Vadasz et al., 2013; Betancur et al., 2014), and that *Pax7* expression in the NB is directly activated by cMyb (Vadasz et al., 2013). Our *cMyb* misexpression experiment in quail embryos ectopically activated the marsupialized *E3* enhancer. Thus, it is reasonable to speculate that the similar mechanism might be used for the heterochronic expression of *Sox9* in the marsupial NB/CNC. It is unclear, however, if cMyb directly activates the marsupial *E3* enhancer, since in our luciferase assays with NIH3T3 cells *cMyb* failed to activate the reporter constructs. Thus, *cMyb*-mediated activation of the marsupialized *E3* enhancer may be context dependent, requiring additional signals/co-factors. In fact, the previous study has hypothesized an involvement of co-factor for the *Pax7* enhancer activation by cMyb (Vadasz et al., 2013). Because the *cMyb* mRNA is expressed broadly in the epiblast of early chicken embryos (Vadasz et al., 2013; Betancur et al., 2014), it is possible to propose such a co-factor responsible for the NB-restricted *Pax7* expression. Alternatively, but not exclusively, post-translational regulations of cMyb activity might also be involved (see Wang et al., 2018, for a review). In fact, both nuclear and cytoplasmic localizations of cMyb protein were observed in the early quail embryonic epiblast/ectoderm, suggesting that a nuclear-cytoplasmic shuttling of the cMyb proteins is controlled by post-translational mechanisms. Further studies are needed to understand how *cMyb* is involved in the NB specification of various vertebrates and in the heterochronic development of the marsupial CNC.

Our studies suggest that a temporal shift of the gene expression and subsequent heterochronic morphogenesis could be achieved by a small sequence alteration in the preexisting enhancer (Wakamatsu et al., 2014 and this study). Our finding seems to be a good contrast, but not contradictive, to the previous notion indicating that changes in signal transduction pathways, such as FGF and Wnt, would contribute to the morphogenetic diversification in evolution (e.g. Liu et al., 2010 for a review). It seems more probable that life has always been opportunistic enough to take advantage of every chance to adopt.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.08.010.

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