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Tissue regulation of somitic *colloid-like1* gene expression

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ABSTRACT

Body skeletal muscles formation starts with somite differentiation, due to signals from surrounding tissues. Somite ventral portion forms the sclerotome while its dorsal fraction constitutes the dermamyotome, and later the dermatome and myotome. Relative levels of BMP activity have been proposed to control several aspects of somite development, namely the time and location of myogenesis within the somite. The fine-tuning of BMP activity is primarily achieved via negative regulation by diffusible BMP inhibitors, such as Noggin and Chordin, and on a secondary level by proteins cleaving these inhibitors, such as BMP1/Tolloid metalloprotease family members. Herein, we carefully described the somitic expression of colloid-like1, one of the chick BMP1/Tolloid homologues, and found that this gene is specifically expressed in the 10 most anterior somites, suggesting that it may be involved in neck muscle formation. By using *in ovo* microsurgery and tridimensional embryo tissue culture techniques we assessed the function of surrounding structures, neural tube, notochord, surface ectoderm and lateral plate mesoderm, on the maintenance of somitic colloid-like1 gene expression. We unveil that a signal coming from the neural tube is responsible for this expression and rule out the main candidate pathway, Wnt. By comparing the somitic colloid-like1 gene expression with that of related signaling partners, such as BMP4, Noggin and Chordin, we propose that colloid-like1 plays a role in the reinforcement of BMP4 activity in the medial portion of the 10 most anterior dermomyotomes, thus belonging to the molecular machinery controlling neck muscle development in the chick.

1. Introduction

Somites are on the basis of the segmental pattern of the adult vertebrate body and give rise to vertebrae, intervertebral discs, ribs, the dermis of the back and all skeletal muscles of the adult body. Somites form along the antero-posterior embryonic axis, bilaterally to the neural tube and notochord, budding off from the cranial end of the presomitic mesoderm (PSM) [1] while new cells are caudally added as a consequence of gastrulation [2]. Initially, the somite is formed by a sphere of epithelial cells but, as development proceeds, its ventral part undergoes an epithelial-to-mesenchymal transition, forming the sclerotome. Meanwhile, its dorsal part remains epithelial and constitutes the dermomyotome, which later originates dermatome and myotome. In the latter, two domains can be defined from which the two sets of the body skeletal muscles arise; a dorsomedial/epaxial and a ventrolateral/hypaxial. Signals from surface ectoderm (SE), neural tube, notochord and lateral plate mesoderm (LPM) have been identified as crucial for somitic axes specification [3]. Bone Morphogenetic Protein 4 (BMP4) is produced by both dorsal neural tube and LPM and was shown to be crucial in several aspects of somite differentiation [4]. Several studies in *Xenopus*, chick and mouse revealed that secreted proteins like Noggin and Chordin create a gradient of BMP4 by directly binding to it, thus preventing an interaction with its receptor [5]. A further level of regulation is introduced by the secreted zinc metalloprotease, Tolloid, which belongs to the conserved family BMP1/Tolloid-like [6]. In early embryo, these proteins have been described as positive regulators of BMP4 activity by proteolytically cleaving Chordin, generating small fragments and thus reducing its affinity to BMP4 [7]. Contrarily to other species, very little is known about the function of these metalloproteases in the chick. Two BMP1/Tolloid family members have already been cloned, chicken BMP1/Tolloid and colloid-like1, and their expression patterns described (*bmp1/Tolloid* [8,9]; *colloid-like1* [10]). In this work, we characterise somitic colloid-like1 gene expression in embryos up to stage 15^{+}HH. We assess the *in vivo* regulation of this expression by the different surrounding embryonic tissues such as the axial structures, neural tube and notochord, SE and flanking LPM. The data obtained allows us to

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propose a function of somitic colloid-like1 gene expression in neck muscle formation.

2. Materials and methods

2.1. Chicken embryos

Fertilised chicken (*Gallus gallus domesticus*) eggs were obtained from commercial sources, stored at 16 °C and incubated in a 45% humidified atmosphere at 37 °C. Incubated embryos were harvested, washed in PBS without Ca^{2+}/Mg^{2+} and staged according to Hamburger and Hamilton classification system [11]. Embryos of stages 7–15^{+}HH were fixed overnight at 4 °C in a 4% formaldehyde solution and then progressively dehydrated in methanol series and stored in 100% methanol at −20 °C.

2.2. RNA probes

Colloid-like1, BMP4, chordin, noggin and myoD anti-sense RNA probes were provided by Fabienne Pituelo, Paul Brickell, Tom Jessel and Richard Harland, respectively [10,12–14].

2.3. Whole-mount in situ hybridization

Whole-mount in situ hybridisation was carried out according to [15]. Stained embryos were photographed using a Leica D200 camera or a Zeiss SteREOLumarV12 Stereomicroscope coupled with a Zeiss Axiocam MRc camera and Zeiss Axiovision software. All images were adjusted for brightness and contrast using Adobe PhotoShop CS3.

2.4. Embryo sectioning procedure

Selected hybridised embryos were progressively dehydrated with ethanol, embedded in resin (Technovit 8100) and sectioned at 20 μm thickness using an ultramicrotome. The slides were then mounted in Neomount (Merck) and photographed using a Zeiss Axio Imager Z2 Fluorescence microscope coupled with a Zeiss ICc3 Axiocam camera. All images were adjusted for brightness and contrast using Adobe PhotoShop CS3.

3. Microsurgical experiments

3.1. In ovo surgical slit

A small window was made in the eggshell and an Indian ink:PBS (1:1) solution was injected into the sub-blastodermic cavity. In embryos ranging from 10HH to 12HH, the right row of somites was separated from axial organs by a slit made through the three germ layers (Fig. 2A), along four to six somites posteriorly to the second to fourth most cranial somites. Operated embryos were reincubated for 12–16 h, harvested, fixed, dehydrated as described above and kept in methanol at −20 °C for in situ hybridisation.

3.2. In vitro Notochord ablation

10HH–12HH staged embryo were removed from the egg yolk as described for the Early Chick (EC) culture technique [16] and placed into resin-coated Petri dishes in PBS without Ca^{2+}/Mg^{2+}, ventral side up to facilitate access to the notochord. A portion of the notochord spanning six somites posteriorly to the first to second most cranial somites was mechanically removed from the neural tube and flanking somites (Fig. 2B) and the embryos were cultured for 12–16 h. Embryos were harvested, fixed and processed for in situ hybridization as described above.

3.3. In ovo neural tube ablation

Embryos were accessed as described above. In 10HH–12HH staged embryos, SE incisions were made bilaterally to the neural tube. After a brief treatment with pancreatin (Sigma), a piece of neural tube with a four to six somite length posterior to the second to fifth most cranial somites (Fig. 2C) was dissected out and removed using a micropipette. The operated area was rinsed with heat-inactivated foetal calf serum and the embryos were reincubated for 12–16 h, then harvested, fixed and processed for in situ hybridization as described above.

3.4. In vitro SE removal

10HH–12HH embryos were surgically removed from the egg yolk into resin-coated Petri dishes in PBS without Ca^{2+}/Mg^{2+} where the microsurgery was performed (Fig. 2D). On the right side of the embryo, a longitudinal incision on the ectoderm overlying the middle of the neural tube was made starting from the second to fourth most cranial somites and spanning along a six somite length. An equivalent incision was made on the ectoderm overlying LPM, and the delimited area was carefully peeled off. By cutting the neural tube down the midline two type of explants were generated: on the right side of the embryo, the exposed area was cultured as an explant deprived of all SE and on the left side of the embryo an equivalent explant (with SE) was cultured as a control.

Explants were cultured for 7.5–9 h on polycarbonate filters (0.8 mm pore size; Millipore) as described in [17], fixed and processed for in situ hybridisation.

3.5. In vitro LPM removal

10HH–12HH embryos were surgically removed from the egg yolk into resin-coated Petri dishes as described above. On the right side of the embryo, a longitudinal incision was made between somites and LPM starting from the second to third most cranial somites and spanning along a six somite length (Fig. 2E). Two explants were generated by cutting the neural tube down the midline: the right portion of the embryo was cultured as an explant deprived of LPM and a left equivalent explant (with SE) was cultured as a control.

Both explants were cultured as described above for 7.5–9 h, harvested, fixed and processed for in situ hybridisation.

3.6. In vitro Wnt inhibition

10HH–12HH embryos were surgically removed from the egg yolk into resin-coated Petri dishes as described above. A six somite length explant containing all embryonic tissues was delimited from the first to third somites (Fig. 3) and cultured as described above in Medium 199 supplemented with two concentrations (2 μl/ml; 3.5 μl/ml) of the commercial available Wnt Pathway Inhibitor VII, Cardamonin (Calbiochem). Control explants were cultured in normal medium as described above for 7.5–9 h, harvested, fixed and processed for in situ hybridisation.

4. Results and discussion

4.1. Somitic colloid-like1 gene expression is restricted to the 10 most rostral somites

We evaluated the somitic expression of *colloid-like1* gene by whole-mount in situ hybridisation and section analysis of embryos ranging from stages 6HH [11] to 15HH (Fig. 1). In embryos from stages 7^{+}HH to 15^{+}HH, *colloid-like1* transcripts are broadly detected

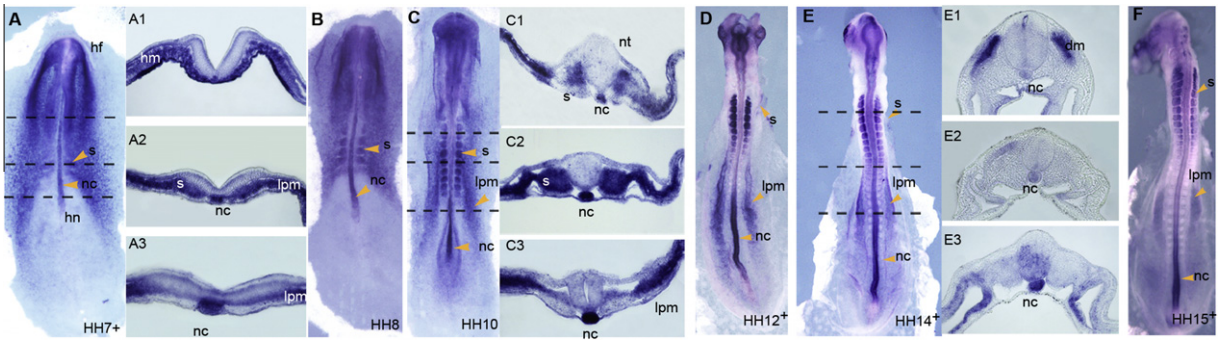

Fig. 1. Colloid-like1 gene is specifically expressed in the 10 most anterior somites. (A–F) Whole-mount in situ hybridised embryos with *colloid-like1* antisense RNA probe. (A) Stage 7^{+}HH embryo, showing expression of *colloid-like1* gene in the headfold, head mesenchyme, newly formed notochord, somites and LPM. (B) In a stage 8HH embryo the expression of *colloid-like1* gene is evident in the epithelial somites, notochord and lateral plate mesoderm. (C) In a stage 10HH embryo, we observe a broad *colloid-like1* expression in all epithelial somites with this expression becoming restricted to their medial part in the most anterior ones. At the level of the notochord, expression starts fading anteriorly being maintained in the LPM. (D–H) Embryos ranging from stage 12^{+}HH–15^{+}HH show that somitic *colloid-like1* gene expression is restricted to the 10 most anterior somites. In these, expression becomes restricted to the dermomyotome region. Notochord expression becomes restricted to its caudal part and LPM transcripts are confined to the region flanking *colloid-like1* negative somites and the most anterior PSM. All embryos are viewed from the dorsal side and the head is directed to top of the picture. Abbreviations: *dm*, dermomyotome *hf*, head fold; *hm*, head mesenchyme; *hn*, Hensen's node; *lpm*, lateral plate mesoderm; *nc*, notochord; *nt*, neural tube *s*, somite.

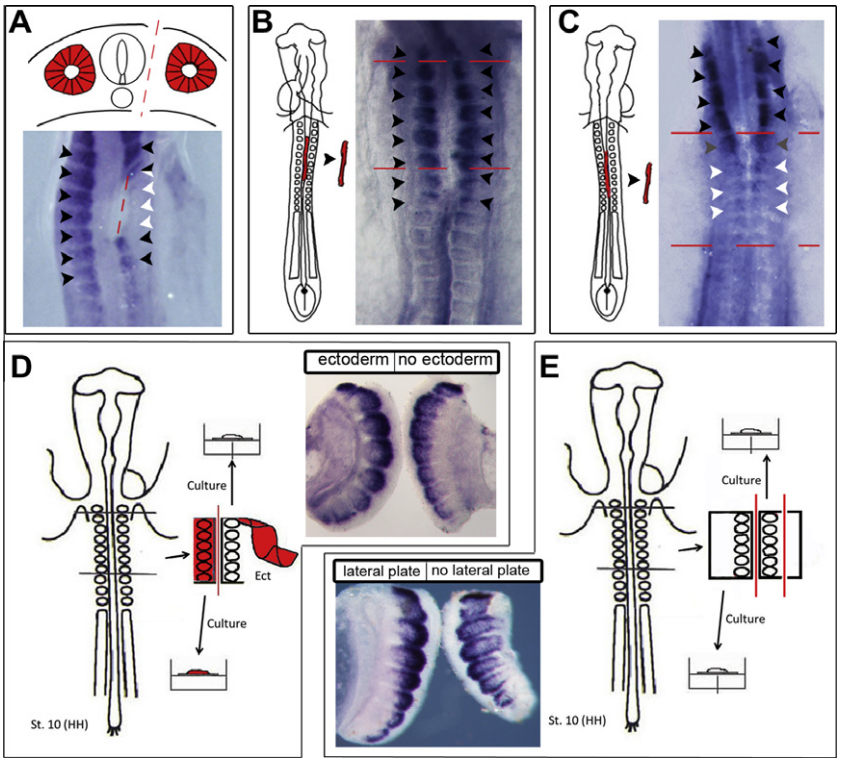

Fig. 2. A neural tube-derived signal maintains somitic *colloid-like1* gene expression. (A) Surgical slit experiment showing down-regulation of *colloid-like1* expression in the somites deprived of contact with the axial (white arrowheads), as opposed to the left side with normal expression (black arrowheads). (B) Notochord removal experiment showing normal expression (black arrowheads) on somites next to the ablated region (between dashed red lines). (C) Neural tube removal experiment showing a down-regulation of *colloid-like1* gene expression in the somites (grey and white arrowheads) next to the ablated area (between dashed red lines). (D) SE removal experiment, showing no difference in somitic *colloid-like1* gene expression between experimental (right) and control (left) halves. (E) LPM removal experiment showing no reduction in somitic expression between experimental (right) and control (left) halves. Anterior is up, posterior is down in all figures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in all epithelial somites since they bud off from PSM (Fig. 1A, A2, B, C, C1, and C2). Surprisingly, from stage 10HH onwards, no *colloid-like1* expression can be observed in forming epithelial somites, with somitic expression always restricted to the 10 most anterior somites (Fig. 1D–F). In these, *colloid-like1* gene expression varies according to somite differentiation, being first medially restricted (Fig. 1C1) and then progressively extending laterally, maybe reflecting somitic mediolateral patterning. A cross section of an older embryo, made at the level of differentiating somites shows that colloid-like1 transcripts are restricted to the dorsomedial part of the differentiating dermomyotome which is already giving rise to the myotome (Fig. 1E1). Furthermore, in stage 7^{+}HH embryos, *colloid-like1* gene expression is detected in all mesodermal tissues, such as the head mesoderm, entire notochord and LPM (Fig. 1A–C). However, as somitogenesis proceeds, notochord and LPM transcripts become posteriorly constrained, with the LPM being specif-

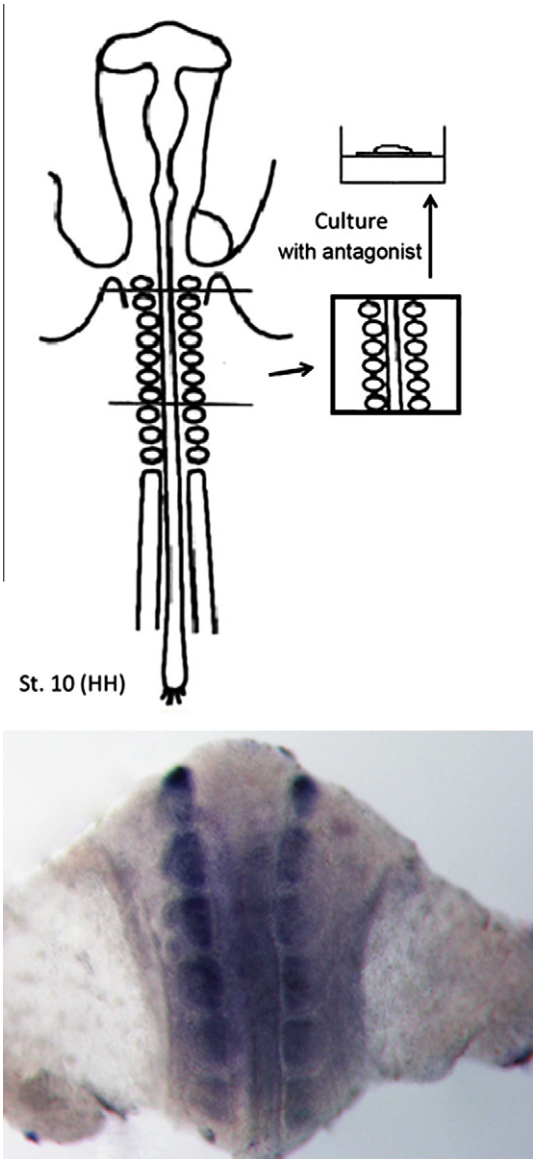

Fig. 3. Somitic colloid-like1 regulation by neural tube is not mediated by Wnt signaling. Explant cultured with a Wnt pathway inhibitor showing no changes in somitic *colloid-like1* expression. Anterior is up, posterior is down.

ically restricted to the region flanked by non-*colloid-like1* expressing somites and presomitic mesoderm (Fig. 1D–E). Throughout the analysed stages, *colloid-like1* gene expression is never observed in the PSM, or in the neural tube. Curiously, somitic *colloid-like1* gene expression is quite distinct from BMP1/Tolloid, the other closely related gene cloned in the chick [8]. Unlike colloid-like1, BMP1/Tolloid is never expressed in newly formed somites; its somitic expression is restricted to the myotome, being absent from the dermomyotome, where *colloid-like1* can be strongly detected. Intriguingly, in Marti et al. we can observe that at stage 17HH, BMP-1/Tolloid is strongly expressed in the myotome of all except the most eight to 10 anterior somites [8]. This comparative analysis suggests that *colloid-like1* might be assuming myotomal BMP1/*Tolloid*'s function in the 10 most anterior dermomyotomes.

4.2. Somitic colloid-like1 gene expression is maintained by a signal coming from the neural tube

Somite differentiation occurs under influence of factors from environmental tissues. Ablation experiments indicate that the dorsal tissues, namely dorsal neural tube and SE, are required for dermomyotome development; neural tube and notochord cooperate to induce epaxial myogenesis, while hypaxial myogenesis depends on signals from the SE and LPM [18]. In order to test whether a signal produced by the axial structures could be responsible for the maintenance of *colloid-like1* in the medial somite, we performed *in ovo* surgical slits between rostral somites and the axial organs ensuring physical separation of these structures (Fig. 2A, scheme). The operated embryos were reincubated during 12–16 h. The results obtained revealed that when somites are separated from the axial structures, somitic *colloid-like1* gene expression is strongly reduced ($n = 23$). This suggested that either the neural tube or notochord, or both, have a function in somitic expression of *colloid-like1*. To further investigate this matter, we started by doing *in vitro* notochord ablations using the Early Chick (EC) culture system [16] culture system. 10HH–12HH stage embryos were collected and a six somite length portion of the notochord posterior to the one to two most cranial somites was removed (Fig. 2B, scheme). In five out of five embryos, there was consistently no reduction in the expression of *colloid-like1* in the somites next to the ablated area (Fig. 2B). This showed that the notochord is not the axial structure responsible for maintaining *colloid-like1* expression in the somites. We next performed a six somite length *in ovo* neural tube ablation, posterior to the two to four most cranial somites (Fig. 2C, scheme). From a total number of eight operated embryos, we observed that the removal of the neural tube causes a strong reduction of *colloid-like1* gene expression in somites next to the ablated region (Fig. 2C). Therefore, we show that the neural tube, but not the notochord, is the axial structure responsible for the maintenance of somitic colloid-like1 gene expression.

In order to determine the role of the SE/LPM in the expression of *colloid-like1* gene at the level of rostral somites, we performed three-dimensional *in vitro* culture explants. In 10HH–12HH stage embryos, we removed either the SE that overlies the right row of rostral somites or the flanking LPM. We then explanted the region deprived of SE/LPM and cultured it for a time period ranging from 7.5 to 9 h (Fig. 2D, 2E, schemes). From the left side of the embryo an equivalent explant with SE/LPM was made and cultured as a control. The analysis of our data reveals that SE/LPM removal (SE, $n = 6$; LPM $n = 10$) does not affect the level of expression of somitic *colloid-like1* (Fig. 2D and 2E). Note that in LPM ablated explants we observe a change in somite morphology, with an expansion of its medio-lateral axis, but no alteration on colloid-like1 expression level. Therefore, we show that neither the SE nor the LPM play an important role on the maintenance of *colloid-like1* expression in the somites.

In summary, our dataset clearly reveals that colloid-like1 expression in the 10 most anterior somites is not regulated by SE, LPM or ventrally located notochord, being maintained by an axial signal coming from the neural tube.

4.3. Wnt signaling is not responsible for somitic colloid-like1 gene maintenance

Members of the Wnt family of secreted glycoproteins are expressed in the dorsal neural tube (Wnt1, 3a and 4) and have been implicated in the formation of the dermomyotome and induction of the embryonic epaxial muscle lineage [19]. These lead us to evaluate if Wnt signaling produced by the dorsal neural tube could be responsible for the maintenance of somitic colloid-like1 gene expression. Embryo explants containing *colloid-like1* expressing somites were generated and incubated in the presence of a Wnt/β-catenin Inhibitor. The data obtained clearly shows that wnt signaling inhibition did not affect somitic colloid-like1 gene expression ($n = 6$). Consequently, dorsal neural tube produced

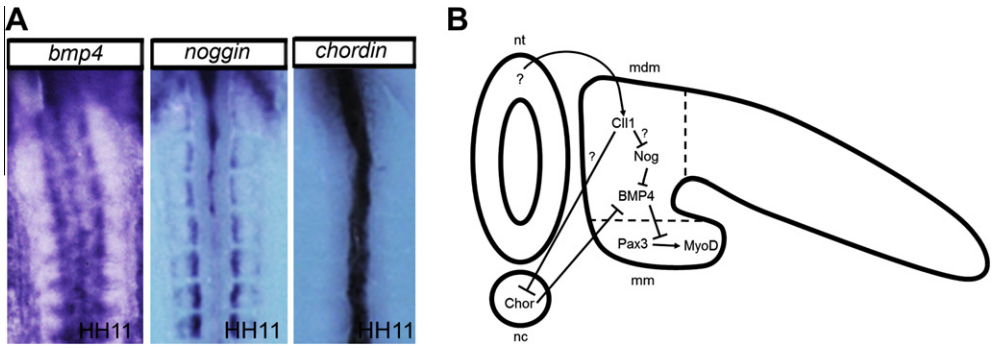

Fig. 4. Proposed model for Colloid-like1 in the molecular machinery underlying neck muscle formation. (A) Whole-mount in situ hybridised embryos for Bmp4, noggin and chordin showing that Bmp4 and noggin are expressed in the medial region of the somite and *chordin* is expressed in the notochord. (B) Schematic representation of the proposed model for *colloid-like1* function in the first 10 somites. A neural tube signal other than Wnt is responsible for *colloid-like1* expression in the medial dermomyotome. There, *colloid-like1* reinforces BMP4 activity by cleaving either locally produced Noggin or Chordin originated from the notochord. This would strengthen the inhibitory effect of BMP4 on the myogenic program of the neck muscle forming somites. Abbreviations: *mdm*, medial dermomyotome; *mm*, medial myotome; *nc*, notochord; *nt*, neural tube.

Wnts do not mediate the positive effect of neural tube on somitic *colloid-like1* gene expression.

4.4. Could colloid-like1 be fine-tuning BMP4 activity during neck muscle formation?

Cross section analysis shows that colloid-like1 transcripts are present in the medial half of the dermomyotome when myotomal cells delaminate from its medial lip, suggesting that this gene may be involved in epaxial muscle formation. In fact, it has been recently proposed that the timing and location of myogenesis within the somite is controlled by relative levels of BMP activity [4]. Taking into account the role of colloid-like1 gene family members on fine-tuning BMP4 activity levels, it could be interesting to compare somitic *colloid-like1*gene expression with the somitic expression of related key players such as BMP4, noggin and chordin (Fig. 4A). This comparison reveals that: (1) both bmp4 and noggin somitic expression starts in the epithelial somite but soon becomes restricted to its medial part, such as *colloid-like1* does [4, our data]; (2) despite chordin is never expressed in somites, it is produced by the neighbouring notochord, known to be involved in epaxial myotome formation [19]. This way, one could argue that *colloid-like1* plays a role in the reinforcement of BMP4 activity in the medial portion of the 10 most anterior dermomyotomes, by cleaving either Noggin locally produced or Chordin secreted by ventrally located notochord (Fig. 4B). In this context it would be important to perform a biochemical study to determine whether *colloid-like1* is able to cleave c-chordin and/or c-Noggin.

Strikingly, *colloid-like1* expression is clearly restricted to the 10 most anterior somites, being totally absent from the following ones. What could be the singularity of this set of somites that would explain such regionalized *colloid-like1* gene expression? Excitingly, we found that the 10 most anterior somites give rise to a set of muscles responsible for the attachment of the head to the thoracic region, by other words, the neck muscles [3]. Additionally, it has been reported that, in chick, somites five to 10 (which form neck muscles) and somites 11–13 (which do not contribute to neck muscles) display differential competence to respond to the muscle-promoting activities of dorsolateral neural tube [19], probably due by differential BMP4 activity along the axis [4]. Could this dissimilar response be related to the expression of colloid-like1 that, by reinforcing BMP4 activity levels, endows these 10 most anterior dermomyotomes with different competences?

Finally, One of the striking lessons from the study of myogenesis is that different myogenic programmes are used to generate the different subsets of skeletal muscles (epaxial, hypaxial, limb, head and neck muscles) [20]. This leads us to propose *colloid-like1* as specifically belonging to the molecular machinery that operates in chick neck muscle formation.

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